ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ
ΤΜΗΜΑ ΒΙΟΧΗΜΕΙΑΣ & ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

«Φυλογένεση και γενετική δομή του λαγού (*Lepus europaeus)*
με τη χρήση μοριακών τεχνικών»

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UNIVERSITY OF THESSALY
DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

Ph.D. Thesis

«Phylogeny and genetic structure of brown hare (*Lepus europaeus*) with the application of molecular methods»

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Larissa, December 2011

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Στους γιους μου και τη σύζυγό μου, Ελένη…

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ΕΥΧΑΡΙΣΤΙΕΣ

Η εκπόνηση της διδακτορικής αυτής διατριβής ήταν ένα προσωπικό στοίχημα, το οποίο είχε, τελικά, θετικό αποτέλεσμα, μετά από επίπονη και χρονοβόρα προσπάθεια. Στη διαδρομή αυτή, ήταν σημαντική η βοήθεια και η συμπαράσταση συγκεκριμένων προσώπων.

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ΠΕΡΙΛΗΨΗ

Ο ευρωπαϊκός λαγός (*Lepus europaeus* Pallas, 1778) αποτελεί ένα ευρέως διαδεδομένο είδος στην Ευρώπη, με μεγάλη ποικιλία ενδιαιτημάτων και ιδιαίτερη οικονομική σημασία, διότι αποτελεί ένα από τα σημαντικότερα θηράματα στην Ευρώπη και ιδιαίτερα στη Βαλκανική χερσόνησο και την Ελλάδα. Η εκτεταμένη κατανομή του και το γεγονός ότι οι πληθυσμοί του ανταποκρίνονται ταχύτατα στις αλλαγές ενδιαιτημάτων, το καθιστούν ένα είδος-μοντέλο, μέσω του οποίου μπορούμε να εξάγουμε χρήσιμα συμπεράσματα για τη συμπεριφορά, τη βιολογία και την πληθυσμιακή δομή των άγριων πληθυσμών πολλών θηλαστικών.

Σε αυτήν τη διδακτορική διατριβή πραγματοποιήθηκε πληθυσμιακή ανάλυση ενός μεγάλου αριθμού δειγμάτων ευρωπαϊκού λαγού σε ένα εκτεταμένο δίκτυο δειγματοληψίας στην Ευρώπη, την Ελλάδα, την Τουρκία και το Ισραήλ. Η μελέτη στηρίχθηκε στην ανάλυση μοριακών δεικτών του μιτοχονδριακού DNA και δεικτών του πυρηνικού DNA (χρωμόσωμα *Y*), χρησιμοποιώντας τεχνικές όπως PCR-RFLP, PCR-SSCP και αλληλούχηση διαφόρων περιοχών DNA.

Οι βασικοί στόχοι της μελέτης αποτυπώνονται ως εξής:

- Μελέτη της γενετικής δομής των φυσικών πληθυσμών του λαγού και της γονιδιακής ροής μεταξύ των περιοχών δειγματοληψίας
- Έλεγχος της υπόθεσης της μετανάστευσης του ευρωπαϊκού λαγού προς την Κεντρική Ευρώπη από καταφύγια της Βαλκανικής χερσονήσου καθώς και της Μικράς Ασίας, κατά την τελευταία Περίοδο των Παγετώνων
- Μελέτη της γενετικής δομής των εκτρεφόμενων πληθυσμών του λαγού, εκτίμηση της γονιδιακής ροής ανάμεσα σε εκτρεφόμενους και φυσικούς πληθυσμούς
- Εύρεση μοριακών δεικτών σε επίπεδο μιτοχονδριακού DNA, για την ταυτοποίηση των εκτρεφόμενων και των φυσικών πληθυσμών.

Τα συμπεράσματα που προέκυψαν από τη μελέτη είναι τα εξής:

- Ο εποικισμός μεγάλων περιοχών της Ευρώπης με ευρωπαϊκούς λαγούς, άρχισε στο τέλος της τελευταίας παγετωνικής περιόδου και στην αρχή του Ολόκαινου, από έναν μόνο αρχικό πληθυσμό των κεντρικών ή νοτιο-κεντρικών Βαλκανίων και υπήρξε σχετικά ταχύτατος. Αυτό έρχεται σε αντίθεση με την υπόθεση

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εποικισμού της Ευρώπης από μερικά μικρά θηλαστικά, κατά το τέλος του Πλειστόκαινου, από μερικά καταφύγια της ανατολικής Ευρώπης και της δυτικής Σιβηρίας.

- Οι μέχρι τώρα αναλύσεις έδειξαν ότι στην Ιβηρική χερσόνησο τα μη υβριδισμένα άτομα ευρωπαϊκού λαγού έχουν τους κλασσικούς απλότυπους στο mtDNA, γεγονός που αποκλείει την περιοχή αυτή από το να έπαιξε το ρόλο καταφυγίου για το *L. europaeus* κατά τον Πλειστόκαινο.
- Στη Βουλγαρία και τη βορειοανατολική Ελλάδα είναι παρούσες όλες οι απλοομάδες, δημιουργώντας μια εκτεταμένη περιοχή επικάλυψης όλων των απλοτύπων.
- Ανιχνεύθηκε γονιδιακή ροή από την Ανατολία προς την Ευρώπη, πιθανότατα κατά τη διάρκεια της τελευταίας περιόδου του Πλειστόκαινου, μέσω της γέφυρας του Βοσπόρου.
- Οι ευρωπαϊκοί λαγοί από τη Μ. Βρετανία εμφάνισαν πολύ χαμηλό πολυμορφισμό, αλλά φαίνεται πως δεν αποτελούν ένα ξεχωριστό υποείδος (*L. e. occidentalis*), όπως είχε θεωρηθεί παλαιότερα. Η βόρεια Γερμανία θα μπορούσε να είναι η περιοχή προέλευσης των σύγχρονων πληθυσμών της Βρετανίας.
- Η υπερθήρευση και οι συνεχείς απελευθερώσεις αλλόχθονων λαγών θα μπορούσαν να εξηγήσουν τη μαζική παρουσία των απλοτύπων της ευρωπαϊκής ομάδας EU-A στην κεντρική και βόρεια Ιταλία. Η πρακτική αυτή ίσως συνέβαλε στην αντικατάσταση των γηγενών απλοτύπων SEE που πιθανώς ήταν διαδεδομένοι αρχικά. Τα δεδομένα μας απορρίπτουν την ύπαρξη ενός διαφορετικού υποείδους (*L. e. meridiei*) στην Ιταλική χερσόνησο.
- Αν και οι πολλές επιχειρήσεις εμπλουτισμού και ανθρωπογενών απελευθερώσεων ευρωπαϊκού λαγού που έχουν πραγματοποιηθεί θα μπορούσαν να εξηγήσουν την παρουσία μη αναμενόμενων απλοτύπων σε ορισμένες περιοχές, εντούτοις ανιχνεύθηκε ένα έντονο φυλογεωγραφικό σήμα σε όλες τις περιοχές που μελετήθηκαν.
- Τα φυλογενετικά δένδρα που προέκυψαν από την ανάλυση των γονιδίων tRNA του mtDNA ήταν συγκρίσιμα με αυτά που έδωσαν τα υπόλοιπα μιτοχονδριακά τμήματα. Είναι πολύ πιθανό ότι τα γονίδια tRNA που αναλύθηκαν, συσσώρευσαν μεταλλάξεις σε θέσεις οι οποίες δεν επηρεάζουν το ρόλο τους στη μιτοχονδριακή σύνθεση των πρωτεϊνών.

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- Οι μοριακές αναλύσεις αυτών των γονιδίων tRNA μπορεί να χρησιμοποιηθούν ως πολύτιμα βοηθητικά εργαλεία για την σύνδεση της ακριβούς βιοχημικής λειτουργίας των μιτοχονδρίων με τις εξελικτικές και φυλογενετικές μελέτες.
- Η μελέτη των tRNAs κατέγραψε εξελικτικές αλλαγές που μπορούν να συνδεθούν άμεσα με τη διακριτή φυλογεωγραφική κατανομή του *L. europaeus*. Το γεγονός ότι οι νουκλεοτιδικές αντικαταστάσεις που ανιχνεύθηκαν εμφανίζονται σε ένα μεγάλο ποσοστό των ατόμων του *L. europaeus* υποδηλώνει ότι είναι ανεκτές από την πίεση της φυσικής επιλογής.
- Σε συμφωνία με τα υπόλοιπα φυλογενετικά δεδομένα του mtDNA, η ανάλυση του γονιδίου Cyt*b* επιβεβαιώνει την ύπαρξη τουλάχιστον τεσσάρων διαφορετικών απλοομάδων με πολύ καλά προσδιορισμένη κατανομή στην Ευρώπη και την Ανατολία.
- Η ανάλυση του Cyt*b* υποστηρίζει επίσης το βαθύ διαχωρισμό των πληθυσμών του *L. europaeus* ανάμεσα στην Ανατολία (Τουρκία και Ισραήλ) και την Ευρώπη σε επίπεδο mtDNA.
- Σε αντίθεση με τους διγονεϊκούς πυρηνικούς μοριακούς δείκτες, τα δεδομένα του *Y*-DNA υποστηρίζουν την ύπαρξη δύο βασικών φυλογενετικών κλάδων για το είδος *L. europaeus* ανάμεσα στην Ευρώπη και την Ανατολία.
- Σε αντίθεση με το mtDNA, ο τύπος του *Y*-DNA της Ανατολίας εντοπίστηκε και σε μερικά άτομα της κεντρικής Ελλάδας. Είναι πολύ πιθανό ότι η τάση για φιλοπατρία των θηλυκών ευρωπαϊκών λαγών, καθιστά την εισδοχή του mtDNA πιο δύσκολη σε σχέση με το *Y*-DNA και καταλήγει σε διαφορετικά πρότυπα κατανομής.
- Οι ελληνικοί πληθυσμοί του *L. europaeus* εμφανίζουν πολύ μεγαλύτερη γενετική ποικιλότητα σε σχέση με τους εκτρεφόμενους αλλά και τους κεντροευρωπαϊκούς πληθυσμούς, όπως συνάγεται από τα δεδομένα του mtDNA και των RAPDs. Επομένως, θα ήταν άστοχο να χρησιμοποιηθούν απελευθερώσεις εκτρεφόμενων ατόμων για να εμπλουτιστεί η ήδη πλούσια γενετική δεξαμενή των ελληνικών πληθυσμών.
- Το επόμενο διαχειριστικό ερώτημα είναι εάν θα υπάρξει το φαινόμενο της γενετικής κατάπτωσης μετά από ανάμιξη των τοπικών γονιδιακών δεξαμενών με μη προσαρμοσμένα γονίδια εισαγόμενων ατόμων. Η Βουλγαρία και πλέον, μετά τις απελευθερώσεις, η βορειοανατολική Ελλάδα είναι οι μόνες περιοχές της

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Ευρώπης όπου συνυπάρχουν και οι τέσσερεις απλοομάδες. Και στις δύο περιπτώσεις δεν υπάρχουν τα κατάλληλα δεδομένα για να αποφανθούμε ως προς τις πιθανές αλληλεπιδράσεις μεταξύ των διαφόρων απλοομάδων. Εάν, μακροχρόνια, τα εισαγόμενα ξένα γονίδια επιβιώσουν, δημιουργώντας νέους γενότυπους με τα ήδη υπάρχοντα, θα αποδειχθεί ότι αυτά μπορούν να προσαρμοστούν. Καθώς τα πυρηνικά γονίδια δεν είναι ιδιαίτερα διαφοροποιημένα μεταξύ των πληθυσμών της Ελλάδας και της υπόλοιπης Ευρώπης, τα ξένα πυρηνικά γονίδια δεν θα αποτελέσουν ένα σοβαρό μειονέκτημα. Επομένως, υπό τις συγκεκριμένες περιπτώσεις, τα προγράμματα απελευθερώσεων θα μπορούσαν να ενταχθούν σε ένα διαχειριστικό σχήμα, με την προϋπόθεση ότι και όλες οι άλλες μη γενετικές παράμετροι λαμβάνονται υπόψη και ελέγχονται αυστηρά.

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ABSTRACT

The brown hare (*Lepus europaeus* Pallas, 1778) is widespread throughout Europe, occurring in a variety of environments. It has considerable economic importance because it is one of the most important game species in Europe and especially in the Balkan Peninsula and Greece. The extensive distribution of this species and the fact that its populations responds rapidly to environmental and habitat changes, make this species a suitable model for the study of the behaviour, biology and genetic structure of wild mammals populations.

In this thesis we analysed the population genetics of the brown hare (*Lepus europaeus*) in European, Greek and Anatolian populations, using sequencing, PCR-RFLP and PCR-SSCP methods, performed on mtDNA and Y-DNA genes, in order to:

- assess the genetic differentiation and the phylogenetic status of brown hare populations
- test the hypothesis that this species migrated into central Europe from a number of late glacial refugia, including some in Asia Minor
- estimate the genetic structure of reared populations and the impact of the releases on the native populations genetic structure
- develop molecular markers for the identification of reared and native populations

The conclusions drawn from the study are the following:

- Colonization of large parts of Europe started from only one late glacial/early Holocene source population in the central or south-central Balkans, and it was relatively quick. This contradicts Bilton *et al.*, (1998), who suggested colonization of central Europe by some small mammals from Late Pleistocene refugees in eastern Europe and western Siberia, as an alternative to colonization from Mediterranean refugees.
- So far, mtDNA of non-introgressed brown hares from Iberia have standard central European haplotypes, suggesting no late Pleistocene refuge in Iberia.
- In Bulgaria and northeastern Greece all haplogroups were present, forming a large introgression zone.
- Gene flow from Anatolia to Europe, across the late Pleistocene Bosporus land bridge, was detected.

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- Brown hares from the British Isles had very low mtDNA polymorphism showing that they do not consitute a separate subspecies (*L. e. occidentalis*), as previously suggested. Northern Germany could be a possible source region of the current populations.
- Over hunting and continuous releases of brown hares could explain the massive presence of EU-A haplotypes in central and northern Italy. This practice might have replaced the native and possibly originally widespread SEE haplotypes. Our data contradict the idea of the existence of the subspecies *L. e. meridiei* in the Italian peninsula.
- Although various restocking operations could be partly responsible for the presence of unexpected haplotypes in certain areas, we nevertheless trace a strong phylogeographic signal throughout all regions under study.
- Phylogenetic trees produced by the comparison of mt tRNA genes are similar compared to those that are produced by the standard methods.
- Molecular investigations on mt tRNA could serve as valuable accessory elements for connecting analysis of precise and unimpeded biochemical function in mitochondria with evolutionary and phylogenetic studies.
- We assume that the tRNA genes that were used for the present analysis accumulated mutations in positions that do not seem to affect their role in mitochondrial protein synthesis.
- These tRNA recorded evolutionary changes that can be directly connected with the distinct phylogeographic distribution of *L. europaeus*. The fact that the observed nucleotide substitutions appear in a large portion of the *L. europaeus* individuals indicates that they are tolerated by natural selection pressure.
- Consistent with previous data, phylogenetic analysis of Cyt*b* gene corroborates the existence of four different haplogroups with a well defined distribution across Europe and Anatolia.
- The analysis consistently supports the deep separation of Anatolian (Turkey and Israel) and European lineages of *L. europaeus* at the mtDNA level.
- Contrary to other biparental nuclear markers, Y-DNA data underline the existence of two major phylogenetic clades within *L. europaeus* species between Anatolia and Europe.

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- Unlike mtDNA, Anatolian Y-DNA extended into central Greece, where it was detected in few individuals. It is very likely, that the tendency toward philopatry of female brown hares makes mtDNA introgression more difficult in comparison with Y-DNA, resulting in different distributional patterns.
- In contrast to natural populations, reared populations showed relatively little genetic variation, as revealed by mtDNA and RAPDs. Greek populations have also higher levels of genetic variation than populations from Central Europe. The only advantage that the released animals could confer is the enrichment of the already rich Greek mtDNA genetic pool, given the absence of the "reared" mtDNA haplotypes from Greece.

In that case there is still a question of "outbreeding depression" from locally maladapted genes imported with the allocthonous animals. Bulgaria and now, after the releases, north-eastern Greece are the only European regions where all four types of mtDNA profile occur. In both cases either there are no data or it is too early to conclude any possible interaction between the different types of haplotypes. If, in the long run, introgressed foreign genes survive, forming new genotypes with indigenous genes, this would in fact demonstrate that they are successful in terms of competition. As nuclear gene pools are not too divergent between Greek and other European hares, foreign nuclear genes should not be a serious handicap. Hence, in certain situations releasing programs might be tolerated under obeying all other non-genetic strict controls.

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- Stamatis C., Suchentrunk F., Sert H., Triantaphyllidis C., Mamuris Z. (2007). Genetic evidence for survival of released captive-bred brown

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hares (*Lepus europaeus*) during restocking operations in Greece. *Oryx* 41(4): 548-551. 25

- Stamatis C., Giannouli S., Suchentrunk F., Sert H., Stathopoulos C., Mamuris Z. (2008). Recruitment of mitochondrial tRNA genes as auxiliary molecular markers for linked functional genomic and genetic analyses: The paradigm of brown hare (*Lepus europaeus*). *Gene* 410: 154-164. 29
- Stamatis C., Suchentrunk F., Moutou K.A., Giacometti M., Haerer G., Djan M., Vapa L., Vucovic M., Tvrtkovic N., Sert H., Alves P., Mamuris Z. (2009). Phylogeography of the Brown hare, *Lepus europaeus*, in Europe: legacy of southeastern Mediterranean refugia? *Journal of Biogeography* 36: 515-528. 40
- Mamuris Z., Moutou K.A., Stamatis C., Sarafidou T., Suchentrunk F. (2010). Y DNA and Mitochondrial lineages in European and Asian populations of the brown hare (*Lepus europaeus*). *Mammalian Biology* 75: 233-242. 63

Πρόδρομα αποτελέσματα

- Mamuris Z., Sfougaris A. I., Stamatis C. (2001). Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA RFLP-PCR analysis: implications for conserving genetic diversity. *Biological Conservation* 101: 187-196. 73
- Mamuris Z., Sfougaris A. I., Stamatis C., Shuchentrunk F. (2002). Assessment of genetic structure of Greek brown hare (*Lepus europaeus*) populations based on variation in Random Amplified Polymorphic DNA (RAPD). *Biochemical Genetics* 40: 323-338. 83
- Shuchentrunk F., Mamuris Z., Sfougaris A. I., Stamatis C. (2003). Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. *Biochemical Genetics* 41: 127-140. 99

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1. ΕΙΣΑΓΩΓΗ

1.1 Πληθυσμιακή γενετική

Η πληθυσμιακή γενετική είναι ο κλάδος ο οποίος μελετά τη γενετική ποικιλομορφία που παρατηρείται τόσο στα άτομα ενός πληθυσμού όσο και μεταξύ διαφορετικών πληθυσμών, δηλαδή την ποικιλότητα αλληλομόρφων και γενοτύπων και τις αντίστοιχες συχνότητές τους καθώς και τα αίτια-μηχανισμούς οι οποίοι μεταβάλλουν τη γενετική δομή των πληθυσμών. Η πληθυσμιακή γενετική είναι άρρηκτα συνδεδεμένη με την εξέλιξη και αποτελεί τη βάση για τη γενετική βελτίωση των ζωικών οργανισμών, τη διατήρηση των ειδών και της βιοποικιλότητας (Russell, 2009). Εφαρμόζοντας τις αρχές της κλασσικής γενετικής σε μεγάλες ομάδες ατόμων, η πληθυσμιακή γενετική μελετάει τα πρότυπα της γενετικής ποικιλομορφίας που εντοπίζονται μέσα σε κάθε ομάδα, δηλαδή τη γενετική δομή των πληθυσμών, τη γεωγραφική τους διαφοροποίηση και τη μεταβολή τους στο χρόνο.

1.2 Γενετική ποικιλομορφία

Η πληθυσμιακή γενετική ποικιλομορφία έχει τη βάση της στη διαφοροποίηση της νουκλεοτιδικής αλληλουχίας. Προϋπόθεση για την ύπαρξη γενετικής ποικιλομορφίας είναι οι νουκλεοτιδικές αλλαγές-μεταλλάξεις. Αυτές οι αλλαγές μπορούν να προκύψουν κατά τη διαδικασία της αντιγραφής του DNA και αν συμβούν στα κύτταρα της γαμετικής σειράς είναι δυνατόν να μεταβιβαστούν στις επόμενες γενιές. Τις τελευταίες δεκαετίες, η εξέλιξη των μοριακών τεχνικών και των μεθόδων αλληλούχησης συνετέλεσε στη ραγδαία αύξηση των δεδομένων της πληθυσμιακής ποικιλομορφίας στο επίπεδο της νουκλεοτιδικής αλληλουχίας. Γενετική ποικιλότητα μπορεί επίσης να προκύψει και από το γενετικό ανασυνδυασμό. Στους ευκαρυωτικούς οργανισμούς υπάρχουν δύο διεργασίες ανασυνδυασμού: Α) Ο διαχρωμοσωματικός ανασυνδυασμός, που γίνεται με βάση τον ελεύθερο συνδυασμό των χρωμοσωμάτων, κατά τον οποίο τα μειωτικά προϊόντα αντιστοιχούν στο 50% του συνόλου των απογόνων και Β) Ο ενδοχρωμοσωματικός ανασυνδυασμός (διασκελισμός), που συμβαίνει κατά τη μείωση ανάμεσα σε μη αδελφές

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χρωματίδες και οδηγεί σε διαφορετικό συνδυασμό αλληλομόρφων. Τα παραγόμενα μειωτικά προϊόντα αυτού του τύπου ανασυνδυασμού αντιστοιχούν σε ποσοστό μικρότερο από το 50% του συνόλου των απογόνων (Τριανταφυλλίδης, 2001).

Η γενετική ποικιλομορφία, που παράγεται μέσα σε έναν πληθυσμό από τις μεταλλάξεις και τον ανασυνδυασμό, τροποποιείται (αυξομειώνεται, καθιερώνεται ή απαλείφεται) μέσα από μηχανισμούς διεργασιών όπως η φυσική επιλογή, η γονιδιακή ροή και η γενετική παρέκκλιση. Η φυσική επιλογή είναι μια ομάδα διεργασιών, μέσω των οποίων, χαρακτηριστικά τα οποία καθιστούν τους οργανισμούς καλύτερα προσαρμοσμένους στο περιβάλλον τους, αυξάνουν δηλαδή την πιθανότητα επιβίωσης και αναπαραγωγής τους, διατηρούνται και μεταβιβάζονται, διαμορφώνοντας ένα μεγάλο μέρος της φαινοτυπικής ποικιλομορφίας που παρατηρείται στην φύση και ταυτόχρονα της υποκείμενης γενετικής παραλλακτικότητας. Η γονιδιακή ροή περιγράφει την είσοδο στη γενετική δεξαμενή ενός πληθυσμού, γονιδίων (ή αλληλομόρφων τους) από έναν ή περισσότερους πληθυσμούς. Συνήθως οι πληθυσμοί αυτοί ανήκουν στο ίδιο είδος, σε μερικές περιπτώσεις, όμως, πραγματοποιείται υβριδισμός ανάμεσα σε διαφορετικά είδη, τα οποία δεν είναι πλήρως αναπαραγωγικά απομονωμένα. Η γονιδιακή ροή πραγματοποιείται με μετακίνηση ατόμων μεταξύ δύο πληθυσμών, μέσω της μετανάστευσης ή μεταφοράς ατόμων από τον άνθρωπο. Εισάγει νέα αλληλόμορφα ή μεταβάλλει τη συχνότητα υπαρχόντων αλληλομόρφων στους πληθυσμούς. Ο όρος γενετική παρέκκλιση αναφέρεται σε αλλαγές από γενιά σε γενιά στις συχνότητες των αλληλομόρφων σε ένα πληθυσμό που οφείλονται σε τυχαία γεγονότα. Πρόκειται για πολύ σημαντική εξελικτική διεργασία, η οποία μπορεί να οδηγήσει σε απότομες αλλαγές των αλληλομορφικών συχνοτήτων σε σχετικά σύντομο χρονικό διάστημα. Μπορεί να συμβάλλει στην εγκαθίδρυση νέων αλληλομόρφων σε ένα πληθυσμό αλλά και στην απώλεια κάποιων, με συνέπεια την σημαντική αλλοίωση της γενετικής ποικιλότητας.

1.3 Μελέτη των εξελικτικών σχέσεων των οργανισμών

Ο όρος φυλογένεση αναφέρεται στη μελέτη των εξελικτικών σχέσεων μεταξύ οργανισμών, πληθυσμών και ειδών όπως αυτές διαμορφώνονται με το πέρασμα του χρόνου. Η εξελικτική διαδικασία είναι δυνατόν να δημιουργήσει διακλαδώσεις, καθώς οι

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πληθυσμοί μεταβάλλονται στο χρόνο, διαχωρίζονται σε ξεχωριστούς κλάδους (ειδογένεση), διασταυρώνονται ή εξαφανίζονται. Ο προσδιορισμός των φυλογενετικών σχέσεων είναι συνδεδεμένος με την ταξινόμηση των οργανισμών, καθώς, από την εποχή του Δαρβίνου ακόμη, στόχος των ειδικών ήταν η ταξινόμηση να αντανακλά όσο πιο πιστά γίνεται τις φυλογενετικές σχέσεις των οργανισμών.

Παλαιότερα, η περιγραφή των σχέσεων μεταξύ των οργανισμών βασιζόταν κυρίως σε φαινοτυπικά χαρακτηριστικά, στη συγκριτική μορφολογία, στη φυσιολογία κ.λπ. Οι μέθοδοι ανάλυσης των γνωρισμάτων (που ονομάστηκαν κλαδιστικές μέθοδοι) βασίζονται εν γένει στις αρχές που περιγράφηκαν από το Γερμανό εντομολόγο Willi Hennig στο βιβλίο του Phylogenetic Systematics (1966). Εφόσον ήταν γνωστό ότι ο γενότυπος ενός ατόμου αντικατοπτρίζεται στο φαινότυπό του, τα μορφολογικά γνωρίσματα ήταν αυτά που χρησιμοποιήθηκαν κατά κύριο λόγο στις κλαδιστικές μελέτες. Επιπροσθέτως, ο φαινότυπος είναι εκείνος που υφίσταται τις πιέσεις της φυσικής επιλογής και εξελίσσεται ορατά. Ένα ακόμη πλεονέκτημα της χρήσης των μορφολογικών γνωρισμάτων, είναι η δυνατότητα χρήσης μεγάλου δείγματος ατόμων στις αναλύσεις, μειώνοντας με τον τρόπο αυτόν την πιθανότητα στατιστικού σφάλματος, καθώς και η δυνατότητα επανάληψης των αναλύσεων με τη χρήση των ίδιων ατόμων, εφόσον είναι δυνατή η διατήρησή τους. Για τους παραπάνω λόγους, η χρήση της μορφολογίας βρισκόταν σε άνθιση μέχρι τη δεκαετία του ’80. Ωστόσο, οι αναλύσεις που στηρίζονται στη μορφολογία των ατόμων έχουν και μειονεκτήματα, όπως για παράδειγμα το γεγονός ότι τα διαφορετικά γνωρίσματα είναι, ορισμένες φορές, δύσκολο να διακριθούν, συνεπώς πρέπει να λαμβάνεται υπόψη η υποκειμενικότητα του ερευνητή. Επιπλέον, τα πρότυπα της κληρονομικότητας των μορφολογικών γνωρισμάτων δεν είναι πάντα σαφή. Τέλος, υπάρχουν περιπτώσεις κατά τις οποίες το πλήθος των δεδομένων (διαθέσιμων γνωρισμάτων) δεν επαρκεί για την αξιόπιστη στήριξη των φυλογενετικών υποθέσεων.

Μια λύση στα προβλήματα αυτά έδωσε η ανάπτυξη των μοριακών μεθόδων, οι οποίες βρίσκουν εφαρμογή σε όλους τους οργανισμούς και σε κάθε μόριο το οποίο φέρει πληροφορία για τον οργανισμό. Με την ανάπτυξη μοριακών τεχνικών, όπως η αλυσιδωτή αντίδραση πολυμεράσης (Polymerase Chain Reaction, PCR), δόθηκε στους ερευνητές η δυνατότητα προσδιορισμού αλληλουχιών DNA σε μεγάλη κλίμακα. Έτσι,

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η μοριακή ανάλυση εντάχθηκε ως απαραίτητο εργαλείο στις φυλογενετικές μελέτες καθώς θεωρήθηκε ότι τα μοριακά δεδομένα, και κυρίως οι αλληλουχίες του DNA παρέχουν αποδείξεις οι οποίες επιτρέπουν τον προσδιορισμό της φυλογένεσης όλων των μορφών ζωής. Επιπλέον, θεωρήθηκε ότι τα μοριακά δεδομένα ήταν ανώτερα από τα μορφολογικά, καθώς αναφέρονται στο επίπεδο του DNA και των γονιδίων, τα οποία παρέχουν τις απαραίτητες αποδείξεις για τις σχέσεις μεταξύ των ατόμων. Ωστόσο, από την αρχή σχεδόν της χρήσης των μοριακών δεδομένων, ήταν γνωστό ότι οι μέθοδοι αυτές, όπως και οι μέθοδοι που χρησιμοποιούσαν μορφολογικά δεδομένα, είχαν τόσο πλεονεκτήματα όσο και μειονεκτήματα.. Η μελέτη του DNA επιτρέπει την άμεση ανάλυση του γενετικού υλικού, το οποίο έχει κληρονομηθεί από τους προγόνους και συνεπώς αντανακλά τη γενεαλογία και είναι αξιόπιστο για τη μελέτη των φυλογενετικών σχέσεων. Αντιθέτως, μια συγκεκριμένη δομή σε έναν οργανισμό, δεν παρέχει πληροφορίες για τον τρόπο με τον οποίο έχει προκύψει, δηλαδή εάν οφείλεται σε άμεσες περιβαλλοντικές επιρροές ή στην κληρονομικότητα. Εφόσον, λοιπόν, για τη μελέτη των φυλογενετικών σχέσεων είναι απαραίτητη η μελέτη των κληρονομούμενων χαρακτηριστικών, η μορφολογία καθίσταται λιγότερο αξιόπιστη για τις φυλογενετικές μελέτες.

Ένα από τα προβλήματα των μορφολογικών μελετών είναι το γεγονός ότι οι υπό μελέτη οργανισμοί μπορεί να μοιάζουν εξαιρετικά μεταξύ τους στη μορφολογία, με συνέπεια οι ομοιότητες αυτές να καθιστούν δυσκολότερη την εύρεση ενός αριθμού χαρακτήρων, ικανού για αξιόπιστη και συστηματική ανάλυση. Αντιθέτως, οι αλληλουχίες DNA είναι ευμετάβλητες ανάμεσα στα είδη και κατά συνέπεια, είναι δυνατόν να βρεθεί μεγάλος αριθμός χαρακτήρων (θέσεις ζευγών βάσεων) οι οποίοι φέρουν φυλογενετικά πληροφοριακή μεταβλητότητα. Το κόστος είναι ένας σημαντικός παράγοντας σε κάθε μελέτη. Η μελέτη της μορφολογίας είναι πολύ πιο οικονομική από τη μελέτη των αλληλουχιών του DNA. Αυτό σημαίνει ότι με το ίδιο κόστος είναι δυνατόν να μελετηθούν περισσότερες δομές ενός οργανισμού, απ’ ότι γονίδια. Στην περίπτωση αυτή η μορφολογία υπερτερεί του DNA. Ωστόσο, η τεχνολογία του DNA γίνεται όλο και πιο οικονομική και γρήγορη, οπότε το συγκεκριμένο πλεονέκτημα της μορφολογίας συνεχώς φθίνει.

Ένα συγκεκριμένο μορφολογικό χαρακτηριστικό είναι δυνατόν να καθορίζεται

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από περισσότερα του ενός γονίδια, με αποτέλεσμα η μελέτη του να αντλεί πληροφορίες για πολλαπλά τμήματα του γενετικού υλικού. Αντιθέτως, συνήθως λόγω οικονομικών περιορισμών, η μοριακή ανάλυση περιορίζεται σε ένα ή σε μικρό αριθμό γονιδίων. Έτσι, δεν λαμβάνεται υπ' όψιν η πιθανή αλληλεπίδρασή του με άλλα άγνωστα γονίδια, με συνέπεια να υπάρχει πιθανότητα χαρακτήρες οι οποίοι αναφέρονται ως ξεχωριστοί, να εξελίσσονται κατά συσχετιζόμενο τρόπο, γεγονός που καθιστά τη φυλογένεση λιγότερο αξιόπιστη. Αντίθετα, εάν η μελέτη αφορά χαρακτηριστικά που κωδικοποιούνται από διαφορετικά γονίδια, είναι λιγότερο πιθανό η εξέλιξή τους να συσχετίζεται. Στην περίπτωση αυτή, οι μορφολογικές μελέτες υπερτερούν. Ωστόσο, αυτή η διαφορά με τις μοριακές μελέτες μειώνεται όσο μεγαλώνει ο αριθμός των υπό μελέτη γονιδίων.

Συμπερασματικά, οι πλέον αξιόπιστες φυλογενετικές μελέτες βασίζονται σε συνδυασμό διαφορετικών ειδών δεδομένων. Αυτή η προσέγγιση τις καθιστά πιο ολοκληρωμένες και μειώνει την πιθανότητα να περιέχουν σφάλματα όσον αφορά τα συμπεράσματα που εξάγονται, ιδιαίτερα δε εάν αυτά συμφωνούν ανάμεσα σε διαφορετικά είδη αναλύσεων (Bininda-Emonds, 2000). Παρ' όλα αυτά, η συμβολή της μοριακής ανάλυσης στη φυλογένεση και ο σημαντικός της ρόλος στη διαλεύκανση των φυλογενετικών σχέσεων ακόμα και μεταξύ ανώτερων ταξινομικών βαθμίδων δεν είναι δυνατόν να αμφισβητηθεί.

1.4 Μοριακοί δείκτες

Οι μοριακοί δείκτες χρησιμοποιούνται ευρέως στην εξελικτική βιολογία και στις φυλογενετικές μελέτες ενώ οι μοριακές μέθοδοι ανάλυσης βελτιώνονται με ταχείς ρυθμούς. Υπάρχει μία πληθώρα μοριακών δεικτών που μελετούν τον πολυμορφισμό είτε σε επίπεδο πρωτεϊνών είτε σε επίπεδο DNA. Οι κυριότεροι από αυτούς είναι:

- Αλλοένζυμα, που βασίζονται στην ποικιλότητα των πρωτεϊνών λόγω μη συνώνυμων νουκλεοτιδικών πολυμορφισμών στην κωδική περιοχή του αντίστοιχου γονιδίου. Τα αλλοένζυμα διαφέρουν ως προς την ηλεκτροφορητική κινητικότητά τους.
- Πολυμορφισμοί μεγέθους τμημάτων περιορισμού (RFLP) (Botstein *et al.,* 1980) που αφορά στην παραγωγή τμημάτων DNA διαφορετικού μήκους, μετά από

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πέψη με περιοριστικά ένζυμα.

- Τυχαία ενισχυμένο πολυμορφικό DNA (RAPD) (Williams *et al.,* 1990) ο οποίος βασίζεται στο διαφορικό πολλαπλασιασμό, τυχαίων αλληλουχιών ενός δείγματος DNA. Η ταυτοποίησή τους επιτυγχάνεται μέσω PCR με ολιγονουκλεοτιδικούς εκκινητές (8-10 βάσεις).
- Μικροδορυφόροι, που αποτελούνται από διαδοχικές επαναλαμβανόμενες αλληλουχίες 1-6 βάσεων οι οποίες φέρουν εκατέρωθεν μοναδιαίες αλληλουχίες. Εμφανίζουν υψηλό βαθμό πολυμορφισμού και εντοπίζονται σε πάρα πολλές διαφορετικές θέσεις του γονιδιώματος (Queller *et al*., 1993).
- Αλληλουχίες DNA. Παλαιότερα, οι παραπάνω μέθοδοι χρησιμοποιούνταν ευρέως στις φυλογενετικές μελέτες κυρίως λόγω του χαμηλού κόστους και της ταχύτητας εκτέλεσής τους σε σύγκριση με την αλληλούχηση του DNA. Ωστόσο, πλέον τόσο το κόστος όσο και ο χρόνος που απαιτείται για την αλληλούχηση μειώθηκαν δραματικά. Έτσι, η αλληλούχηση του DNA εφαρμόζεται πολύ συχνά στις φυλογενετικές μελέτες, καθώς συνιστά μια λεπτομερή και αναλυτική μέθοδο για την εκτίμηση των γενετικών αποστάσεων γονιδιακών τμημάτων και γονιδίων.

Παρόλα αυτά, σε μία συγκεκριμένη φυλογενετική ανάλυση είναι δυνατόν να απαιτείται η ανάλυση πολλαπλών μοριακών δεικτών που παρέχουν συμπληρωματικές πληροφορίες.

1.4.1 Το μιτοχονδριακό DNA (mtDNA) στις φυλογενετικές μελέτες

Οι μοριακές φυλογενετικές μελέτες στηρίζονται συνήθως στη χρήση μιτοχονδριακών γονιδίων. Το μιτοχονδριακό DNA, ως μοριακός δείκτης, χρησιμοποιείται ευρύτατα στις φυλογενετικές μελέτες. Τα περισσότερα μιτοχονδριακά γονιδιώματα είναι κυκλικά, δίκλωνα, υπερελικωμένα μόρια DNA ενώ γραμμικά μόρια εντοπίζονται μόνο σε ορισμένα πρωτόζωα και μύκητες. Σε πολλές περιπτώσεις, το περιεχόμενο του mtDNA σε γουανίνες/κυτοσίνες (GC) διαφέρει σημαντικά από το πυρηνικό DNA και για αυτό είναι δυνατόν να διαχωριστεί από το πυρηνικό με φυγοκέντρηση σε διαβάθμιση πυκνότητας χλωριούχου καισίου (αναφορά). Το mtDNA δε συνδέεται με ιστόνες ή παρόμοιες πρωτεΐνες (Russell, 2009). Τα μιτοχονδριακά γονιδιώματα διαφορετικών ειδών παρουσιάζουν πολλές ομοιότητες ως προς τον αριθμό και τις λειτουργίες των γονιδίων

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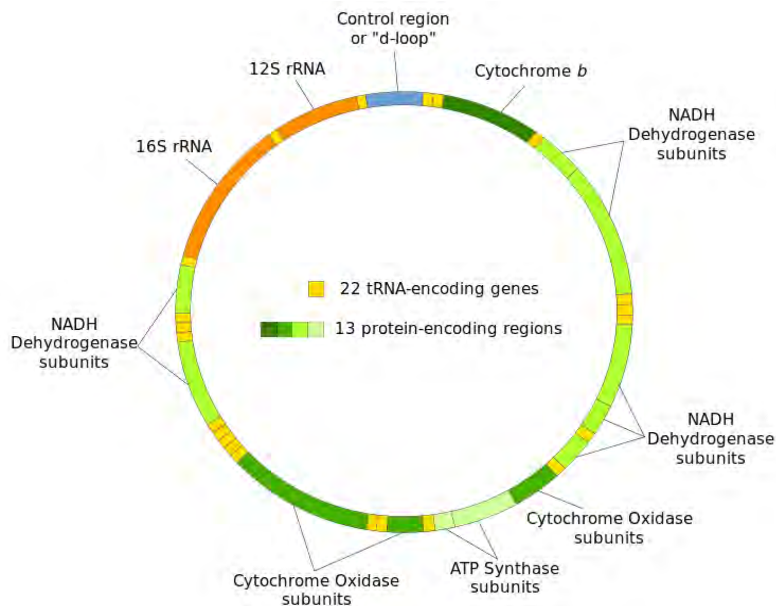
τους. Το μιτοχονδριακό DNA των ζώων (Εικόνα 1) είναι ένα μικρό, εξωχρωμοσωματικό γονιδίωμα, που έχει συνήθως μέγεθος της τάξης των 16-17 kb και περιλαμβάνειτα ίδια 37 γονίδια: 2 για ριβοσωμικά RNAs (rRNAs), 13 για πρωτεΐνες και 22 για μεταφορικά RNAs (tRNAs). Επιπλέον, υπάρχει μια μεγάλη μη κωδική περιοχή (D-loop), η οποία είναι γνωστό ότι περιέχει στοιχεία ελέγχου της αντιγραφής και της μεταγραφής (Krzywinski *et al.,* 2006). Δεν είναι ξεκάθαρο κατά πόσο αυτές οι περιοχές ελέγχου είναι ομόλογες μεταξύ απόμακρων ζωικών ειδών ή, εναλλακτικά, έχουν προκύψει ανεξάρτητα, από διαφορετικές μη κωδικές αλληλουχίες σε ξεχωριστές εξελικτικές γενεαλογίες, δεδομένου ότι δεν παρουσιάζουν νουκλεοτιδική ομολογία εκτός από τις περιπτώσεις που πρόκειται για στενά συγγενικά είδη (Boore, 1999).

Τα προϊόντα αυτών των γονιδίων, μαζί με μόρια RNA και πρωτεΐνες που εισάγονται από το κυτταρόπλασμα, παρέχουν στο μιτοχόνδριο το δικό του σύστημα αντιγραφής και μεταγραφής του DNA και μετάφρασης των πρωτεϊνών. Η μελέτη του mtDNA παρέχει πολύτιμες πληροφορίες για την γονιδιωματική εξέλιξη. Επιπλέον, η σύγκριση της οργάνωσης των μιτοχονδριακών γονιδίων είναι δυνατόν να οδηγήσει στην εξαγωγή αρχαίων εξελικτικών σχέσεων, καθώς η διάταξη των γονιδίων σε απόμακρες ταξινομικά ομάδες είναι μοναδική και έτσι είναι σχεδόν αδύνατο να συμβεί αυτό το γεγονός με τον ίδιο τρόπο σε ανεξάρτητες εξελικτικά γενεαλογίες (Boore, 1999).



Εικόνα 1: Μιτοχονδριακό DNA (mtDNA)

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Τα γονιδιώματα των οργανιδίων αντιγράφονται και κληρονομούνται με διαφορετικό τρόπο απ' ότι τα γονίδια του πυρήνα και ως εκ τούτου η δυναμική των νουκλεοτιδικών αντικαταστάσεων είναι πολύ διαφορετική. Ο μέσος ρυθμός συνώνυμων αντικαταστάσεων στα μιτοχονδριακά γονίδια των θηλαστικών είναι περίπου 5,7 $x10^{-8}$ ανά θέση ανά έτος, είναι δηλαδή περίπου δεκαπλάσιος από το μέσο ρυθμό συνώνυμων αντικαταστάσεων των γονιδίων του πυρήνα. Ο ρυθμός μη συνώνυμων αντικαταστάσεων ποικίλλει σημαντικά μεταξύ των γονιδίων του μιτοχονδρίου αλλά σε κάθε περίπτωση είναι σημαντικά υψηλότερος από το μέσο ρυθμό συνώνυμων αντικαταστάσεων που παρατηρείται στα πυρηνικά γονίδια. Αυτό σχετίζεται με την χαμηλότερη αξιοπιστία του μηχανισμού αντιγραφής του DNA, γεγονός που έχει ως συνέπεια τον υψηλότερο ρυθμό εισαγωγής σφαλμάτων κατά την αντιγραφή και την επιδιόρθωση του mtDNA. Σε αντίθεση με τις πολυμεράσες DNA του πυρήνα, οι πολυμεράσες DNA του μιτοχονδρίου δεν έχουν ικανότητα επιδιορθωτικού ελέγχου. Επίσης, οι υψηλότερες συγκεντρώσεις μεταλλαξιγόνων, όπως οι ελεύθερες ρίζες οξυγόνου, οι οποίες προκύπτουν από μεταβολικές διεργασίες που διεξάγονται στα μιτοχόνδρια, είναι δυνατόν να παίζουν ρόλο στους υψηλότερους ρυθμούς νουκλεοτιδικών αντικαταστάσεων. Επιπλέον, είναι πιθανόν, η πίεση της φυσικής επιλογής, που φυσιολογικά εξαλείφει πολλές μεταλλαγές στα γονίδια του πυρήνα, να είναι λιγότερο έντονη στα μιτοχόνδρια, επειδή τα περισσότερα κύτταρα περιέχουν πολλές δεκάδες μιτοχόνδρια καθένα από τα οποία περιέχει μέχρι και δώδεκα αντίγραφα του μιτοχονδριακού γονιδιώματος. Τέλος, οι μεταλλαγές στα γονίδια του μιτοχονδριακού γονιδιώματος που κωδικοποιούν πρωτεΐνες, tRNA και rRNA φαίνεται να μην επηρεάζουν τόσο αρνητικά την προσαρμοστικότητα του ατόμου όσο οι αντίστοιχες μεταλλαγές στα πυρηνικά γονίδια.

Παρότι είναι γνωστό ότι τα μιτοχονδριακά γονιδιώματα των ζώων εξελίσσονται ταχύτατα, η οργάνωση των γονιδίων φαίνεται να είναι απαράλλακτη για μεγάλες χρονικές περιόδους της εξελικτικής ιστορίας. Με ορισμένες εξαιρέσεις, η οργάνωση των γονιδίων είναι σχετικά σταθερή μέσα στις κύριες ομάδες, αλλά διαφέρει μεταξύ αυτών, και η σύγκριση αυτής της οργάνωσης παρέχει σημαντικές πληροφορίες για τις φυλογενετικές σχέσεις στους βαθύτερους κλάδους των μεταζώων. Ο μεγάλος αριθμός των πιθανών γονιδιακών διατάξεων καθιστά σχεδόν αδύνατο το να αποκτούν διαφορετικές ταξινομικές βαθμίδες το ίδιο μοντέλο οργάνωσης. Η σύγκριση της

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οργάνωσης των μιτοχονδριακών γονιδίων παρείχε πειστικές φυλογενετικές σχέσεις σε αρκετές περιπτώσεις κατά τις οποίες τα υπόλοιπα δεδομένα ήταν αμφίβολα, όπως π.χ. στις περιπτώσεις των μεγάλων ομάδων των εχινόδερμων και των αρθροπόδων.

Η ευκολία στον πολλαπλασιασμό, οι σχετικά γρήγοροι εξελικτικοί ρυθμοί, καθώς και η απουσία γενετικού ανασυνδυασμού, έκαναν το mtDNA αναπόσπαστο κομμάτι της συστηματικής και της γενετικής πληθυσμών (Avise, 1986, Awadalla *et al.,* 1999, Simon *et al.,* 2006). Επειδή όλο το μιτοχονδριακό DNA κληρονομείται ως μία μονάδα, ή απλότυπος, η σύγκριση του μιτοχονδριακού DNA από διαφορετικά άτομα μπορεί να παρουσιαστεί με τη μορφή φυλογενετικού δένδρου. Παρ’ όλα αυτά, το μιτοχονδριακό DNA αντανακλά μόνο την ιστορία των θηλυκών ατόμων του πληθυσμού και έτσι μπορεί να μην αντιπροσωπεύει την εξελικτική ιστορία όλου του πληθυσμού. Για αυτό το λόγο χρησιμοποιούνται συνήθως συμπληρωματικά και μελέτες του πυρηνικού DNA ή περιοχή του χρωμοσώματος *Y*, το οποίο διακρίνεται από πατρική κληρονόμηση (Garrigan & Hammer, 2006).

1.4.2 Πυρηνικοί γενετικοί τόποι

Παρόλα τα πλεονεκτήματα που παρουσιάζει το μιτοχονδριακό γονιδίωμα, ως πηγή μοριακών δεικτών για φυλογενετικές μελέτες, το γεγονός ότι κληρονομείται ως ενιαία μονάδα, έχει ως συνέπεια να μην είναι δυνατόν να θεωρηθούν τα γονίδια που περιλαμβάνει ως ανεξάρτητες πηγές φυλογενετικής πληροφορίας. Αντίθετα, το πυρηνικό γονιδίωμα περιλαμβάνει περιοχές που κωδικοποιούν πρωτεΐνες και RNA, ενώ φέρει και μη κωδικές περιοχές προσφέροντας έτσι μια πληθώρα ανεξάρτητων δεικτών, οι οποίοι εξελίσσονται με διαφορετικούς ρυθμούς, καθιστώντας το χρήσιμο εργαλείο για τη μελέτη των φυλογενετικών σχέσεων μεταξύ συγγενών ειδών (Dolman & Phillips, 2004, Willows-Munro *et al.,* 2005).

Οι πυρηνικοί γενετικοί τόποι που κωδικοποιούν πρωτεΐνες συνήθως δεν εμφανίζουν πολυμορφισμό μεγέθους ή, εάν εμφανίσουν, αυτός αντιστοιχεί σε αριθμό πολλαπλάσιο των τριών νουκλεοτιδίων, με αποτέλεσμα η ομοπαράθεση των αλληλουχιών να μην παρουσιάζει δυσκολίες. Ωστόσο, οι συγκεκριμένοι γενετικοί δείκτες είναι δυνατόν να δημιουργήσουν δυσκολίες κατά την επεξεργασία των αποτελεσμάτων, λόγω των ιντρονίων που περιέχουν (Downie & Gullan, 2004) και για αυτό το λόγο, σε

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ορισμένες φυλογενετικές μελέτες χρησιμοποιούνται μεμονωμένες περιοχές εξονίων. Αυτοί οι δείκτες αξιοποιούνται συνήθως σε φυλογενετικές μελέτες υψηλότερων ταξινομικών επιπέδων. Από την άλλη πλευρά, οι αλληλουχίες και η τοπολογία των ιντρονίων είναι δυνατόν να παρέχουν χρήσιμες πληροφορίες για την εξελικτική ιστορία και τη φυλογένεση μιας ομάδας ειδών. Έτσι, οι αλληλουχίες των ιντρονίων, οι οποίες εξελίσσονται ταχέως, χρησιμοποιούνται συχνά για φυλογενετικές μελέτες που αφορούν στενά σχετιζόμενα είδη ενώ η εξέλιξη της γονιδιακής οργάνωσης, μέσω απώλειας ή προσθήκης ιντρονίων, γεγονός που φαίνεται να συμβαίνει με πολύ αργούς ρυθμούς σε ορισμένες γενεαλογίες, χρησιμοποιείται συχνά για τη διερεύνηση βαθύτερων φυλογενετικών σχέσεων για τις οποίες από μόνη της η αλληλουχία, λόγω μεγάλων αποκλίσεων, δεν παρέχει αξιόπιστα συμπεράσματα (Irimia & Roy, 2008). Άλλοι πυρηνικοί γενετικοί τόποι που μπορούν να χρησιμοποιηθούν για μελέτες φυλογένεσης είναι οι μικροδορυφορικές αλληλουχίες. Πρόκειται για επαναλαμβανόμενες αλληλουχίες ενός έως πέντε νουκλεοτιδίων που υπάρχουν σε σημαντικό ποσοστό στο γονιδίωμα των ευκαρυωτικών οργανισμών. Εμφανίζουν υψηλά επίπεδα πολυμορφισμού, λόγω του υψηλού ρυθμού μεταλλάξεων. Η αξιοποίησή τους σε μελέτες πληθυσμιακής γενετικής συνέβαλαν στον καθορισμό της γενετικής δομής πολλών ειδών, συγκρίνοντας τις εξελικτικές σχέσεις και μελετώντας την πρόσφατη ιστορία σε επίπεδο πληθυσμών (Buburuzan *et al.,* 2007).

Τέλος, στις φυλογενετικές αναλύσεις χρησιμοποιούνται επίσης οι γενετικοί τόποι που κωδικοποιούν rRNA. Αυτοί οι γενετικοί τόποι βρίσκονται συγκεντρωμένοι σε γειτονικές περιοχές του γονιδιώματος και οργανώνονται σε μια ενιαία λειτουργική μονάδα. Κάθε τέτοια μονάδα περιλαμβάνει γονίδια, τα οποία κωδικοποιούν τη μεγάλη (28S) και τη μικρή (18S) υπομονάδα του ριβοσώματος, καθώς και το 5,8S rRNA, τα οποία διαχωρίζονται από μη κωδικές περιοχές (ETS, ITS1, ITS2). Το κύριο πλεονέκτημα που καθιστά το πυρηνικό ριβοσωμικό DNA κατάλληλο φυλογενετικό δείκτη, είναι το μεγάλο μέγεθος της αλληλουχίας που συνεπάγεται και μεγάλη ποσότητα φυλογενετικής πληροφορίας. Η επιλογή του κατάλληλου γενετικού δείκτη στην εκάστοτε φυλογενετική μελέτη εξαρτάται από τον αντίστοιχο εξελικτικό ρυθμό ενώ, κατά περίπτωση, οι εναλλακτικοί γενετικοί δείκτες μπορούν να χρησιμοποιηθούν και συνδυαστικά (Olsen & Woese, 1993).

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1.4.3 Το χρωμόσωμα *Y*

Το χρωμόσωμα *Y* είναι ένα από τα δύο φυλετικά χρωμοσώματα (*XY*), τα οποία διαθέτουν σε κάθε κύτταρο τα περισσότερα θηλαστικά και περιέχει μεταξύ άλλων το γονίδιο *SRY* (sex determination region- φυλο-καθοριστικός παράγοντας), το οποίο όταν είναι παρόν, ενεργοποιεί την ανάπτυξη των όρχεων και καθοδηγεί τη δημιουργία αρσενικών φαινοτύπων. Το *Y* χρωμόσωμα περιέχει και μερικά άλλα γονίδια που είναι απαραίτητα για την παραγωγή σπέρματος. Το χρωμόσωμα *Y* του ανθρώπου αποτελείται από 60 εκατομμύρια ζεύγη βάσεων. Το γεγονός ότι το DNA του χρωμοσώματος *Y* μεταβιβάζεται από πατέρα σε γιο, η ανάλυση του μπορεί να οδηγήσει σε διερεύνηση των πατρογραμμικών σειρών σε φυλογενετικές αναλύσεις.

Ο ανασυνδυασμός ανάμεσα στα χρωμοσώματα *X* και *Y* αποδείχτηκε επιζήμιος δεδομένου ότι κατέληγε σε αρσενικά χωρίς τα απαραίτητα γονίδια που προηγουμένως βρίσκονταν στο *Y* και σε θηλυκά με αχρείαστα ή ακόμη και βλαβερά γονίδια, που προηγουμένως βρισκόταν μόνο στο χρωμόσωμα *Y*. Ως αποτέλεσμα τα χρήσιμα γονίδια για τα αρσενικά συγκεντρώθηκαν κοντά στο γονίδιο *SRY* και ο ανασυνδυασμός καταστάλθηκε, προκειμένου να διατηρηθεί αυτή η ειδική περιοχή ανέπαφη (Graves, 2006).

Το χρωμόσωμα *Y* είναι εκτεθειμένο σε σχετικά υψηλούς ρυθμούς μεταλλαξιγένεσης σε σχέση με το υπόλοιπο πυρηνικό γονιδίωμα. Το χρωμόσωμα *Y* μεταβιβάζεται αποκλειστικά από το σπέρμα, το οποίο υποβάλλεται σε πολλαπλές κυτταρικές διαιρέσεις κατά τη διάρκεια της γαμετογένεσης. Κάθε κυτταρική διαίρεση αποτελεί μια επιπλέον ευκαιρία για συσσώρευση μεταλλάξεων. Επιπλέον, το σπέρμα αποθηκεύεται στο ιδιαίτερα όξινο περιβάλλον των όρχεων, το οποίο ενθαρρύνει περαιτέρω την μεταλλαξιγένεση. Οι δύο αυτοί παράγοντες δημιουργούν τις συνθήκες ώστε το χρωμόσωμα *Y* να διατρέχει ένα κίνδυνο μεταλλάξεων 4,8 φορές μεγαλύτερο από το υπόλοιπο γονιδίωμα (Graves, 2006).

Το πληθυσμιακό μέγεθος του χρωμοσώματος *Y* είναι περιορισμένο κληρονομικά στο 1/4 σε σχέση με αυτό των αυτοσωμάτων. Οι διπλοειδείς οργανισμοί περιέχουν δύο αντίγραφα των αυτοσωμικών χρωμοσωμάτων, ενώ μόνο ο μισός περίπου πληθυσμός περιέχει ένα χρωμόσωμα *Y*. Έτσι, το φαινόμενο της γενετικής παρέκκλισης είναι μια

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ιδιαίτερα έντονο στο χρωμόσωμα *Y*. Μέσω της τυχαίας διευθέτησης, ένα ενήλικο αρσενικό μπορεί να μη μεταβιβάσει ποτέ το χρωμόσωμα εάν αποκτήσει μόνο θηλυκούς απογόνους. Έτσι, αν και το αρσενικό μπορεί να έχει ένα καλά προσαρμοσμένο χρωμόσωμα *Y*, απαλλαγμένο από υπερβολικές μεταλλάξεις, μπορεί ποτέ να μην το περάσει στην επόμενη γενετική δεξαμενή (Graves, 2006).

Για όλους τους παραπάνω λόγου, το χρωμόσωμα *Y* αποτελεί έναν άκρως ενδιαφέροντα μοριακό δείκτη, ο οποίος θα μπορούσε να δώσει πολύτιμές πληροφορίες για την γενετική πληθυσμιακή δομή και την καταγωγή των σύγχρονων γονιδιακών δεξαμενών στα διάφορα είδη θηλαστικών, συμπεριλαμβανομένου και του ευρωπαϊκού λαγού.

1.5 Ο ευρωπαϊκός λαγός (*Lepus europaeus* Pallas, 1778)

1.5.1. Συστηματική κατάταξη του είδους *Lepus europaeus*

Βασίλειο:	Ζώα
Φύλο:	Χορδωτά
Υπο-φύλο:	Σπονδυλωτά
Κλάση:	Θηλαστικά
Τάξη:	Λαγόμορφα
Οικογένεια:	Leporidae
Γένος:	*Lepus*
Είδος:	*europaeus*

Από: http://www.ncbi.nlm.nih.gov/taxonomy/

1.5.2 Φυσική περιγραφή

Εικόνα 2: *Lepus europaeus*

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Ο ευρωπαϊκός λαγός έχει συνολικό μήκος που κυμαίνεται από 60 έως 75 cm και ζυγίζει από 3 έως 5 κιλά (Hall & Kelson, 1959, Peterson, 1966). Το χρώμα του είναι καστανόγκριζο έως γκρίζο καφέ, εκτός από την περιοχή της κοιλιάς που είναι γκρι-άσπρη. Το κεφάλι έχει καφέ αποχρώσεις με πιο ανοιχτόχρωμους κύκλους γύρω από τα μάτια. Έχει μακριά αυτιά, τα πίσω πόδια του είναι πιο μακριά από τα μπροστινά, ενώ τα πέλματά του καλύπτονται από παχύ τρίχωμα για να εμποδίζουν την ολίσθηση. Η ουρά, με μήκος συνήθως 7-11 cm, είναι μαύρη στο επάνω μέρος της και άσπρη στο κάτω. Η διάρκεια ζωής του φτάνει τα 7-8 χρόνια (Peterson, 1966, Bansfield, 1974). Το χειμώνα το τρίχωμα του ευρωπαϊκού λαγού γίνεται ελαφρώς πιο γκρίζο. Επίσης, δεν έχει παρατηρηθεί αξιοσημείωτος διμορφισμός ανάμεσα στα δύο φύλα. Η μοναδική διαφορά που έχει παρατηρηθεί είναι ότι τα αρσενικά έχουν συνήθως πιο κοντό και παχύ λαιμό σε σχέση με τα θηλυκά άτομα (Bansfield, 1974, Dragg, 1974).

1.5.3. Συμπεριφορά

Ο ευρωπαϊκός λαγός θεωρείται νυκτόβιο ζώο. Γενικά προτιμά τη μοναχική ζωή αν και έχουν σημειωθεί εμφανίσεις του σε μικρές ομάδες, κυρίως την περίοδο του ζευγαρώματος. Χαρακτηριστικό του γνώρισμα είναι η μεγάλη ταχύτητα που αναπτύσσει, η οποία μπορεί να φτάσει τα 70 km/h. Έχει διαμορφωμένα πόδια έτσι ώστε να πηδά, να περπατά και να τρέχει. Διαθέτει ιδιαίτερα ανεπτυγμένη την αίσθηση της όρασης, όσφρησης και ακοής. Όταν ακούσει θόρυβο στέκεται στα πίσω πόδια με σηκωμένα τα αυτιά του (Hamilton & Whitaker, 1943).

1.5.4. Γεωγραφική εξάπλωση

Ο ευρωπαϊκός λαγός συναντάται στο μεγαλύτερο τμήμα της Ευρώπης και ανατολικά της Μ. Ασίας έως την Κεντρική Ασία (Εικ. 3), (Lincoln, 1974, Broekhuizen & Maaskamp, 1980, Caillol & Meunier, 1989, Poli *et al.,* 1991). Επίσης, έχει εισαχθεί στην Αμερική, Αυστραλία και Νέα Ζηλανδία (Lincoln, 1974, Caillol & Meunier, 1989, Bonino & Montenegro, 1997).

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Εικόνα 3: Παγκόσμια κατανομή του *Lepus europaeus* (από: Flux & Angermann, 1990).

1.5.5. Βιότοπος

Ο ευρωπαϊκός λαγός ζει σε πεδιάδες και σε βουνά μέχρι το υψόμετρο των 1800 m. Συνήθως δραστηριοποιείται σε ανοιχτές περιοχές, οριοθετημένες από δασικές εκτάσεις ενώ αποφεύγει τα μέρη με πολλή υγρασία. Ο λαγός έχει ανάγκη από μια ζωτική περιοχή 300 στρεμμάτων, στην οποία υπάρχουν αρκετές διαδρομές διαφυγής. Η φωλιά του, την οποία αναζητά σε φυσικά προστατευμένα σημεία χωρίς να την διαμορφώνει ο ίδιος όπως π.χ. το συγγενικό του κουνέλι, βρίσκεται σε κοιλώματα κάτω από ψηλά χόρτα ή θάμνους, καλά καμουφλαρισμένη (Peterson 1966, Bansfield 1974).

1.5.6. Διατροφικές συνήθειες

Την ημέρα, ο ευρωπαϊκός λαγός κρύβεται ενώ τις νυχτερινές ώρες αφήνει τη φωλιά για να αναζητήσει την τροφή του. Αυτή αποτελείται από μεγάλη ποικιλία και εξαρτάται από τις εποχές του έτους. Γενικά, τρέφεται με κάθε είδους φυτά, χόρτα, σιτηρά, λαχανικά, φύλλα θάμνων και μικρά κλαδιά. Επίσης, χαρακτηριστικό του είναι η κοπροφαγία, καθώς συχνά καταναλώνει μαλακές κουτσουλιές πουλιών που είναι πλούσιες σε βιταμίνες, αμινοξέα και βακτήρια τα οποία του είναι απαραίτητα (Hamilton & Whitaker, 1943, Bansfield, 1974).

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1.5.7. Αναπαραγωγή

Αν και ο ευρωπαϊκός λαγός είναι γενικά μοναχικό ζώο, κοινωνικοποιείται ιδιαίτερα κατά την περίοδο του ζευγαρώματος, κατά τη διάρκεια της οποίας επιδίδεται σε θεαματικά άλματα και μάχες διεκδικώντας το ταίρι του. Μέχρι πρόσφατα, αυτές οι μάχες, κατά τις οποίες ο ένας λαγός χτυπούσε τον αντίπαλο με τις πατούσες του, θεωρούνταν μια μορφή ανταγωνισμού μεταξύ των αρσενικών. Ωστόσο, μια πιο λεπτομερής παρακολούθηση έδειξε ότι πολλές φορές επρόκειτο για θηλυκά άτομα που χτυπούσαν αρσενικά είτε για να δείξουν πως δεν ήταν ακόμα έτοιμα για ζευγάρωμα είτε για να δοκιμάσουν την αποφασιστικότητα των αρσενικών. Η αναπαραγωγική περίοδος του λαγού είναι συνήθως εν μέσω χειμώνα και εν μέσω καλοκαιριού. Το θηλυκό γεννά τρεις ως τέσσερις φορές το χρόνο, έπειτα από κυοφορία 30-40 ημερών. Η τελευταία κυοφορία συνήθως είναι στο τέλος του καλοκαιριού (Peterson, 1966, Bansfield, 1974, Bonino & Montenegro, 1997).

1.5.8. Κίνδυνοι και απειλές

Ο ευρωπαϊκός λαγός αποτελεί θήραμα πολλών αρπακτικών και για αυτό το λόγο θεωρείται αρκετά σημαντικός οικολογικός παράγοντας σταθερότητας του οικοσυστήματος. Τον κυνηγούν σχεδόν όλα τα σαρκοφάγα, όπως ο λύκος, ο σκύλος, το κουνάβι, η αλεπού και ο άνθρωπος αλλά και τα αρπακτικά πτηνά, όπως οι αετοί και τα γεράκια (Peterson, 1966, Bansfield, 1974). Ο λαγός αμύνεται τρέχοντας, χρησιμοποιώντας ταυτόχρονα ταχύτατους ελιγμούς για να διαφύγει ή μένοντας κρυμμένος στο έδαφος.

Ο πληθυσμός του παρουσιάζει έντονες και ακανόνιστες αυξομειώσεις που μπορεί να οφείλονται σε κλιματικούς παράγοντες, αλλά και σε παράγοντες όπως ο ανταγωνισμός για την εξεύρεση τροφής, η θήρευση και διάφορες ασθένειες. Οι παραπάνω λόγοι έχουν ως αποτέλεσμα αφ’ ενός τη μείωση του ρυθμού της αναπαραγωγής και αφ’ ετέρου την ελάττωση της αντοχής και της προσαρμοστικότητας τους σε ασθένειες (Smith & Johnston, 2008).

1.5.9. Οικονομική σημασία για τον άνθρωπο

Ο ευρωπαϊκός λαγός, λόγω της εξαιρετικής ποιότητας του κρέατός του, αποτελεί

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θηρεύσιμο είδος στην Ευρώπη και στην Αμερική (Bansfield, 1974). Ωστόσο, σε πολλές περιοχές, όπως η Αργεντινή και η Αυστραλία, ο ευρωπαϊκός λαγός, λόγω της γρήγορης αναπαραγωγής του, προκαλεί εκτεταμένες καταστροφές στις γεωργικές καλλιέργειες στην προσπάθειά του να τραφεί (Smith & Johnston, 2008).

1.5.10. Ερευνητική σημασία

Ο ευρωπαϊκός λαγός αποτελεί ένα κυρίαρχο στοιχείο της πανίδας των ανοικτών εκτάσεων με ιδιαίτερη οικονομική σημασία, διότι αποτελεί ένα από τα πιο δημοφιλή θηράματα στην Ευρώπη και ιδιαίτερα στη Βαλκανική χερσόνησο και την Ελλάδα. Η εκτεταμένη κατανομή του και το γεγονός ότι ανταποκρίνεται ταχύτατα στις αλλαγές ενδιαιτημάτων του το καθιστούν ένα είδος-μοντέλο μέσω του οποίου μπορούμε να εξάγουμε χρήσιμα συμπεράσματα για τη συμπεριφορά, τη βιολογία και την πληθυσμιακή δομή των άγριων πληθυσμών πολλών θηλαστικών.

1.6 Μοριακές πληθυσμιακές μελέτες του *Lepus europaeus* στην Ευρώπη

Η γνώση της γενετικής ποικιλότητας του ευρωπαϊκού λαγού αποτελεί ένα πολύ σημαντικό εργαλείο για την κατανόηση της πληθυσμιακής δομής και δυναμικής αυτού του είδους υπό διαφορετικές περιβαλλοντικές συνθήκες. Παρόλο που το είδος *L. europaeus* είναι αρκετά διαδεδομένο στην Ευρώπη, οι μελέτες που σχετίζονται με την πληθυσμιακή δομή τόσο αυτού του είδους, όσο και άλλων ειδών της οικογένειας *Leporidae* και βασίζονται σε μοριακούς δείκτες, είναι περιορισμένες. Στη συνέχεια αναλύονται οι μοριακές μελέτες, τόσο σε επίπεδο πρωτεϊνών όσο και σε επίπεδο DNA, που αφορούν τον ευρωπαϊκό λαγό και είχαν δημοσιευθεί μέχρι την έναρξη της συγκεκριμένης διατριβής.

1.6.1 Πληθυσμιακές μελέτες σε επίπεδο πρωτεϊνών

Οι μελέτες των Suchentrunk *et al*., (1998, 2001), σε επίπεδο αλλοενζύμων, σε πληθυσμούς της Αγγλίας και της Νέας Ζηλανδίας, έδειξαν χαμηλότερα επίπεδα ετεροζυγωτίας και γενετικής ποικιλότητας σε σχέση με τα αντίστοιχα επίπεδα σε πληθυσμούς της Αυστρίας, που παρουσιάστηκαν στις μελέτες των Hartl *et al*., (1993,

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1995). Οι πληθυσμοί της Νέας Ζηλανδίας παρουσίασαν μικρή μείωση γενετικής ποικιλότητας σε σχέση με τους πληθυσμούς της Αγγλίας, από τους οποίους θεωρείται ότι προέρχονται. Επίσης, από τα δεδομένα προέκυψε ότι οι πληθυσμοί της Αγγλίας προήλθαν από τους πληθυσμούς της κεντρικής Ευρώπης και εμφανίζουν χαμηλότερα επίπεδα γενετικής ποικιλότητας σε σύγκριση με τους πληθυσμούς της Αυστρίας. Τα αποτελέσματα ενίσχυσαν την υπόθεση περί εισαγωγής λαγών στα Βρετανικά νησιά από τους Ρωμαίους (Arnold, 1993) ή, εναλλακτικά, της εισόδου λαγών στην Αγγλία μέσω της φυσικής γέφυρας που ένωνε τα Βρετανικά νησιά με τη σημερινή ηπειρωτική Ευρώπη, πριν από περίπου 8.000 χρόνια (Suchentrunk *et al*., 1998).

Αυξημένη διαφοροποίηση σε επίπεδο αλλοενζύμων αποκαλύφθηκε και από τη μελέτη των Suchentrunk *et al*., (2000) σε πληθυσμούς του ευρωπαϊκού λαγού της Βουλγαρίας, σε σύγκριση με πληθυσμούς της κεντρικής Ευρώπης. Τα αποτελέσματα, έδειξαν ότι οι γενετικές δεξαμενές της κεντρικής Ευρώπης προήλθαν από περιοχές της νοτιοανατολικής Ευρώπης και ότι η κατανομή της γενετικής ποικιλομορφίας στις περιοχές αυτές δεν επηρεάζεται σε σημαντικό βαθμό από τις μεγάλες γεωγραφικές αποστάσεις, λόγω της πιθανής ύπαρξης ενός παμμικτικού δικτύου τοπικών πληθυσμών. Υπάρχει, ωστόσο, τάση για διαφοροποίηση των τοπικών γενετικών δεξαμενών των πληθυσμών της νοτιοανατολικής (Βουλγαρία) και κεντρικής (Αυστρία) Ευρώπης. Το γεγονός αυτό μπορεί εν μέρει να οφείλεται σε γονιδιακή ροή από άλλες περιοχές της νοτιοανατολικής Ευρώπης προς την κεντρική Ευρώπη.

Η μελέτη των Alves *et al*., (2000) με βάση πρωτεΐνες του πλάσματος, αποκάλυψε υψηλότερο επίπεδο γενετικής ποικιλότητας στον ιβηρικό λαγό (*Lepus granatensis*), σε σχέση με τον ευρωπαϊκό λαγό σε περιοχές της Γαλλίας και της Αυστρίας. Η διαφορά αυτή μπορεί να οφείλεται στο γεγονός ότι οι πληθυσμοί του ευρωπαϊκού λαγού, που αναλύθηκαν, προέρχονταν από πληθυσμούς της Γαλλίας και της Αυστρίας, που βρίσκονταν σε κατάσταση αιχμαλωσίας. Επίσης, πληθυσμιακές μελέτες στις περιοχές επαφής των δύο ειδών (*L. granatensis* και *L. europaeus*) στην Ιβηρική χερσόνησο, αποκάλυψαν την ύπαρξη ισχυρής γενετικής απομόνωσης των ειδών αυτών χωρίς να αποκλείουν την πιθανότητα γονιδιακής ροής σε μικρό ποσοστό (Bonhomme *et al*., 1986, Alves and Ferrand, 1999).

Οι Suchentrunk *et al*., (2003) μελέτησαν την ποικιλότητα, στο επίπεδο των

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αλλοενζύμων, 91 *L. europaeus* από επτά περιοχές της Ελλάδας. Τα αποτελέσματα συγκρίθηκαν με αντίστοιχα αποτελέσματα από πληθυσμούς της Βουλγαρίας, με σκοπό να εξεταστεί η υπόθεση της ύπαρξης συγκεκριμένων αλληλόμορφων στην Ελλάδα, τα οποία, πιθανόν, να προήλθαν από έναν απομονωμένο πληθυσμό στη νότια Βαλκανική χερσόνησο κατά τη διάρκεια της τελευταίας περιόδου των Παγετώνων. Αυτή η υπόθεση ενισχύεται και από την αυξημένη γενετική διαφοροποίηση στο επίπεδο του mtDNA σε ελληνικούς πληθυσμούς λαγού (Mamuris *et al.,* 2001). Η ανάλυση 35 γενετικών τόπων αποκάλυψε τρία αλληλόμορφα σε χαμηλή συχνότητα, τα οποία δεν βρέθηκαν σε κανένα πληθυσμό από τη Βουλγαρία ή άλλη ευρωπαϊκή χώρα. Αντιθέτως, ορισμένα αλληλόμορφα που εντοπίστηκαν στη Βουλγαρία και σε περιοχές της Ευρώπης, απουσίαζαν από τους ελληνικούς πληθυσμούς. Επίσης, από την ανάλυση των αποτελεσμάτων προέκυψε μεγαλύτερη γενετική διαφοροποίηση στους ελληνικούς πληθυσμούς, καθώς και μία μικρή γονιδιακή ροή μεταξύ των πληθυσμών. Τα αποτελέσματα συμφωνούν με την αρχική υπόθεση περί ύπαρξης καταφυγίου στη νότια Βαλκανική, την περίοδο του Ανώτερου Πλειστόκαινου, καθώς βρέθηκαν συγκεκριμένα αλληλόμορφα στις περιοχές αυτές, με μικρή, όμως, επίδραση στη γενετική διαφοροποίηση των πληθυσμών λαγού της Ελλάδας και της Βουλγαρίας.

Η γενετική ποικιλότητα των ευρωπαϊκών λαγών της Ανατολής και η σχέση τους με τους ευρωπαϊκούς πληθυσμούς σε εξελικτικό επίπεδο, μελετήθηκαν από τους Sert *et al.,* (2005) με ανάλυση ισοενζύμων. Από τα αποτελέσματα παρατηρήθηκε αυξημένη γενετική διαφοροποίηση στους λαγούς της Ανατολής, σε σχέση με τη μέση διαφοροποίηση των πληθυσμών της νότιας και νοτιοανατολικής Βαλκανικής και τη χαμηλή διαφοροποίηση των πληθυσμών της κεντρικής Ευρώπης. Η αυξημένη αυτή γενετική διαφοροποίηση ενδέχεται να είναι αποτέλεσμα της γεωγραφικής θέσης της περιοχής αυτής καθώς και της αυξημένης γονιδιακής ροής που δέχεται από γειτονικές περιοχές, αλλά και της μακροχρόνιας παρουσίας πληθυσμών λαγού κατά τη διάρκεια της τελευταίας περιόδου των Παγετώνων, όταν βορειότερες περιοχές, την ίδια περίοδο, δεν παρείχαν κατάλληλα ενδιαιτήματα για τους πληθυσμούς του λαγού.

1.6.2 Πληθυσμιακές μελέτες με χρήση δεικτών μιτοχονδριακού DNA

Ο προσδιορισμός της αλληλουχίας ολόκληρου του μιτοχονδριακού γονιδιώματος του

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ευρωπαϊκού λαγού (17734 bp) πραγματοποιήθηκε από τους Arnason *et*. *al*., (2002), στο πλαίσιο της μελέτης των φυλογενετικών σχέσεων 60 ειδών θηλαστικών διαφόρων ταξινομικών επιπέδων.

Η μελέτη των Thulin *et al*., (1997) σε πληθυσμούς *L. europaeus* και *L. timidus* της Σουηδίας βασίστηκε στην ανάλυση του mtDNA με τη μέθοδο RFLP. Στους πληθυσμούς του *L. europaeus*, ανιχνεύθηκε χαμηλή απλοτυπική διαφοροποίηση, το οποίο οφείλεται στο ότι οι πληθυσμοί του *L. europaeus* στις Σκανδιναβικές χώρες εισήχθηκαν από περιοχές της κεντρικής και βόρειας Ευρώπης, στις οποίες παρατηρείται χαμηλό επίπεδο γενετικής ποικιλότητας. Το συμπέρασμα αυτό ενισχύεται και από την έρευνα των Hartl *et al.*, (1993), στην οποία βρέθηκε χαμηλή διαφοροποίηση, σε επίπεδο mtDNA, σε πληθυσμούς *L. europaeus* της Αυστρίας. Επίσης τα αποτελέσματα της έρευνας των Thulin *et al.*, (1997) ανέδειξαν τον υβριδισμό φυσικών πληθυσμών των ειδών *L. europaeus* και *L. timidus*, γεγονός που ήδη είχε παρατηρηθεί σε κατάσταση αιχμαλωσίας.

Υψηλός βαθμός ενδοειδικής διαφοροποίησης του mtDNA στο είδος *L. europaeus* παρατηρήθηκε στην Ιβηρική χερσόνησο, από τους Pérez-Suárez *et al*., (1994), το οποίο αποτελεί ένδειξη υβριδισμού του *L. europaeus* με τα είδη *L. castroviejoi* και *L. granatensis*, που συνυπάρχουν στην περιοχή.

Οι Fickel *et al.,* (2008), αναλύοντας αλληλουχίες της περιοχής ελέγχου (control region, CR) του mtDNA, σε πληθυσμούς ευρωπαϊκού λαγού από επτά ευρωπαϊκές χώρες, απέδειξαν ότι η Ιταλική χερσόνησος αποτέλεσε ένα επιπλέον καταφύγιο για τους λαγούς, εκτός από τη Βαλκανική και τη Μικρά Ασία, κατά την τελευταία Περίοδο των Παγετώνων, από τις οποίες προέκυψε η επανεποίκιση των χωρών της κεντρικής Ευρώπης.

Οι Kasapidis *et al*., (2005) μελέτησαν τη φυλογεωγραφική κατάσταση του ευρωπαϊκού λαγού, αναλύοντας αλληλουχίες της περιοχής ελέγχου του mtDNA από 98 λαγούς από την ηπειρωτική και νησιωτική Ελλάδα, τη Βουλγαρία, την Κύπρο και το βόρειο Ισραήλ καθώς και 44 δημοσιευμένες αλληλουχίες που προέρχονταν από λαγούς της κεντρικής Ευρώπης και της Ιταλίας. Τα αποτελέσματα της μελέτης υποδεικνύουν δύο διακριτούς τύπους μιτοχονδριακού DNA, έναν «ανατολικό τύπο» και ένα «δυτικό τύπο», με μέση νουκλεοτιδική διαφοροποίηση 6,6%. Ο «ανατολικός τύπος» περιλαμβάνει

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απλοτύπους από τα νησιά του Ανατολικού Αιγαίου, την Κύπρο, το βόρειο Ισραήλ, τη Θράκη και την ανατολική Μακεδονία. Στον «δυτικό τύπο» περιλαμβάνονται οι απλότυποι από την Κρήτη, τη Λευκάδα, τη Νάξο, τα Κύθηρα και την ηπειρωτική Ελλάδα καθώς και οι απλότυποι της Αυστρίας, της Γερμανίας, της Ουγγαρίας, της Σερβίας, της Ρουμανίας, της Βουλγαρίας και της Ιταλίας,. Μία ζώνη επικάλυψης των δύο τύπων μιτοχονδριακού DNA εντοπίζεται στην περιοχή της Θράκης και της Βουλγαρίας.

Οι Mamuris *et al.,* (2001) μελέτησαν τη γενετική διαφοροποίηση και τη φυλογενετική κατάσταση πληθυσμών του *L. europaeus* της κεντρικής Ελλάδας, καθώς και την επίδραση των απελευθερώσεων εκτρεφόμενων ατόμων στη γενετική δομή των φυσικών πληθυσμών. Για αυτό το σκοπό αναλύθηκαν τμήματα του mtDNA με τη μέθοδο RFLP-PCR. Η ανάλυση των δεδομένων αποκάλυψε εκτεταμένη απλοτυπική ποικιλότητα εντός και μεταξύ των φυσικών πληθυσμών (42 από τους 56 απλότυπους ήταν μοναδικοί). Η απλοτυπική ποικιλότητα ήταν εξ ίσου κατανεμημένη εντός και μεταξύ των γεωγραφικών περιοχών, ενώ η ετερογένεια των απλοτυπικών συχνοτήτων, υπέδειξε σημαντική γενετική διαφοροποίηση μεταξύ των περιοχών δειγματοληψίας. Επιπλέον, προσδιορίστηκαν συγκεκριμένα πρότυπα mtDNA, τα οποία διαφοροποιούσαν εντελώς τους εκτρεφόμενους από τους φυσικούς πληθυσμούς και αποδείκνυαν, σε μεγάλο βαθμό, την ύπαρξη εκτρεφόμενων λαγών εντός των φυσικών πληθυσμών, πιθανόν από παλαιότερες απελευθερώσεις. Επίσης, η ανάλυση του mtDNA απέδειξε τη διείσδυση αλλόχθονων γονιδιακών αποθεμάτων στους φυσικούς πληθυσμούς. Έτσι, για να διατηρηθούν οι γηγενείς γονότυποι και να προληφθεί η μείωση της γενετικής ποικιλότητας προτάθηκε να σταματήσουν τα προγράμματα εμπλουτισμού και να αναπτυχθούν κατάλληλα προγράμματα διαχείρισης, προσαρμοσμένα στη δυναμική των τοπικών πληθυσμών.

1.6.3 Πληθυσμιακές μελέτες με χρήση δεικτών του πυρηνικού γονιδιώματος

Οι Fickel *et al.,* (1999) χρησιμοποίησαν τέσσερις μικροδορυφορικούς γενετικούς τόπους καθώς και την περιοχή ελέγχου του μιτοχονδριακού DNA για να μελετήσουν τη γενετική δομή και ποικιλότητα δύο πληθυσμών του *L. europaeus* στην περιοχή της Βεστφαλίας της Γερμανίας. Από την κατανομή των αλληλομόρφων των μικροδορυφορικών τόπων δεν προέκυψε καμία διαφοροποίηση μεταξύ των πληθυσμών. Αντιθέτως, από την

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κατανομή των απλοτύπων του mtDNA, οι δύο πληθυσμοί εμφανίζονται να είναι γενετικά δομημένοι και σημαντικά διαφοροποιημένοι μεταξύ τους, αποδεικνύοντας ότι η μητρική γονιδιακή ροή είναι πολύ περιορισμένη. Εφόσον τα θηλυκά άτομα, βάσει της απλοτυπικής κατανομής, είναι αποκλεισμένα από τη γονιδιακή ροή, συμπέραναν ότι μόνο τα αρσενικά άτομα είναι υπεύθυνα για την ανταλλαγή αλληλομόρφων μικροδορυφορικών τόπων μεταξύ των δύο πληθυσμών. Το γεγονός αυτό οφείλεται στο ότι, ενώ το mtDNA χαρακτηρίζεται από μητρική κληρονομικότητα, το μικροδορυφορικό DNA, κληρονομείται τόσο μητρικά όσο και πατρικά σε ίσες αναλογίες. Τα αποτελέσματα αυτά μπορεί να δικαιολογηθούν από τη φιλοπατρική συμπεριφορά των θηλυκών ατόμων αλλά και από τον υψηλότερο βαθμό διασποράς και χωροκράτειας (home range) των αρσενικών ατόμων.

Οι Putze *et al.,* (2007) χρησιμοποίησαν δέκα νέους μοριακούς δείκτες στο χρωμόσωμα Y του ευρωπαϊκού λαγού, οι οποίοι είναι κατάλληλοι για τον προσδιορισμό του φύλου στα άτομα ενός πληθυσμού, καθώς και για τη διερεύνηση της γενετικής δομής και της γονιδιακής ροής στους πληθυσμούς του ευρωπαϊκού λαγού, στο επίπεδο του πυρηνικού DNA και κυρίως, σε γονίδια που κληρονομούνται πατρικά. 1.8 Μοριακές πληθυσμιακές μελέτες του *L. europaeus* στην Ελλάδα

Η μέθοδος RAPD χρησιμοποιήθηκε από τους Mamuris *et al.,* (2002), για να εκτιμηθεί η γενετική διαφοροποίηση πληθυσμών του ευρωπαϊκού λαγού από την κεντρική Ελλάδα. Οι ελληνικοί φυσικοί πληθυσμοί συγκρίθηκαν με δείγματα από την Αυστρία, την Πολωνία, τη Γερμανία, τη Γαλλία και τη Βουλγαρία, καθώς επίσης και με τους εκτρεφόμενους/απελευθερωμένους λαγούς, για να διερευνηθεί η επίδραση των απελευθερώσεων στη γενετική σύνθεση των γηγενών πληθυσμών. Η απουσία διαγνωστικών ζωνών, που θα μπορούσαν να διαχωρίσουν πληθυσμούς λαγού, επιβεβαίωσε την ύπαρξη υψηλής πυρηνικής γονιδιακής ροής μεταξύ, αρκετά απομακρυσμένων, πληθυσμών λαγού. Τα φυλογενετικά δέντρα, που προέκυψαν από τις γενετικές αποστάσεις, οι οποίες υπολογίστηκαν από τις συχνότητες των ζωνών RAPD, υπέδειξαν ένα κύριο σημείο διαχωρισμού της γενεαλογίας του πυρηνικού DNA. Τα εκτρεφόμενα άτομα ομαδοποιήθηκαν με τους πληθυσμούς της Πολωνίας, της Αυστρίας, της Γερμανίας και της Γαλλίας, ενώ οι πληθυσμοί της Ελλάδας διαφοροποιήθηκαν και ομαδοποιήθηκαν με τον πληθυσμό της Βουλγαρίας. Η διαφοροποίηση των φυσικών

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πληθυσμών στην Ελλάδα δεν ακολούθησε κάποια γεωγραφική τάση, δεδομένου ότι η γενετική απόκλισή τους δεν φάνηκε να συσχετίζεται με τις γεωγραφικές αποστάσεις. Ωστόσο, ορισμένα πρότυπα RAPD εκτρεφόμενων και άγριων λαγών ήταν πολύ διαφορετικά από το κοινό πρότυπο που βρέθηκε στη συντριπτική πλειοψηφία των λαγών που εξετάστηκαν, γεγονός που αντανακλά, ενδεχομένως, την ανάμιξη γενετικά διαφοροποιημένων ατόμων. Η ανάλυση RAPD έδειξε ότι οι απελευθερώσεις είχαν ήδη αρχίσει να επηρεάζουν τη γενετική δομή των ελληνικών πληθυσμών και ενίσχυσαν την άποψη ότι ήταν απαραίτητη μια κατάλληλη διαχείριση, προσαρμοσμένη στη βιολογία και την οικολογία των τοπικών πληθυσμών.

1.7 Σκοπός της Διδακτορικής Διατριβής

Στην εργασία αυτή πραγματοποιήθηκε πληθυσμιακή ανάλυση ενός μεγάλου αριθμού δειγμάτων ευρωπαϊκού λαγού σε ένα εκτεταμένο δίκτυο δειγματοληψίας, καλύπτοντας, συνολικά, 33 περιοχές της Ελλάδας, διαφόρων ευρωπαϊκών χωρών, της Τουρκίας και του Ισραήλ. Η μελέτη στηρίχθηκε στην ανάλυση μοριακών δεικτών του μιτοχονδριακού DNA και δεικτών του πυρηνικού DNA (*Y* χρωμόσωμα), χρησιμοποιώντας τεχνικές όπως PCR-RFLP, PCR-SSCP και αλληλούχηση διαφόρων περιοχών DNA.
Οι βασικοί στόχοι της μελέτης αποτυπώνονται ως εξής:

- Μελέτη της γενετικής δομής των φυσικών πληθυσμών του λαγού και της γονιδιακής ροής μεταξύ των περιοχών δειγματοληψίας
- Έλεγχος της υπόθεσης της μετανάστευσης του ευρωπαϊκού λαγού προς την Κεντρική Ευρώπη από καταφύγια της Βαλκανικής χερσονήσου καθώς και της Μικράς Ασίας, κατά την τελευταία Περίοδο των Παγετώνων
- Μελέτη της γενετικής δομής των εκτρεφόμενων πληθυσμών του λαγού, εκτίμηση της γονιδιακής ροής ανάμεσα σε εκτρεφόμενους και φυσικούς πληθυσμούς
- Εύρεση μοριακών δεικτών σε επίπεδο μιτοχονδριακού DNA, για την ταυτοποίηση των εκτρεφόμενων και των φυσικών πληθυσμών.

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2. ΑΠΟΤΕΛΕΣΜΑΤΑ

2.1 Δημοσιευμένες εργασίες

Τα αποτελέσματα που προέκυψαν από την παρούσα διατριβή αποτέλεσαν το υλικό τεσσάρων δημοσιεύσεων σε διεθνή επιστημονικά περιοδικά, ενώ πρόδρομα αποτελέσματα της διδακτορικής διατριβής δημοσιεύτηκαν σε άλλες τρεις εργασίες σε διεθνή περιοδικά. Οι εργασίες οι οποίες δημοσιεύθηκαν (και επισυνάπτονται στη συνέχεια) είναι οι εξής:

- **Stamatis C**., Suchentrunk F., Sert H., Triantaphyllidis C., Mamuris Z. (**2007**). Genetic evidence for survival of released captive-bred brown hares (*Lepus europaeus*) during restocking operations in Greece. ***Oryx*** 41(4): 548-551.

- **Stamatis C**., Giannouli S., Suchentrunk F., Sert H., Stathopoulos C., Mamuris Z. (**2008**). Recruitment of mitochondrial tRNA genes as auxiliary molecular markers for linked functional genomic and genetic analyses: The paradigm of brown hare (*Lepus europaeus*). ***Gene*** 410: 154-164.

- **Stamatis C**., Suchentrunk F., Moutou K.A., Giacometti M., Haerer G., Djan M., Vapa L., Vucovic M., Tvrtkovic N., Sert H., Alves P., Mamuris Z. (**2009**). Phylogeography of the Brown hare, *Lepus europaeus*, in Europe: legacy of southeastern Mediterranean refugia? ***Journal of Biogeography*** 36: 515-528.

- Mamuris Z., Moutou K.A., **Stamatis C**., Sarafidou T., Suchentrunk F. (**2010**). Y DNA and Mitochondrial lineages in European and Asian populations of the brown hare (*Lepus europaeus*). ***Mammalian Biology*** 75: 233-242.

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Πρόδρομα αποτελέσματα

- Mamuris Z., Sfougaris A. I., **Stamatis C**. (2001). Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA RFLP-PCR analysis: implications for conserving genetic diversity. ***Biological Conservation*** 101: 187-196.

- Mamuris Z., Sfougaris A. I., **Stamatis C**., Shuchentrunk F. (2002). Assessment of genetic structure of Greek brown hare (*Lepus europaeus*) populations based on variation in Random Amplified Polymorphic DNA (RAPD). ***Biochemical Genetics*** 40: 323-338.

- Shuchentrunk F., Mamuris Z., Sfougaris A. I., **Stamatis C**. (2003). Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. ***Biochemical Genetics*** 41: 127-140.

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Short Communication

Genetic evidence for survival of released captive-bred brown hares *Lepus europaeus* during restocking operations in Greece

Costas Stamatis, Franz Suchentrunk, Hakan Sert, Costas Triantaphyllidis and Zissis Mamuris

Abstract To prevent the decline of the brown hare *Lepus europaeus*, especially as a result of hunting pressure, restocking programmes, using hares from elsewhere, were carried out in Greece up to 2001. Using diagnostic RFLP mitochondrial DNA markers we traced the origins of released captive-bred brown hares. We provide evidence that released animals survived long enough to have at least one reproductive cycle and to transmit their genome. If, in the long-term, introgressed foreign genes survive, forming new genotypes with indigenous genes, this would demonstrate that they are successful in terms of competition. As nuclear gene pools are not markedly divergent between Greek and other European hares foreign nuclear genes should not be a serious handicap. Hence, in certain situations release programmes may be appropriate.

Keywords Brown hare, conservation, Greece, *Lepus europaeus*, mtDNA, restocking, RFLP.

The brown hare *Lepus europaeus* is an important small game species in Europe (Pielowski, 1976). It is thought to have evolved on the open steppe grasslands of Eurasia and has adapted to mixed arable agriculture (Frylestam, 1980). European hare hunting bags indicate a dramatic decline during 1960-1980 (Smith *et al.*, 2005). Changes in agricultural management, heavy hunting pressure and diseases are the most likely factors responsible for this long-term decline. As a consequence, although the brown hare is not on the IUCN Red List (IUCN, 2006), it is protected under Appendix III of the Convention of the Conservation of European Wildlife and Natural Habitats (Bern Convention; Smith *et al.*, 2005), and is classified as a 'priority species of conservation concern' by the UK government (Smith *et al.*, 2005).

To stabilize population declines, especially as a result of previously heavy hunting pressure, restocking programmes using allochthonous individuals have been carried out in several European countries. While simple in concept, restocking as a management tool remains controversial (Booth, 1988; Conant, 1988; Griffith *et al.*, 1989; Kleiman, 1989; Storfer, 1998). Criticisms focus on the lack of long-term quantitative information on post-release impact (Scott & Carpenter, 1987), difficulty of establishing success or failure criteria (Seddon, 1999), and concerns that extensive gene flow can interfere with local adaptations (Storfer, 1998).

Breeding stations in Bulgaria, Slovakia, Hungary and Poland have traditionally functioned as source populations for restocking operations in Central and Western Europe. These practices may have affected the historical distribution and genetic integrity of indigenous hare species in several European countries (Thulin *et al.*, 1997; Pierpaoli *et al.*, 1999). In Greece, the brown hare is still legally hunted but restocking programmes were abandoned from 2001. The records of the Ministry of Agriculture and of hunting associations show that 2,000 reared individuals (bought from private Greek breeding stations but previously imported mainly from Italy, Yugoslavia and Bulgaria) were released during 1991-2001. However, until 1998, restocking operations were uncontrolled and there was no monitoring of released animals.

In a previous study (Mamuris *et al.*, 2001) we examined wild brown hares from Greece collected during the hunting seasons of 1998 and 1999, and reared brown hares from two farms in 1999. Extensive restriction fragment length polymorphism (RFLP) analysis of three different mitochondrial DNA (mtDNA) segments revealed three groups of individuals: (1) reared brown hares, (2) wild brown hares, and (3) wild individuals with

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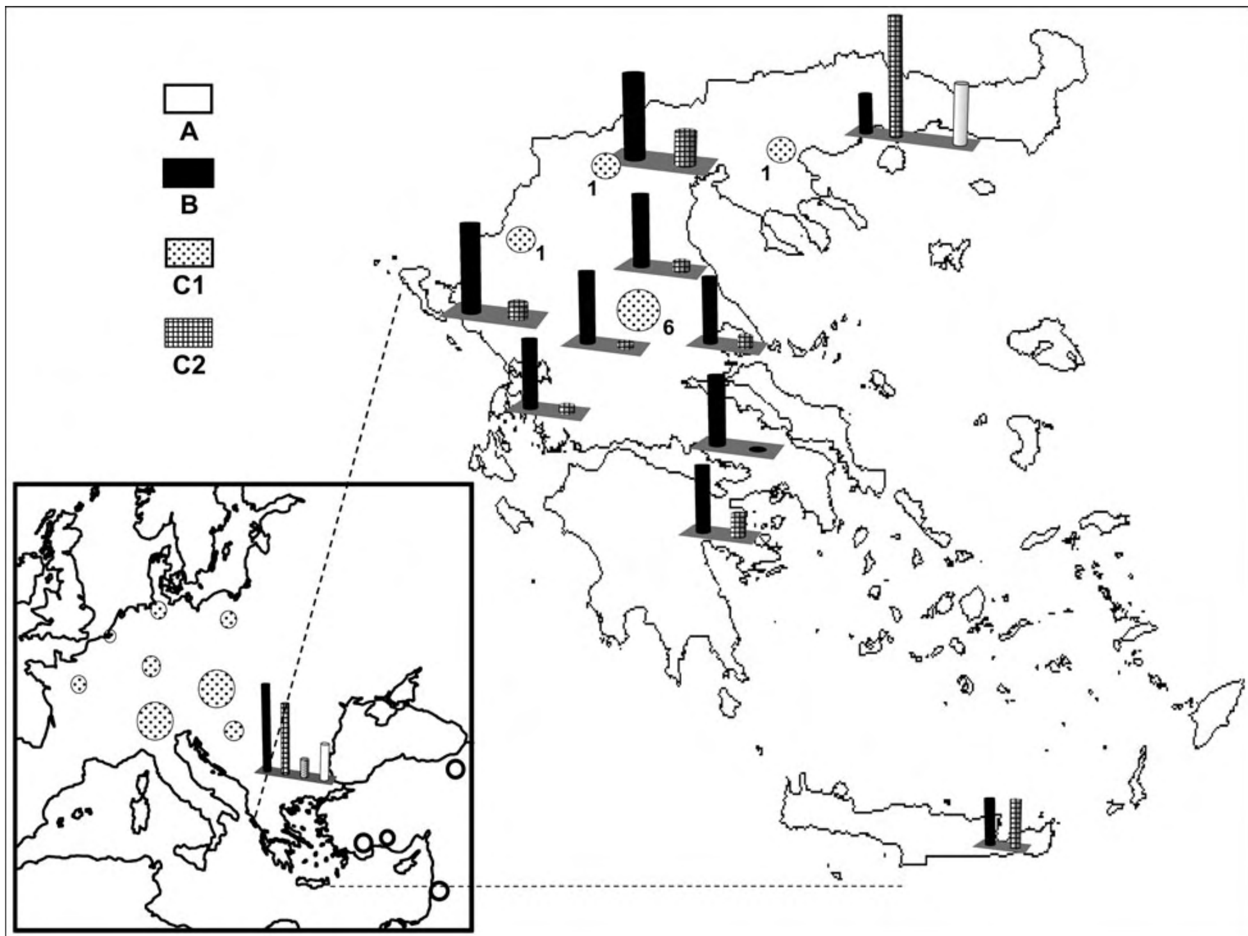

Fig. 1 Sites from which European brown hares were sampled and the occurrence of the mtDNA profiles A, B, C1 and C2 (Fig. 2). Circles and bar charts depict the occurrence of one and multiple haplogroups respectively, at the sampled sites. Numbers correspond to the nine brown hares with profile C1 detected in Greece (see text for details).

mtDNA haplotypes closely related, but not identical, to those observed in the reared group of hares (Mamuris *et al.*, 2001). To trace the origins of the reared individuals and determine the degree of genetic introgression of released brown hares into existing populations in 1999-2004 we collected 187 brown hare samples from Epirus and Thessaly, 42 from north Greece, 53 from north-east Greece, 28 from south Greece and eight from Crete (Fig. 1). The samples were either from hunted hares or from dead individuals found in the wild. We also collected a total of 323 samples from France, the Netherlands, Germany, Poland, Austria, Switzerland, Serbia and Bulgaria, and 60 samples from Turkey and Israel (Fig. 1). Based on Mamuris *et al.* (2001) we identified diagnostic RFLP markers on the mtDNA that could rapidly and unambiguously differentiate the three previously defined groups of brown hare mtDNA haplotypes: a segment of the cytochrome b (Cytb) gene amplified by the primers L14841 and H15149 (Palumbi *et al.*, 1991) and digested simultaneously by the *Alu*I and the *Hinf*I restriction enzymes and a segment of the cytochrome oxydase I gene (COI) amplified by the primers L5950 and H7196 (Palumbi *et al.*, 1991) and digested by the *Hha*I restriction enzyme. Both segments, Cytb and COI, were part of the three regions screened by Mamuris *et al.* (2001). Segment COI was exactly the same and was amplified with the same primers, whereas Cytb is part of the region D-loop/Cytb screened in Mamuris *et al.* (2001). Double strand DNA amplifications were performed in 100 μl volumes, containing 3 units of Taq polymerase, 1x reaction buffer (500 mM KCl, 200 mM Tris-HCl pH 9.0), 0.2 mM dNTPs, 0.5 mM of each primer, 2 mM $MgCl_2$ and approximately 500 ng of DNA. Polymerase chain reaction (PCR) amplification conditions were as follows: one preliminary denaturation at 95°C for 5 minutes, followed by strand denaturation at 94°C for 1 minute, annealing at 52°C for 30 seconds (Cytb) or 1 minute (COI), and primer extension at 72°C for 30 seconds (Cytb) or 1.5 min (COI).

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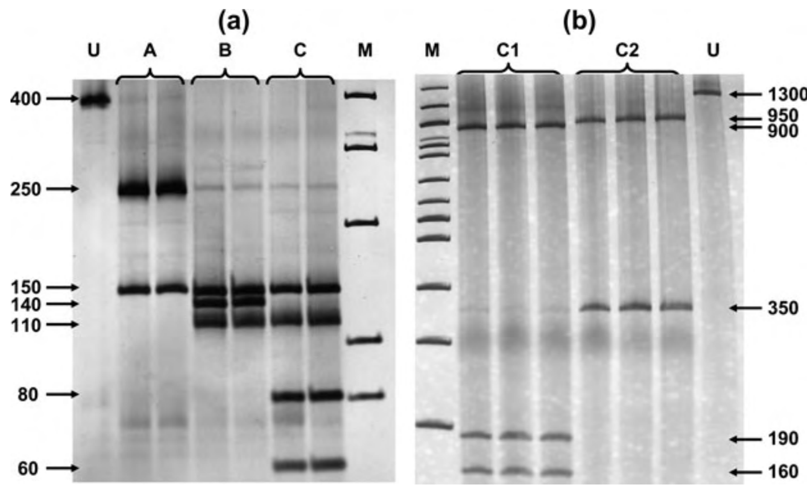

Fig. 2 (a) Digestion of Cytb segment with *Alu*I and *Hinf*I produced three profiles, referred to as A, B and C. (b) Individuals with profile C were further differentiated as C1 and C2 after the digestion of COI segment with *Hha*I. U = undigested DNA, M = 100bp ladder. Numbers indicate base pairs for each band.

Digestion profiles are shown in Fig. 2, and their geographical occurrence in Fig. 1. The diagnostic enzymes permit the allocation of individuals to the different haplogroups. The results were validated by using both types of analysis (full set of restriction enzymes on the three mtDNA, segments as in Mamuris *et al.*, 2001, vs only diagnostic enzymes) in a large proportion of the samples. Profile A was found in all brown hares hunted in Turkey and Israel and a percentage of brown hares from north-eastern continental Greece (15.4%) and Bulgaria (21.8%). Profile B occurred exclusively in Greece and Bulgaria. Hares with the C2 profile apparently occurred in different places both in Greece and Bulgaria but were never traced in Turkey, Israel or in any other European country screened. Analysis of the reared brown hares from the two breeding stations revealed an identical profile (C1) to that traced in all individuals from France, the Netherlands, Germany, Poland, Austria, Switzerland, Serbia and a percentage of hares from Bulgaria. Until 2003, after the analysis of more than 400 individuals (Mamuris *et al.*, 2001, and this study), we had never detected the profile C1 in wild brown hares, even in central Greece, which was intensively surveyed. However, monitoring in 2003 and 2004 detected nine brown hares (four females, one male and four of unidentified sex) from central (seven) and northern Greece (two) with the profile C1. Some of those hares previously released in Europe may also have had profile B because hares that were reared in eastern European breeding stations may have been supplied from Bulgaria. However, our genetic data for brown hares from central or north-western Europe did not trace any individual with profile B.

In February 1999 four released brown hares (two males, two females), born in captivity and hard-released, were radio-tracked during a restocking operation (A.I. Sfougaris, pers. comm.). Three died during the first 6 days and the fourth one after 13 days; all four were probably killed by red foxes *Vulpes vulpes* (A.I. Sfougaris, pers. comm.). Other surveys (Pepin & Cargnelutti, 1985; Angelici *et al.*, 1999) also indicate that survival of released captive-bred individuals is low. However, given that restocking operations in Greece were halted in 2001 and that brown hares with profile C1 were not traced in Greece before 2003, the presence of nine individuals with the profile C1 is the first indication that a percentage of the released farm-bred brown hares survived long enough to have had at least one reproductive cycle and transmit their genome.

There are two major management objectives of restocking programmes: to prevent the decline or even the extinction of local populations and/or to increase genetic diversity, reducing the degree of relatedness and inbreeding. Hare densities in central and western Greece, ranging from 1.1 to 2.4 individuals per 100 ha, are much lower than in other European countries (Smith *et al.*, 2005). Furthermore, during the years 1986-1990, the records of the Ministry of Agriculture show that European brown hare syndrome caused severe mortality (40-90%). In these circumstances, captive breeding and restocking programmes followed by strict genetic control should be considered and evaluated.

In contrast to natural populations, reared populations showed relatively little genetic variation (Mamuris *et al.*, 2001, 2002). From the genetic point of view the only advantage that released animals could confer is the enrichment of the Greek mtDNA genetic pool with the ‘reared’ mtDNA haplotypes. According to our data Bulgaria, and now, after the releases, north-eastern continental Greece, are the only European regions where animals with all four types of mtDNA profile occur. In both cases either there are no data or it is too early to check for possible locally maladapted individuals or genes (Hodder & Bullock, 1997).

Studies revealing the richness of the genetic diversity of brown hare populations in Greece in comparison

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with other European countries (Mamuris *et al.*, 2001, 2002; Suchentrunk *et al.*, 2003) led to the cessation of releases to protect this genetic diversity. Our data indicate that regional gene pools are differentiating, with a detectable change in the genetic structure of Greek brown hare populations resulting from restocking operations. If, in the long-term, introgressed foreign genes survive, forming new genotypes with indigenous genes, this would demonstrate that they are successful in terms of competition. As nuclear gene pools are not markedly divergent between Greek and other European hares (Mamuris *et al.*, 2002; Suchentrunk *et al.*, 2003), foreign nuclear genes should not be a serious handicap. Hence, in certain situations release programmes may be appropriate.

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References

Angelici, F.M., Riga, F., Boitani, L. & Luiselli, L. (1999) Use of dens by radiotracked brown hares *Lepus europaeus*. *Behavioural Processes*, **47**, 205–209.
Booth, W. (1988) Reintroducing a political animal. *Science*, **241**, 156–158.
Conant, S. (1988) Saving endangered species by translocation. Are we tinkering with evolution? *BioScience*, **38**, 254–257.
Frylestam, B. (1980) Reproduction in the European hare in southern Sweden. *Holartic Ecology*, **3**, 74–80.
Griffith, B., Scott, J.M., Carpenter, J.W. & Reed, C. (1989) Translocations as a species conservation tool: status and strategy. *Science*, **245**, 477–480.
Hodder, K.H. & Bullock, J.M. (1997) Translocation of native species in the UK: implications for biodiversity. *Journal of Applied Ecology*, **34**, 547–565.
IUCN (2006) *2006 IUCN Red List of Threatened Species*. IUCN, Gland, Switzerland [http://www.redlist.org, accessed 1 August 2007].
Kleiman, D.G. (1989) Reintroduction of captive mammals for conservation. *BioScience*, **39**, 152–161.
Mamuris, Z., Sfougaris, A.I. & Stamatis, C. (2001) Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA-PCR analysis: implications for conserving genetic diversity. *Biological Conservation*, **101**, 187–196.
Mamuris, Z., Sfougaris, A.I., Stamatis, C. & Suchentrunk, F. (2002) Genetic structure of Greek brown hare (*Lepus europaeus*) populations based on the Random Amplified Polymorphic DNA (RAPD) method. *Biochemical Genetics*, **40**, 323–338.
McNeely, J.A., Miller, K.R., Reid, W.V., Mittermeier, R.A. & Werner, T.B. (1990) *Conserving the World's Biological Diversity*. IUCN/World Resources Institute/Conservation International/WWF-US/World Bank, Washington, DC, USA.
Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L. & Grabowski, G. (1991) *The Simple Fool's Guide to PCR*. University of Hawaii, Honolulu, USA.
Pepin, D. & Cargnelutti, B. (1985) Dispersion and home range acquisition of restocked hares (*Lepus europaeus*). *Biology of Behaviour*, **10**, 353–365.
Pielowski, Z. (1976) The role of foxes in the reduction of the European hare population. In *Ecology and Management of European Hare Populations* (eds Z. Pielowski & Z. Pucek), pp. 135–148. Polish Hunting Association, Warsaw, Poland.
Pierpaoli, M., Riga, F., Trocchi, V. & Randi, E. (1999) Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. *Molecular Ecology*, **8**, 1805–1817.
Scott, J.M. & Carpenter, J.W. (1987) Release of captive-reared or translocated endangered birds: what do we need to know? *Auk*, **104**, 544–545.
Seddon, P.J. (1999) Persistence without intervention: assessing success in wildlife reintroductions. *Trends in Ecology and Evolution*, **14**, 503.
Smith, R.K., Vaughan Jennings, N. & Harris, S. (2005) A quantitative analysis of the abundance and demography of European hares *Lepus europaeus* in relation to habitat type, intensity of agriculture and climate. *Mammal Review*, **35**, 1–24.
Storfer, A. (1998) Gene flow and endangered species translocations: a topic revisited. *Biological Conservation*, **87**, 173–180.
Suchentrunk, F., Mamuris, Z., Sfougaris, A.I. & Stamatis, C. (2003) Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. *Biochemical Genetics*, **41**, 127–140.
Thulin, C.-G., Jaarola, M. & Tegelstrom, H. (1997) The occurrence of mountain hare mitochondrial DNA in wild brown hares. *Molecular Ecology*, **6**, 463–467.

Biographical sketches

Costas Stamatis is currently working on the phylogeography, population genetics and management of brown hares in Europe. Franz Suchentrunk has a long-term interest in wildlife population genetics and ecology and particularly in mammals, studying, among other things, the evolution of hares. Hakan Sert's main interests are the biodiversity and genetic resources of Anatolian animals, the phylogeography of the Middle East, and ecology and genetics of various animal species in the Middle East. Costas Triantaphyllidis' interests are in animal population genetics, evolution and phylogeography. Zissis Mamuris's research interests include the evolution, phylogeography, population genetics and conservation of animal populations.

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Recruitment of mitochondrial tRNA genes as auxiliary variability markers for both intra- and inter-species analysis: The paradigm of brown hare (*Lepus europaeus*)

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Abstract

We sequenced and analyzed the mitochondrial $tRNA^{Thr}$ and $tRNA^{Pro}$ genes from brown hare (*Lepus europaeus*) individuals of different geographic distribution and we investigated the role of various nucleotide substitutions that were detected. We compared these tRNAs with the respective available mitochondrial tRNA genes sequences within *Lepus* species and among mammals. The mutations that were detected represent specific and conserved polymorphisms that do not seem to affect the structural and functional features that are required for participation of tRNA molecules in mitochondrial protein synthesis. These changes however, possibly reflect on the evolutionary background of the species, which is based on the high intra-genomic variability and the evolutionary dynamic of the mitochondrial DNA. In an attempt to compare the phylogeny that is based on these specific tRNA genes with the phylogeny that is produced from sequencing data of the mitochondrial variable loop, we came up with results that indicate similar phylogeographic clusters. This observation implies that the tRNA mutations that were used for the present study have been well tolerated during evolution and they define an additional genetic and biochemical tag that can be used for such studies. Based on this notion and according to our results, we propose that mitochondrial tRNA genes can be used as valuable auxiliary molecular markers for contemporaneous and linked biochemical and genetic analyses.
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Keywords: mt tRNA; Mitochondria; Phylogeny; *Lepus europaeus*

1. Introduction

Mitochondrial tRNAs are essential co-factors of mitochondrial translation and at the same time represent a family of ancient molecules (Di Gulio, 2004). Their accurate aminoacylation is catalyzed by aminoacyl-tRNA synthetases (aaRSs) that are imported to the mitochondrion from the cytosol (Ibba and Söll, 2004) and this process is governed by the presence of specific tRNA identity elements, both determinants and anti-determinants (Beuning and Musier-Forsyth, 1999). These elements consist of specific nucleotides in specific positions that are distributed throughout the surface of the tertiary L-shaped tRNA structure

Abbreviations: aaRSs, Aminoacyl-tRNA synthetases; CR region, Control region.

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and are characteristic for each tRNA species and in many cases for each organism (McClain, 1993). To fulfill their biological role, tRNAs depend also on three-dimensional L-shaped form based on long-range tertiary interactions. Although tRNAs in general are evolutionary conserved, some parts of the mitochondrial tRNA molecules appear to be variable enough to provide critical signals for evolutionary and phylogenetic analyses (Helm et al., 2000). It is worth-mentioning that in humans the 22 mt tRNAs are of particular interest because although they span only 10% of the mitochondrial genome yet they harbor more than 50% of all known mitochondrial mutations related to pathogenic phenotypes, possibly by affecting the preservation of tRNA's higher structure and functional integrity (Zifa et al., 2007). Comparison of available sequence data has revealed that mitochondrial tRNAs deviate, at the structural level, from their nuclear-encoded counterparts (Dirheimer et al., 1995). The most common differences are changes in the number of nucleotides at the connectors as well as shortening of the D- and/or T-stems and loops, and/or elongated anticodon stems (Steinberg et al., 1997).

Comparative studies on the tRNA genes from mammalian mitochondrial and nuclear genomes have demonstrated that the mitochondrial tRNAs accumulate nucleotide substitutions more rapidly than their nuclear counterparts. Haplotypes carrying such mutations are kept at low frequency by their mildly deleterious effects but they are not eliminated from the population immediately (McFarland et al., 2004). Phylogenetic studies that have reported so far for other organisms have included mt tRNA genes but with caution against reliance for refined phylogenetic reconstructions (Lavrov and Lang, 2005). In general, such studies are based on sequencing results mainly from the mitochondrial variable D-loop (CR region) and from conserved mitochondrial genes (cytochrome *b*, cytochrome oxidase I, 12-16S rRNA etc.). However, detailed analyses of mitochondrial tRNA genes in an appropriate phylogenetic context could possibly emerge mt tRNA genes as an accessional tool and they could be combined with biochemical data on the functional integrity of these molecules (Kern and Kondrashov, 2004; Ruiz-Pesini and Wallace, 2006). For example, nucleotides in the mt tRNA molecules that do not correspond to critical identity elements or are not involved in the L-shaped structure may be mutated without any great influence. These positions seem to be unaffected by evolutionary forces and natural selection and they can be used for the computation of phylogenetic relationships.

In the present study we used our data deriving from sequencing of specific mitochondrial tRNA genes from brown hare (*Lepus europaeus*) individuals and we followed inter- or intra-species alterations that could explain phylogenetic differences or different geographic distribution. We chose to focus on the genes encoding for $tRNA^{Thr}$ and $tRNA^{Pro}$, since both are located in close proximity of the mitochondrial D-loop, which is mainly used to draw evolutionary, phylogenetic and phylogeographic relationships among different species or among the same species. In addition, they encode representative tRNA molecules one being transcribed from the "light" mtDNA strand (C-rich strand encoding 14 tRNAs) thus resulting a "heavy" $tRNA^{Pro}$ while the other is transcribed from the "heavy" mtDNA strand (G-rich strand encoding 8 tRNAs) thus resulting a "light" $tRNA^{Thr}$ (Anderson et al., 1981). We wanted to detect whether we could use them as molecular markers to trace useful phylogenetic or phylogeographic information and to simultaneously extract results on biochemical level. Based solely on our sequencing data deriving from mitochondrial tRNA genes we focused on positions of the tRNA molecules that could not affect the overall structure of the molecule, but could possibly contribute to a different phylogeny within *L. europaeus* that exhibit a different geographic distribution or even among variable *Lepus* species. Finally, we compared these results with phylogenetic data that were produced in previous studies (Kasapidis et al., 2005) by standard methods, based on the mitochondrial D-loop analysis and we extended our survey among representative mammalian mitochondrial genomes.

2. Materials and methods

2.1. Sampling

Sampling locations are given in Table 1 and Fig. 4. The Greek samples that we obtained were either from hunted hares from the local hunting associations or from dead individuals found in the wild. All hares had the typical brownish coat colour with blackish tinge dorsally and variably greyish thighs. All other coat pattern characteristics (nape, flanks, tail, ear etc.) conformed to those of typical brown hares. Tissues were stored frozen at −20 °C or preserved in alcohol until DNA extraction (Miller et al., 1988) and sequencing analyses. Samples from European countries, Turkey and Israel were obtained in the course of previous studies on genetic variability of brown hares (Suchentrunk et al., 2000; Sert et al., 2005). According to previous reports (Kasapidis et al., 2005) the samples used in the present study were representative of *L. europaeus* individuals from habitats of distinct phylogeographic distribution throughout Europe and Middle East. A detailed list of the *L. europaeus* individuals and *Lepus* species and the corresponding GenBank accession numbers that were deposited is presented in Tables 1 and 2, respectively. Representative mammal species used for the present phylogenetic analysis are given in Table 3.

2.2. Sequencing data analysis

Bioinformatic analysis of mitochondrial $tRNA^{Pro}$ and $tRNA^{Thr}$ genes of *L. europaeus* individuals and different *Lepus* species was obtained from direct sequencing analysis of mitochondrial DNA after PCR amplification, using the primer pair LepCyb2L (5′-GAAACTGGCTCCAATAACCC-3′ and LepD2H (5′-ATTTAAGAGGAACGTGTGGG-3′) (Pierpaoli et al., 1999). The amplified fragment included the genes encoding for $tRNA^{Pro}$, $tRNA^{Thr}$, the mt variable region (D-loop region) and a ~400 bp fragment of mitochondrial cytochrome *b*. Double strand DNA amplifications were performed in 50 μL volumes, containing 2 units of Taq DNA polymerase, 5 μL of 10× reaction buffer (500 mM KCl, 100 mM Tris–HCl pH 9.0), 5 mM dNTPs, 50 pmol of each primer, 2.5 mM $MgCl_2$ and approximately 500 ng of DNA. PCR amplification conditions were as follows: one initial step of denaturation at 95 °C for 5 min, followed, by

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Table 1
List of *Lepus europaeus* individuals used in the present study with the corresponding GenBank accession numbers (new entries from the present study)

Deposit code		Region	Samples	GenBank accession no	
tRNA^{Pro}	tRNA^{Thr}				
PEU-01	TEU-01	Aberdeenshire, Scotland (N-UK)	1	EU160602	EU160611
		Wiltshire, South England (S-UK)	1	EU160602	EU160611
		Niebüll, North Germany (N-D)	1	EU160599	EU160608
		Offenburg, South-West Germany (SW-D)	2	EU160599	EU160608
		Membrolles, France (F)	2	EU160601	EU160610
		Airport Schipol, The Netherlands (NL)	1	EU160603	EU160612
		Bulgaria (BL)	1	EU160606	EU160615
		Poland (PL)	2	EU160605	EU160614
		Switzerland (CH)	3	EU160604	EU160613
		Eastern Austria (A)	3	EU160600	EU160609
		Southern Greece (S-GR)	2	EF515857	EF515859
		Crete (Cr-GR)	2	EF515857	EF515859
		North-Eastern Greece (NE-GR)	2	EF515857	EF515859
		Spilia (Sp-GR)	3	EF515857	EF515859
		Elassona (El-GR)	3	EF515857	EF515859
		Velestino (Ve-GR)	3	EF515857	EF515859
		Western Greece, Vradeto (Vr-GR)	5	EF515857	EF515859
		Central Greece, Pyrra (Py-GR)	2	EF515857	EF515859
PEU-02	TEU-02	South Israel (S-ISR)	6	EF515858	EF515860
		North Israel (N-ISR)	10	EF515858	EF515860
		Turkey (TR)	15	EU160607	EU160616
		Thrace, North-Eastern Greece (NE-GR)	3	EU293197	EU293199
		North-Eastern Greece (NE-GR)	1	EU293197	EU293199
		Bulgaria (BL)	1	EU293198	EU293200

35 cycles of amplification; each cycle being 95 °C for 40 s, 52 °C for 50 s and 72 °C for 1.30 min and a final extension step at 72 °C for 10 min. The PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosiences) and directly sequenced by Macrogen Inc., using an automated ABI Prism 3730XL DNA sequencer (Perkin Elmer Corporation). All products were sequenced in both directions. The structural and functional integrity of the tRNA genes was predicted using tRNAscan-SE Search Server (http://selab.janelia.org/tRNAscan-SE). Apart from tRNA^{Pro} and tRNA^{Thr} genes, the D-loop mitochondrial locus was aligned to compare tRNA-based phylogenies both at intra-species and intra-genus levels. For intra-genus analysis we used also a fragment of the mitochondrial cytochrome *b* gene. Hybridization and mtDNA introgression are common within genus *Lepus* (Thulin et al., 1997; Melo-Ferreira et al., 2005; Alves et al., 2006). Therefore, several *Lepus* species could be introgressed by foreign mtDNA, making species identification questionable, if based solely on mtDNA. To avoid any misinterpretation, instead of using various Genbank sequences for comparison, we confined only to our own *Lepus* species results.

Genomic survey was performed based on available sequences of known mitochondrial tRNA genes and of complete mitochondrial genomes that are deposited in public databases (GenBank: http://www.ncbi.nlm.nih.gov/Genbank, RNABase: http://www.rnabase.org, MITOMAP: http:// www.mitomap.org, mtDB: http://www.genpat.uu.se/mtDB, Compilation of mammalian mitochondrial tRNA genes: http://mamit-trna.u-strasbg.fr, Compilation of tRNA sequences and sequences of tRNA genes http://www.uni-bayreuth.de/departments/biochemie/trna).

2.3. Genomic data analysis

Alignments of the tRNA sequences were produced using ClustalW and were based on the existing numbering and nomenclature of the respective human mitochondrial tRNA genes database (MITOMAP and mtDB). The mutations that were detected are presented as the positions on both the mitochondrial genome and the tRNA sequence (positions 1–73).

Table 2
List of *Lepus* species and *Oryctolagus cuniculus* used in the present study with the corresponding GenBank accession numbers (new entries from the present study)

Deposit code	*Lepus* species	GenBank accession no
D-loop		
L.tim-Dloop-01	*L. timidus* (Swiss)	EF515861
L.tim-Dloop-02	*L. timidus* (Ural)	EF515862
L.co-Dloop	*L. corsicanus*	EF515867
L.cas-Dloop	*L. castroviejoi*	EF515868
L.gran-Dlooop-01	*L. granatensis*	EF515865
L.gran-Dloop-02	*L. granatensis*	EF515866
L.sax-Dloop	*L. saxatilis*	EF515869
L.cap-Dloop-01	*L. capensis*	EF515863
L.cap-Dloop-02	*L. capensis*	EF515864
L.e-Dloop-EU-A07	*L. europaeus*	DQ469657
L.e-Dloop-EU-A10	*L. europaeus*	DQ469669
L.e-Dloop-PTSEE-13	*L. europaeus*	DQ469649
L.e-Dloop-PTSEE-14	*L. europaeus*	DQ469682
L.e-Dloop-PTAM13	*L. europaeus*	DQ469704
L.e-Dloop-PTAM21	*L. europaeus*	DQ469705
Or.cun-Dloop	*O. cuniculus*	EF515870
Cytochrome b		
L.tim-CYTB-01	*L. timidus* (Swiss)	EU285246
L.tim-CYTB-02	*L. timidus* (Ural)	EU285247
L.co-CYTB	*L. corsicanus*	EU285248
L.cas-CYTB	*L. castroviejoi*	EU285249
L.gran-CYTB-01	*L. granatensis*	EU285250
L.gran-CYTB-02	*L. granatensis*	EU285251
L.sax-CYTB	*L. saxatilis*	EU285252
L.cap-CYTB-01	*L. capensis*	EU285253
L.cap-CYTB-02	*L. capensis*	EU285254
L.e-CYTB-EU-A07	*L. europaeus*	EU285261
L.e-CYTB-EU-A10	*L. europaeus*	EU285258
L.e-CYTB-PTSEE-13	*L. europaeus*	EU285259
L.e-CYTB-PTSEE-14	*L. europaeus*	EU285260
L.e-CYTB-PTAM13	*L. europaeus*	EU285256
L.e-CYTB-PTAM21	*L. europaeus*	EU285257
Or.cun-CYTB	*O. cuniculus*	EU285255

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Table 3
List of representative mammalian species used for the present phylogenetic analysis

Taxon	Species	Common name	GenBank accession no
Primates	*Gorilla gorilla*	Gorilla	D38114
	Homo sapiens	Human	AF347015
	Pan paniscus	Pygmy chimpanzee	D38116
	Pongo pygmaeus	Orangutan	D38115
	Hylobates lar	Gibbon	X99256
	Macaca mulatta	Rhesus monkey	AY612638
	Macaca sylvanus	Barbary ape	AJ309865
	Papio hamadryas	Baboon	Y18001
Artiodactyla	*Bos taurus*	Cow	AY526085
	Bos grunniens	Domestic yak	AY684273
	Bos indicus	Zebu cattle	AY126697
	Ovis aries	Sheep	AF010406
	Hippopotamus amphibius	Hippopotamus	AJ010957
	Lama pacos	Alpaca	Y19184
Cetacea	*Balaenoptera physalus*	Fin whale	X61145
	Balaenoptera musculus	Blue whale	X72204
	Balaenoptera bonaerensis	Antarctic minke whale	AP006466
Carnivora	*Hydrurga leptonyx*	Leopard seal	AM181026
	Mirounga leonina	Southern elephant seal	AM181023
	Phoca caspica	Caspian seal	AM181033
	Canis familiaris	Dog	U96639
	Canis lupus	Gray wolf	DQ480505
	Canis latrans	Coyote	DQ480509
	Eumetopias jubatus	Steller sea lion	AJ428578
	Halichoerus grypus	Gray seal	X72004
	Phoca vitulina	Harbor seal	X63726
	Ursus arctos	Brown bear	AF303110
	Ursus maritimus	Polar bear	AF303111
	Ursus americanus	Black bear	AF303109
Perissodactyla	*Equus caballus*	Horse	X79547
	Rhinoceros unicornis	Indian rhinoceros	X97336
	Equus asinus	Ass	X97337
	Ceratotherium simum	White rhinoceros	Y07726
Chiroptera	*Chalinolobus tuberculatus*	New Zealand long-tailed bat	AF321051
	Pteropus scapulatus	Little red flying fox	AF321050
	Pteropus dasymallus	Ryukyu flying fox	AB042770
Macroscelidae	*Macroscelides proboscideus*	Elephant shrew	AJ421452
Proboscidea	*Loxodonta africana*	African elephant	AJ224821
	Elephas maximus	Asiatic elephant	DQ316068
Xenarthra	*Dasypus novemcinctus*	Armadillo	Y11832
Muridae	*Mus musculus*	Mouse	AY172335
Muridae	*Rattus norvegicus*	Rat	X14848
Lagomorpha	*Lepus europaeus*	European hare	AJ421471
	Oryctolagus cuniculus	Rabbit	AJ001588
	Ochotona princeps	American pika	AJ537415a
	Ochotona collaris	Collared pika	AF348080
Soricidae	*Sorex unguiculatus*	Long-clawed shrew	AB061527
Talpidae	*Talpa europaea*	European mole	Y19192
Marsupialia	*Macropus robustus*	Wallaroo	Y10524
	Trichosurus vulpecula	Brushtail possum	AF357238
	Didelphis virginiana	Opossum	Z29573
	Isoodon macrourus	Bandicoot	AF358864
	Vombatus ursinus	Common wombat	AJ304826
Monotremata	*Ornithorhynchus anatinus*	Platypus	X83427
	Tachyglossus aculeatus	Australian echidna	AJ303116

For all haplotypes, base composition, nucleotide variation, polymorphic and parsimony informative sites were assessed using MEGA version 3.0. For all data, phylogenetic and molecular evolutionary analyses were conducted using both genetic distance and Bayesian analyses. Phylogenetic associations among lineages were assessed with PAUP* 4.0 beta 10 version. To determine the appropriate model of sequence evolution and statistically compare successively nested more parameter-rich models for this data set, the program MODELTEST Version 3.6 was used (Posada and Crandall 1998). The HKY85 + γ correction model (Hasegawa et al., 1985) obtained the best likelihood score and was thus selected for the Neighbour-Joining analysis. Maximum Parsimony (MP) trees were also constructed under the heuristic search option with 100 random-taxon-addition replicates and tree bisection-reconnection branch swapping, using PAUP*. Node support was assessed on the basis of 1000 bootstrap replicates. Phylogenetic trees were rooted, using mountain hare (*Lepus timidus*) sequence.

The program MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) was used to conduct Bayesian analyses with two substitution types and a gamma rate distribution, run for 2×10^6 generations, sampling a tree every 100 generations. Burn-in frequency was set to the first 25% of the sampled trees. Direct examination of the sampled log-likelihood values showed that values had reached a stationary equilibrium by this point. All trees preceding this cut-off were discarded when calculating posterior nodal probabilities, mean log-likelihood scores, and a summary phylogeny including estimates of branch lengths. The mt tRNA regions were analyzed separately and combined. Homogeneity of phylogenetic signal between regions was assessed using the partition-homogeneity test implemented in PAUP* v40b10 (Swofford, 1998).

3. Results

3.1. Comparison of structural and functional features of L. europaeus mt tRNA^{Pro} and tRNA^{Thr}

An important preliminary condition to individual nucleotide comparison in the present work was the analysis of the primary and secondary structures as well as interactions important for the tertiary structure that these specific mt tRNAs form. We based on our own sequencing information from *L. europaeus*

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Fig. 1. A. Alignment of cytoplasmic and mitochondrial $tRNA^{Pro}$ and $tRNA^{Thr}$ sequences from *Homo sapiens* and *Lepus europaeus*. B. Percentage of mismatches and G–U, C–A base pairs present in mitochondrial $tRNA^{Pro}$ and $tRNA^{Thr}$ from *Homo sapiens* and *Lepus europaeus*.

mitochondrial tRNA genes and we compiled our findings according to the commonly used nomenclature and numbering from human (and other mammals) that are available in public databases. We first observed that both tRNAs are slightly smaller in length compared to their nuclear-encoded counterparts (66nt for $tRNA^{Pro}$ and 67nt for $tRNA^{Thr}$ compared to 73nt for both tRNAs that are encoded in the nuclear genome). The alignment that we initially produced indicated that this major difference is localized mainly in the D-loop and the T-loop of the tRNAs where certain nucleotides are missing (Fig. 1A). This observation coincides with previous reports showing that the mitochondrial tRNA genes encompass a smaller number of nucleotides, as well as nucleotide substitutions that affect interactions between parts of the L-shaped structure of the tRNA molecule (Steinberg and Cedergren, 1994). More specifically, a striking feature of mt tRNAs that distinguish them from the tRNAs that act in the cytoplasm is the unusual high percentage of mismatches and G–U base pairs. Moreover, it has been previously reported that in mammals there is a high degree of "skew" between the heavy (GC rich) and the light (AT rich) strand of mt DNA (Reyes et al., 1998). Therefore "light" mt tRNAs (transcribed from the heavy strand mt DNA) are globally poor in G–U and rich in mismatches whereas "heavy" tRNAs are G–U rich and poor in mismatches. The G-richness or G-poverty is maintained and fits with the fact that G-rich sequences allow for higher numbers of G–U base pairs than G-poor sequences (Helm et al., 2000).

In our study we calculated the respective percentage and we found that indeed the "light" $tRNA^{Thr}$ exhibits 10% mismatches whereas only 1.21% corresponds to G–U base pairs. On the other hand the heavy $tRNA^{Pro}$ exhibits 13.8% for G–U base pairs while mismatches are not represented in this molecule. These results are in good agreement with the general structural idiosyncrasy of the mt tRNA genes (Fig. 1B).

The question raised from our preliminary survey was whether this peculiar feature of the mt tRNA's primary structure may not have a striking effect on the secondary structure (cloverleaf structure) but could potentially influence the essential integrity of the tRNA's L-shape. Our bioinformatic analysis revealed mutations that were distributed along the secondary structure of the tRNAs under investigation. When we compared mitochondrial $tRNA^{Thr}$ and $tRNA^{Pro}$ sequences from *L. europaeus* individuals, we noticed that $tRNA^{Thr}$ is the one that encompasses the majority of various mutations (the same exists for different *Lepus* species and representative mammals; data not shown). Extensive alignments divulged that there are 42 different mutations into $tRNA^{Pro}$ and 51 different mutations into $tRNA^{Thr}$ (data not shown). Firstly, we focused on the positioning of the observed polymorphisms. All our analyses point towards the assumption that none of these mutated positions affect the overall 3D structure and stabilization of the tRNA's L-shape, based on the structural information that are available on human mt tRNAs (Helm et al., 2000). However, since no 3D structure is available for *Lepus* mt tRNAs we cannot exclude the possibility that these mutations might play a role in tRNA's L-shape. The majority of the polymorphisms of mt $tRNA^{Pro}$ molecules from *L. europaeus* were observed in the T-loop. It has been already reported that the T-loop is the most polymorphic site of mt tRNAs (Fig. 2A). On the other hand the anticodon loop seems to be the most conserved among all parts of the tRNA molecule. Even when more mammalian mt $tRNA^{Pro}$ sequences were aligned the anticodon loop was shown to be 100% conserved (data not shown).

The mt $tRNA^{Thr}$ sequences alignment from *L. europaeus* revealed more polymorphisms (Fig. 2B). Their distribution was not identical to those of $tRNA^{Pro}$. The polymorphisms of mt $tRNA^{Thr}$ were mainly found in the T-loop and the acceptor stem and the only common feature that the two tRNAs had was the conservation of their anticodon loop. Even in this case there was no polymorphism on this part of the $tRNA^{Thr}$ molecule and the alignment of more mammalian mt $tRNA^{Thr}$ sequences resulted in the same observations.

It has been well established by numerous studies on tRNA identity that specific conserved nucleotides between tRNAs of a same specificity in the same or within different mammalian species, can be used not only to preserve special structural features (as mentioned above), but they also mediate specific interactions with many important key-proteins (including maturation, modification and editing enzymes, initiation and elongation factors etc.) (Spermulli et al., 2004; Sakurai et al., 2005, 2006). Among all these interactions one of the most essential is the specific recognition by the cognate aaRSs that catalyze the precise charging of the tRNAs with the cognate amino acid (Ibba and Söll, 2000). Aminoacylation tRNA identity elements have been well deciphered only for nuclear-encoded tRNAs and are considered conserved during evolution (McClain, 1993; Giegé et al., 1998). On the other hand,

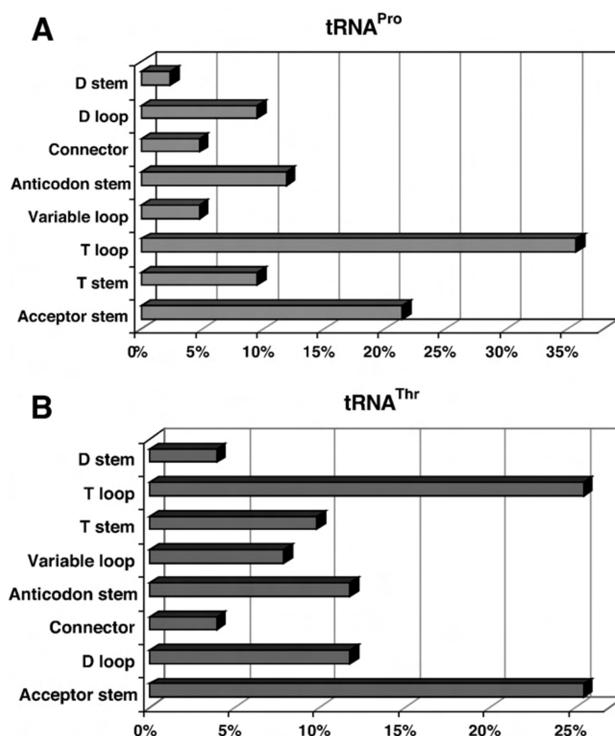

Fig. 2. Percentage of polymorphisms observed in different parts of mitochondrial $tRNA^{Pro}$ (A) and $tRNA^{Thr}$ (B) molecules from *Lepus europaeus*. Polymorphisms have been assigned based on the respective human sequences.

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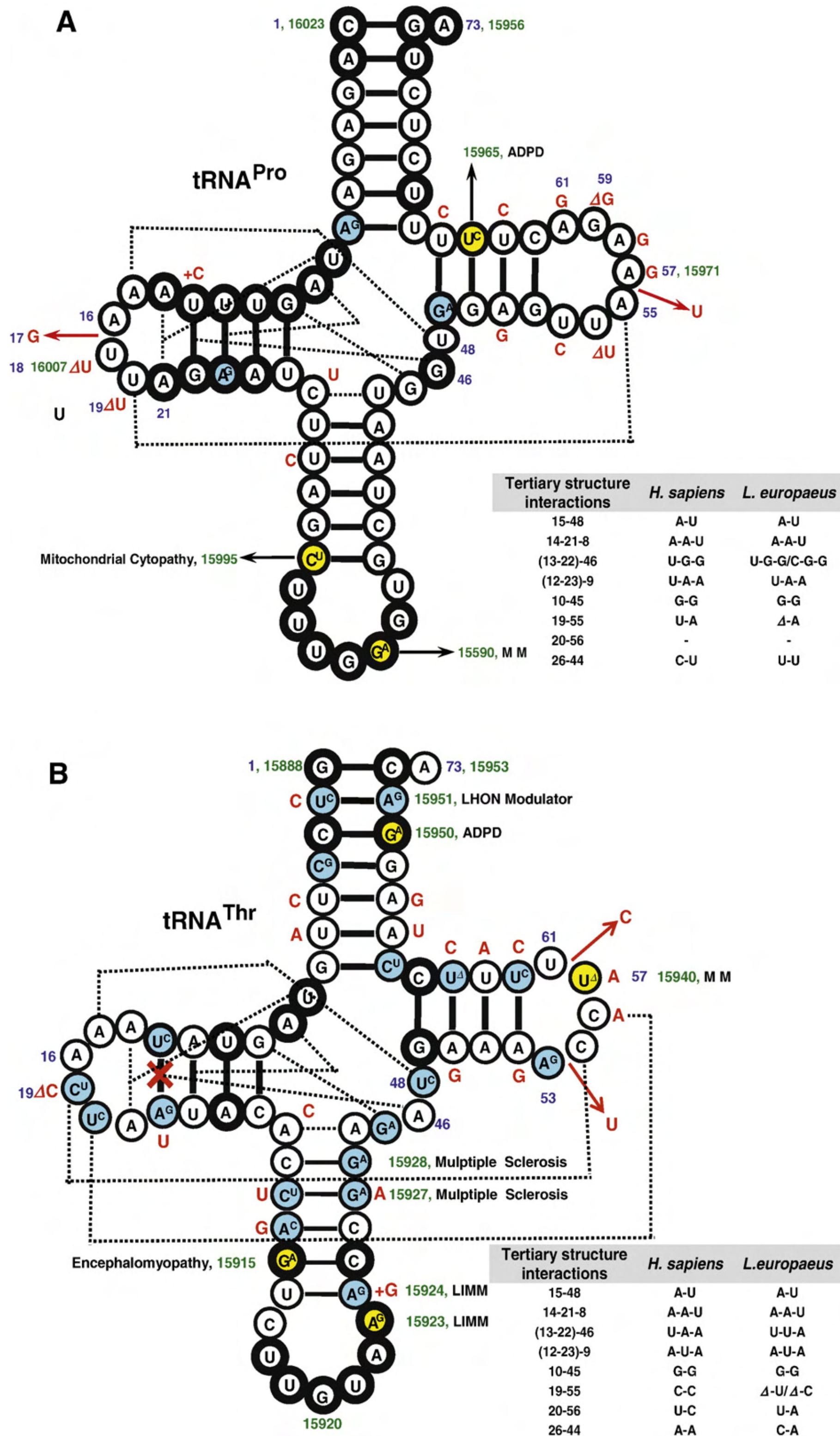

Tertiary structure interactions	*H. sapiens*	*L. europaeus*
15-48	A-U	A-U
14-21-8	A-A-U	A-A-U
(13-22)-46	U-G-G	U-G-G/C-G-G
(12-23)-9	U-A-A	U-A-A
10-45	G-G	G-G
19-55	U-A	Δ-A
20-56	-	-
26-44	C-U	U-U

Tertiary structure interactions	*H. sapiens*	*L.europaeus*
15-48	A-U	A-U
14-21-8	A-A-U	A-A-U
(13-22)-46	U-A-A	U-U-A
(12-23)-9	A-U-A	A-U-A
10-45	G-G	G-G
19-55	C-C	Δ-U/Δ-C
20-56	U-C	U-A
26-44	A-A	C-A

Fig. 3. Superimposition of secondary structures of mitochondrial tRNA^{Pro} (A) and tRNA^{Thr} (B) molecules from *Homo sapiens* and *Lepus europaeus*. The polymorphisms observed in *Lepus europaeus* are indicated with red letters. Polymorphisms indicated with + correspond to those observed in a subset of individuals. The light blue circles correspond to polymorphisms reported for human and are indicated in superscript. The superscript nucleotides in yellow circles correspond to mutations that have been associated with specific diseases in human (LHON, Leber Hereditary Optic Neuropathy; ADPD, Alzheimer's Disease and Parkinson's Disease; MM, Mitochondrial Myopathy; LIMM, Lethal Infantile Mitochondrial Myopathy). Nucleotides in bold outlined circles have been reported as conserved. Tertiary structure interactions are indicated in tables and with dashed lines. The numbering of tRNA molecules is presented according to both their mitochondrial location and the standard tRNA nomenclature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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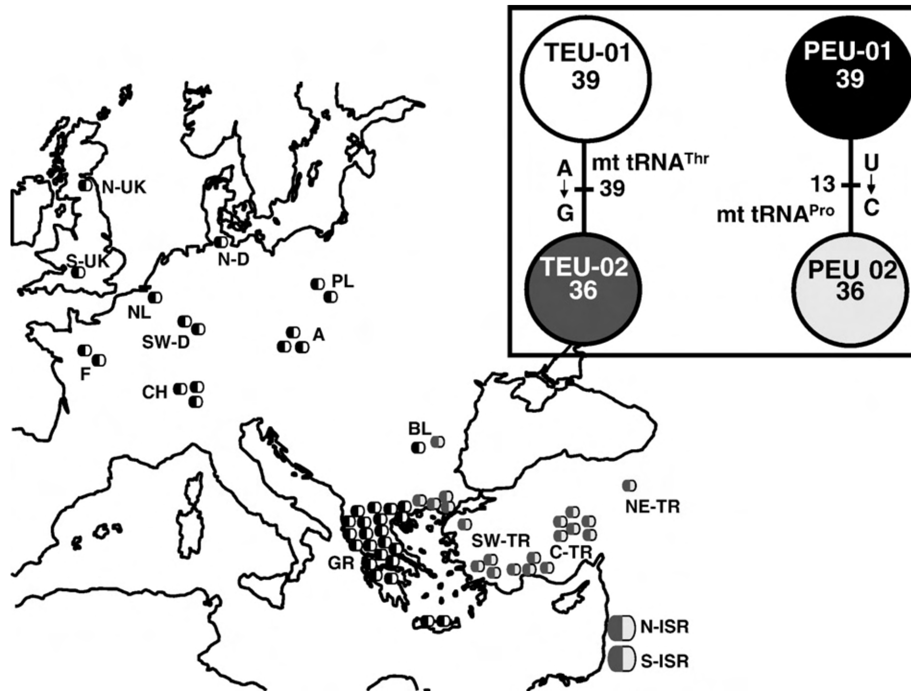

Fig. 4. Map showing the sampling sites and haplotype distribution within the surveyed area. The mutational relationships between haplotypes for the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes are shown in the plate. Each circle in the map represents the approximate sampling position of each individual apart from north and south Israel where the sampling sites are grouped. Black and white circles represent brown hares with haplotype TEU-1 for the $tRNA^{Thr}$ and the haplotype PEU-1 for the $tRNA^{Pro}$, whereas deep grey and light grey circles represent brown hares with haplotype TEU-2 for the $tRNA^{Thr}$ and the haplotype PEU-2 for the $tRNA^{Pro}$ (for sample abbreviations see Table 1).

aminoacylation identity elements for mammalian mt tRNAs are only known for $tRNA^{Asp/Gly}$ (Börner et al., 1996) and $tRNA^{Ser}$ (Ueda et al., 1992). Therefore, it was tempting to search for mutations that could possibly affect the identity elements within the tRNA species that we analyzed here and subsequently could affect their recognition by their cognate aaRSs (Fig. 3).

The majority of the polymorphisms found were not previously reported or studied for their effect on the function of the tRNA molecules. Three of the polymorphisms found only in the mt

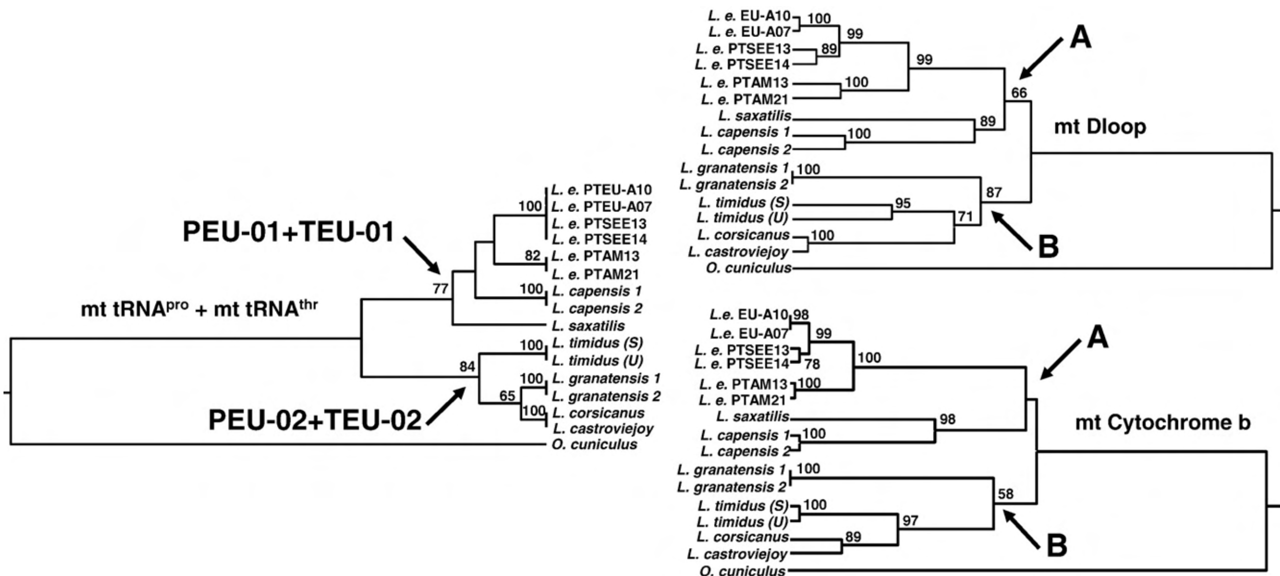

Fig. 5. Phylogenetic trees based on Bayesian analysis of the combined sequences of the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes (left side) and part of the D-loop and cytochrome *b* regions (right side) from different *Lepus* species. The topology for the major clusters was identical for Bayesian, Maximum Parsimony (MP) and Neighbour-Joining (NJ) analyses. Numbers at the internodes of the clusters indicate percentage posterior probabilities from the Bayesian analysis. Probabilities below 50% are omitted. Similar values for MP and NJ bootstrap (1000 replicates) are not shown. *Oryctolagus cuniculus* was used as outgroup. Letters A and B in both trees indicate the two major clusters.

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tRNA^{Thr} were related to pathogenesis in human from some sources while from others were just recorded as neutral mutations. These polymorphisms correspond to positions 15924, 15927 and 15928 according to human mt DNA numbering. However, these mutations have not been reported to be pathogenic in other mammals including *L. europaeus*. Concerning tRNA^{Pro} only one of the observed polymorphisms has been previously reported (A16017G) in human (Seneca et al., 2000). All the other nucleotide substitutions detected, are reported for the first time in literature (located mainly on the T-loop) (Fig. 3A). The tRNA^{Thr} molecule on the other hand is more polymorphic compared to the tRNA^{Pro}. In this case the nucleotide substitutions are equally distributed in the T-loop and the acceptor stem and in lower frequency they are present in the other parts of the molecule. Again, the majority of these nucleotide substitutions (80%) are reported for the first time (Fig. 3B).

3.2. Phylogeographic analysis of L. europaeus individuals based on mt tRNA genes

After the clustering of the nucleotide changes that were identified, we used these tRNA sequences for phylogenetic analysis of the different *L. europaeus* individuals and among *Lepus* species. We identified only one mutation in position 13 (U13C) in the tRNA^{Pro}, which is located at the D-stem and one mutation (A39G) in tRNA^{Thr} located in the anticodon stem (Fig. 3). As mentioned above, we assume that these mutations are not inhibitory for recognition by aaRSs and they possibly don't affect functional integrity. Therefore, we were positive that these tRNA sequences that we identified satisfy the structural and functional requirements of these specific tRNA molecules.

Both tRNA^{Pro} and tRNA^{Thr} analyses revealed that *L. europaeus* individuals can be clustered into two major phylogeographic subgroups based on mt tRNA mutations: one from Northern, Central and South-Eastern Europe and another from Anatolia (Turkey and Israel). These two groups overlapped in Bulgaria and North-Eastern Greece (Fig. 4). A previous phylogenetic study based on D-loop sequencing from the same *L. europaeus* individuals examined in the present study, has shown the same phylogeographic distribution (Stamatis et al., unpublished data).

Further inter-species comparative phylogenetic analysis was performed between *L. europaeus* (Table 1) and six other *Lepus* species (Table 2, Fig. 5). Trees were rooted with *Oryctolagus cuniculus*. As already stated, to overcome the problem of species misidentification we restrained the analysis to the data produced in our laboratory. With some minor differences, for

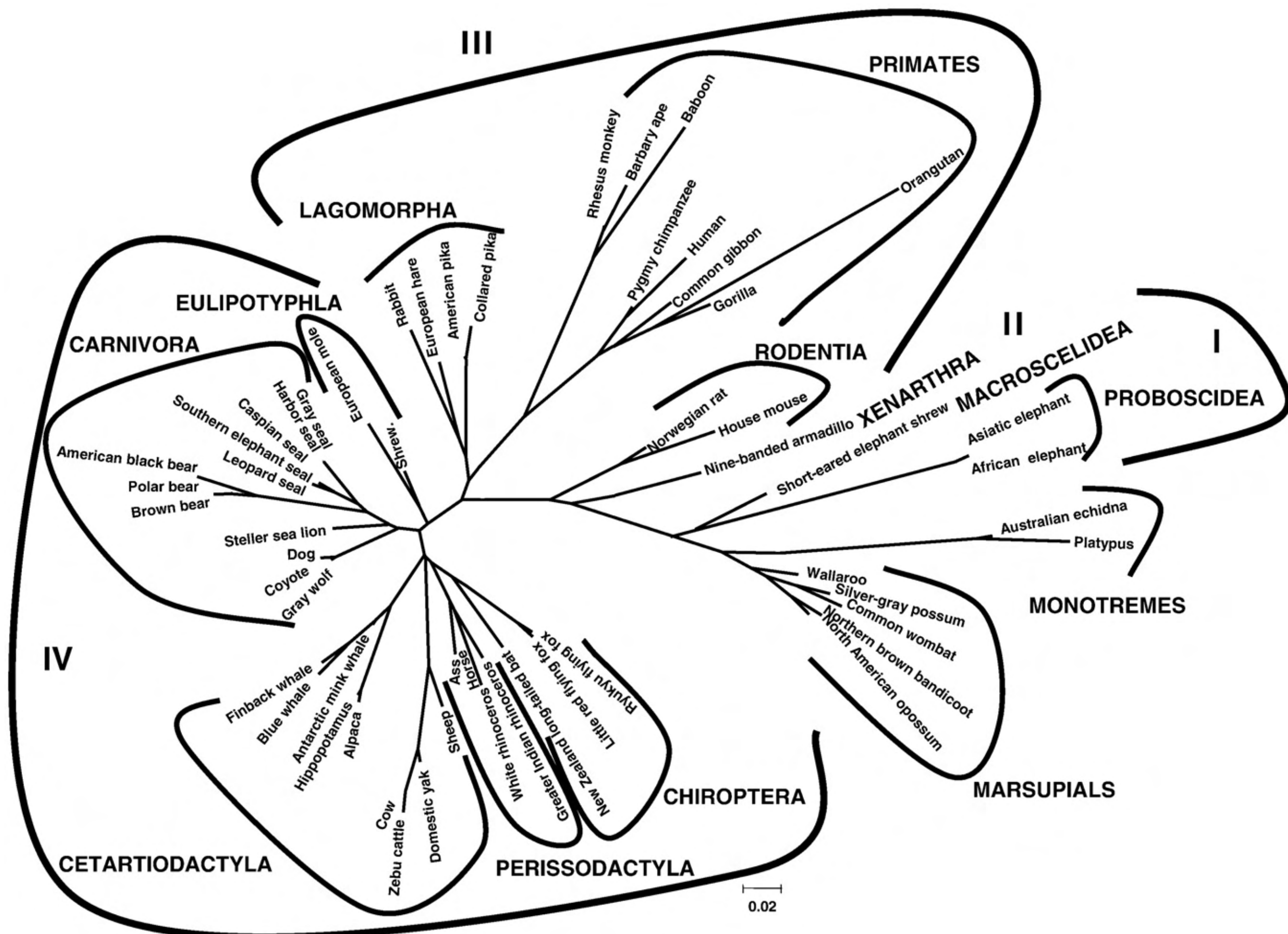

Fig. 6. Phylogenetic tree based on Bayesian analysis of the combined sequences of the tRNA^{Thr} and tRNA^{Pro} genes from 55 different species retrieved in the GenBank (see Table 3).

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both tRNA genes, trees resulted from the Neighbour-Joining (NJ), the Maximum Parsimony (MP) and the Bayesian analyses (trees not shown, available on request) showed in essence the same topology. There were two major clusters grouping the seven species, separated with high bootstrap value, according to Bayesian high posterior probabilities. *L. europaeus* clustered with Cape hare (*Lepus capensis*) and scrub hare (*Lepus saxatilis*), while the second cluster grouped *L. timidus*, Iberian hare (*Lepus granatensis*), Corsican hare (*Lepus corsicanus*) and broom hare (*Lepus castroviejoi*). Given that the partition-homogeneity test revealed homogeneity of phylogenetic signals between the two regions, we combined the two gene sequences in one and we compared the resulted trees with the trees based on the analysis of the D-loop and the cytochrome *b* (Cytb) of the same species. The tRNA-based trees were in good agreement with both the D-loop and Cytb analyses (Fig. 5).

Finally, based on available sequences of complete mitochondrial genomes retrieved from GenBank, we aligned the combined $tRNA^{Pro}$ and $tRNA^{Thr}$ sequences from 55 mammalian species (Table 3), covering 17 orders of placental mammals, *Marsupialia* and *Monotremata*. Interestingly, the topography of the resultant tree (Fig. 6), based on the Bayesian analysis, was in general agreement with trees derived both from nuclear (Madsen et al., 2001; Murphy et al., 2001a,b) and mitochondrial (Hudelot et al., 2003) genes, using large data sets.

All the above results strongly indicate that the tRNA sequences used for the present study could simultaneously provide facile information on both biochemical function and phylogenetic relationships within a species or even among different species, at higher taxonomic levels of mammals.

4. Discussion

We present here a comprehensive study on the variability and putative role of the nucleotide substitutions in mt tRNA genes from *L. europaeus*. Several studies have recently attempted to evaluate the functional significance of tRNA mutations based on analyses of tRNA secondary structure and folding free energy (Kern and Kondrashov, 2004; Vilmi et al., 2005). Population data has also been used to examine differential polymorphism frequencies in tRNAs (Vilmi et al., 2005; Kondrashov, 2005; Kivisild et al., 2006). In addition, previous reports have been extended on the analysis of putative adaptive mtDNA polypeptide variants (Mishmar et al., 2003; Ruiz-Pesini et al., 2004) to demonstrate that at least 19% of ancient stem and 13% of ancient loop tRNA polymorphisms are also adaptive. A number of the tRNA and rRNA sequence polymorphisms have been observed to occur at the base of geographically associated branches of the mtDNA tree (Pesole et al., 1999; Hudelot et al., 2003). Since all possible mtDNA sequence polymorphisms are not found in the mtDNA tree, the fact that specific mtDNA sequence variants are found repeatedly strongly implies that some selective factor results in the repeated retention of these mtDNA sequence mutants.

We show herein that phylogenetic trees produced only by the comparison of few mt tRNA genes are similar compared to those that are produced by the standard methods (analysis of mt variable loop and/or cytochrome *b*). Since tRNA genes are highly conserved throughout evolution, changes that affect mitochondrial tRNA molecules could reflect important evolutionary adaptations among phylogenetically close species or within the same species. From a broader perspective, molecular investigations on mitochondrial tRNA molecules could serve as valuable accessory elements for connecting analysis of precise and unimpeded biochemical function in mitochondria with evolutionary and phylogenetic studies. We assume that the tRNA genes that were used for the present analysis accumulated mutations in positions that do not seem to affect their role in mitochondrial protein synthesis. More importantly it seems that these tRNA species recorded evolutionary changes that can be directly connected with the distinct phylogeographic distribution of *L. europaeus*. The fact that the observed nucleotide substitutions appear in a large portion of the *L. europaeus* individuals indicates that they are tolerated by natural selection pressure.

So far, the majority of the evolutionary, phylogenetic and evolutionary studies have been carried out based on the mitochondrial D-loop, on the conserved genes that encode the respiratory chain components or on the ribosomal RNA subunits. Although many reports include mt tRNA sequences in their phylogenetic analyses, they refer to tRNA molecules as minor factors. The major reason is that the study of mitochondrial D-loop can reveal numerous polymorphisms that can be useful for drawing a more detailed phylogeny. The tRNA genes on the other hand include a significantly lower number of polymorphisms which is in some cases is not adequate to support detailed phylogenetic studies. These polymorphisms however, are included in much smaller genetic pieces and they highly influence the biochemistry and the viability of the organisms. Therefore, screening of mt tRNAs can provide rapidly a quite accurate picture on the evolution, phylogeny and, as we report in the present study, the phylogeographic distribution of species. Extensive molecular investigations on mitochondrial tRNA genes could serve as the interface between biochemical and genetic analyses and they can be used as dependable auxiliary molecular markers for deciphering both evolution and phylogeny in most of the cases.

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References

Alves, P.C., et al., 2006. Hares on thin ice: introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. Mol. Phylogen. Evol. 16, 605–618.

Anderson, S., et al., 1981. Sequence and organization of the human mitochondrial genome. Nature 290, 457–465.

Beuning, P.J., Musier-Forsyth, K., 1999. Transfer RNA recognition by aminoacyl-tRNA synthetases. Biopolymers 52, 1–28.

Börner, G.V., Mörl, M., Janke, A., Pääbo, S., 1996. RNA editing changes the identity of a mitochondrial tRNA in marsupials. EMBO J. 15, 5949–5957.

Di Gulio, M., 2004. The origin of the tRNA molecule: implications for the origin of protein synthesis. J. Theor. Biol. 226, 89–93.

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Dirheimer, G., Keith, G., Dumas, P., Westhof, E., 1995. Primary, secondary and tertiary structures of tRNAs. In: Söll, D., Rajbandary, U.L. (Eds.), tRNA: Structure, Biosynthesis and Function. ASM Press, Washington DC, pp. 93–126.

Giegé, R., Sissler, M., Florentz, C., 1998. Universal rules and idiosyncratic features in tRNA identity. Nucleic Acids Res. 26, 5017–5035.

Hasegawa, M., Kishino, M., Yano, T., 1985. Dating the human–ape split by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Helm, M., Brule, H., Friede, D., Giege, R., Pütz, J., Florentz, C., 2000. Search for characteristic structural features of mammalian mitochondrial tRNAs. RNA 6, 1356–1379.

Hudelot, C., Gowri-Shankar, V., Jow, H., Rattray, M., Higgs, P.G., 2003. RNA-based phylogenetic methods: application to mammalian mitochondrial RNA sequences. Mol. Phylogen. Evol. 28, 241–252.

Huelsenbeck, J.P., Ronquist, F., 2001. Mrbayes: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Ibba, M., Söll, D., 2000. Aminoacyl-tRNA synthesis. Annu. Rev. Biochem. 69, 617–650.

Ibba, M., Söll, D., 2004. Aminoacyl-tRNAs: setting the limits of the genetic code. Genes Dev. 18, 731–738.

Kasapidis, P., Suchentrunk, F., Magoulas, A., Kotoulas, G., 2005. The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations. Mol. Phylogenet. Evol. 34, 55–66.

Kern, A.D., Kondrashov, F.A., 2004. Mechanisms and convergence of compensatory evolution in mammalian mitochondrial tRNAs. Nat. Genet. 36, 1207–1212.

Kivisild, T., et al., 2006. The role of selection in the evolution of human mitochondrial genomes. Genetics 172, 373–387.

Kondrashov, F.A., 2005. Prediction of pathogenic mutations in mitochondrially encoded human tRNAs. Hum. Mol. Genet. 14, 2415–2419.

Lavrov, D.V., Lang, B.F., 2005. Transfer RNA gene recruitment in mitochondrial DNA. Trends Genet. 21, 129–133.

Madsen, O., et al., 2001. Parallel adaptive radiations in two major clades of placental mammals. Nature 409, 610–614.

McClain, W.H., 1993. Transfer RNA identity. FASEB J. 7, 72–78.

McFarland, R., Elson, J.L., Taylor, R.W., Howell, N., Turnbull, D.M., 2004. Assigning pathogenicity to mitochondrial tRNA mutations: when “definitely maybe” is not good enough. Trends Genet. 20, 591–596.

Melo-Ferreira, J., Boursot, P., Suchentrunk, F., Ferrand, N., Alves, P.C., 2005. Invasion from the cold: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. Mol. Ecol. 14, 2459–2464.

Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215.

Mishmar, D., et al., 2003. Natural selection shaped regional mtDNA variation in humans. Proc. Natl. Acad. Sci. U. S. A. 100, 171–176.

Murphy, W.J., Eizirik, E., Johnson, W.E., Zhang, Y.P., Ryder, A., O’Brien, S.J., 2001a. Molecular phylogenetics and the origins of placental mammals. Nature 409, 614–618.

Murphy, W.J., et al., 2001b. Resolution of the early placental mammal radiation using Bayesian phylogenetics. Science 294, 2348–2351.

Pesole, G., Gissi, C., De Chirico, A., Saccone, C., 1999. Nucleotide substitution rate of mammalian mitochondrial genomes. J. Mol. Evol. 48, 427–434.

Pierpaoli, M., Riga, F., Trocchi, V., Randi, E., 1999. Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. Mol. Ecol. 8, 1805–1817.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Reyes, A., Gissi, C., Pesole, G., Saccone, C., 1998. Asymmetrical directional mutation pressure in the mitochondrial genome of mammals. Mol. Biol. Evol. 15, 957–966.

Ruiz-Pesini, E., et al., 2004. Effects of purifying and adaptive selection on regional variation in human mtDNA. Science 303, 223–226.

Ruiz-Pesini, E., Wallace, D.C., 2006. Evidence for adaptive selection acting on the tRNA and rRNA genes of human mitochondrial DNA. Human Mutat. 10, 1–10.

Sakurai, M., Ohtsuki, T., Watanabe, K., 2005. Modification at position 9 with 1-methyladenosine is crucial for structure and function of nematode mitochondrial tRNAs lacking the entire T-arm. Nucleic Acids Res. 33, 1653–1661.

Sakurai, M., Watanabe, Y., Watanabe, K., Ohtsuki, T., 2006. A protein extension to shorten RNA: elongated elongation factor Tu recognizes the D-arm of T-armless tRNAs in nematode mitochondria. Biochem. J. 399, 249–256.

Seneca, S., Lisens, W., Ceuterick-De Groote, C., Van Coster, R., De Meirler, L., 2000. A novel mitochondrial transfer RNA proline mutation. J. Inherit. Metab. Dis. 23, 853–854.

Sert, H., Suchentrunk, F., Erdogăn, A., 2005. Genetic diversity within Anatolian brown hares (*Lepus europaeus* Pallas, 1778) and differentiation among Anatolian and European populations. Mamm. Biol. 70, 171–186.

Spermulli, L.L., Coursey, A., Navratil, T., Hunter, S.E., 2004. Initiation and elongation factors in mammalian mitochondrial protein biosynthesis. Prog. Nucleic Acid Res. Mol. Biol. 77, 211–261.

Steinberg, S., Cedergren, R., 1994. Structural compensation in atypical mitochondrial tRNAs. Nat. Struct. Biol. 1, 507–510.

Steinberg, S., Leclerc, F., Cedergen, R., 1997. Structural rules and conformational compensations in the tRNA L-form. J. Mol. Biol. 266, 269–282.

Suchentrunk, F., Michailov, C., Markov, G., Haiden, A., 2000. Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoogeographical crossroads. Acta Theriol. 45, 1–12.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts, U.S.A.

Thulin, C.-G., Jaarola, M., Tegelstrom, H., 1997. The occurrence of mountain hare mitochondrial DNA in wild brown hares. Mol. Ecol. 6, 463–467.

Ueda, T., Yotsumoto, Y., Ikeda, K., Watanabe, K., 1992. The T-loop region of animal mitochondrial $tRNA^{Ser(AGY)}$ is a main recognition site for homologous seryl-tRNA synthetase. Nucleic Acids Res. 20, 2217–2222.

Vilmi, T., Moilanen, J.S., Finnila, S., Majamaa, K., 2005. Sequence variation in the tRNA genes of human mitochondrial tRNAs. J. Mol. Evol. 60, 587–597.

Zifa, E., Giannouli, S., Theotokis, P., Stamatis, C., Mamuris, Z., Stathopoulos, C., 2007. Mitochondrial tRNA mutations: clinical and functional perturbations. RNA Biol. 4, 38–66.

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Phylogeography of the brown hare (*Lepus europaeus*) in Europe: a legacy of south-eastern Mediterranean refugia?

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ABSTRACT

Aim We analysed the population genetics of the brown hare (*Lepus europaeus*) in order to test the hypothesis that this species migrated into central Europe from a number of late glacial refugia, including some in Asia Minor.

Location Thirty-three localities in Greece, Bulgaria, Italy, Croatia, Serbia, Poland, Switzerland, Austria, France, Germany, the Netherlands, Spain, the United Kingdom, Turkey and Israel.

Methods In total, 926 brown hares were analysed for mitochondrial DNA (mtDNA) variation by restriction fragment length polymorphism (RFLP) performed on polymerase chain reaction-amplified products spanning cytochrome *b* (cyt *b*)/control region (CR), cytochrome oxidase I (COI) and 12S–16S rRNA. In addition, sequence analysis of the mtDNA CR-I region was performed on 69 individuals, and the data were compared with 137 mtDNA CR-I sequences retrieved from GenBank.

Results The 112 haplotypes detected were partitioned into five phylogeographically well-defined major haplogroups, namely the 'south-eastern European type haplogroup' (SEEh), 'Anatolian/Middle Eastern type haplogroup' (AMh), 'European type haplogroup, subgroup A' (EUh-A), 'European type haplogroup, subgroup B' (EUh-B) and 'Intermediate haplogroup' (INTERh). Sequence data retrieved from GenBank were consistent with the haplogroups determined in this study. In Bulgaria and north-eastern Greece numerous haplotypes of all five haplogroups were present, forming a large overlap zone.

Main conclusions The mtDNA results allow us to infer post-glacial colonization of large parts of Europe from a late glacial/early Holocene source population in the central or south-central Balkans. The presence of Anatolian/Middle Eastern haplotypes in the large overlap zone in Bulgaria and north-eastern Greece reveals gene flow from Anatolia to Europe across the late Pleistocene Bosporus land-bridge. Although various restocking operations could be partly responsible for the presence of unexpected haplotypes in certain areas, we nevertheless trace a strong phylogeographic signal throughout all regions under study. Throughout Europe, mtDNA results indicate that brown hares are not separated into discernable phyletic groups.

Keywords

Brown hare, Europe, *Lepus europaeus*, mtDNA, phylogeography, population genetics, post-glacial colonization.

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INTRODUCTION

The brown hare (*Lepus europaeus* Pallas, 1778) is widespread throughout Europe and occurs in a variety of environments ranging from Mediterranean to subarctic regions. It is present both in habitats at sea level and in the Alps, to an altitude of *c.* 2200 m. It has successfully been introduced into exotic temperate environments (e.g. in Argentina, North America and New Zealand) and thrives on tropical and sub-Antarctic islands (Barbados, the Falkland Islands; Flux & Angermann, 1990). This wide geographic range indicates a considerable ecological plasticity of this species. However, during the Late Glacial Maximum (LGM), large parts of central and northern Europe provided frozen habitats (Frenzel *et al.*, 1992) inhospitable for brown hares. At the same time, southern and south-eastern European landscapes, characterized by steppe, open woodland, or scattered tree stands (Bennett *et al.*, 1991), could have served as refugia for brown hares (Corbet, 1986), as indicated to a certain extent by fossil records from the late Pleistocene (see references in Suchentrunk *et al.*, 2003). Temporary land-bridges connecting Asia Minor and south-eastern Europe during the late Pleistocene and the early Holocene (Gökaşan *et al.*, 1997) might have enabled significant gene flow of brown hare populations from south-western Asia to south-eastern Europe. This palaeogeographic situation, together with the likely persistence of long-term late-Pleistocene refugium populations in this part of the Mediterranean, might have led to a particularly rich genetic diversity in hares from the south-eastern Balkans.

Various isolated Pleistocene refugia of brown hares might have existed over millennia in the Balkan Peninsula (Kasapidis *et al.*, 2005), and under the absence of significant gene flow such refugium populations could have acquired differentiated gene pools. In fact, brown hares from south-eastern and eastern Europe have been described as various subspecies (e.g. *Lepus europaeus carpathus*, *L. e. creticus*, *L. e. cyrensis*, *L. e. ghigii*, *L. e. meridiei*, *L. e. niethammeri*, *L. e. parnassius*, *L. e. rhodius*, *L. e. transsylvanicus*), based on fur coloration and patterns, body size, external body measurements, and skull and tooth characteristics (overview in De Beaufort, 1991). All these classifications used morphological characters of questionable taxonomic value, often based on a few individuals. However, given the large intra- and inter-specific variation in the genus *Lepus*, assignment to subspecies should be based on robust criteria and a sufficient number of samples (Angermann, 1983; Flux & Angermann, 1990).

All available population genetic data on brown hares from several regions in Europe indicate fairly high gene flow at the nuclear DNA level across large ranges, but a higher degree of spatial partitioning of mitochondrial DNA (mtDNA) (Hartl *et al.*, 1993; Fickel *et al.*, 1999, 2005; Suchentrunk *et al.*, 2000, 2003; Mamuris *et al.*, 2001, 2002; Ben Slimen *et al.*, 2005; Sert *et al.*, 2005). In addition to these studies, which suggest enhanced male dispersion and a more philopatric behaviour of females, a recent study by Kasapidis *et al.* (2005), based on control region hypervariable domain 1 (CR-I) mtDNA analysis, found that haplotypes from Greek brown hares were separated into two distinct clades, probably corresponding to late-Pleistocene refugia in the central/southern Balkans and in Anatolia. However, more comprehensive data are needed to assess the evolutionary history and relationships among brown hares from different parts of Europe, their systematic status, and the geographic ranges of evolutionary units. Such an evaluation should increase our ability to identify locations of late-Pleistocene refugia and to understand the post-glacial colonization history of brown hares in Europe.

In this study we analysed mtDNA variability in 926 brown hares from Europe, Asia Minor and the Middle East as obtained by a polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) approach. In addition, we performed sequence analysis of the mtDNA CR-I region on 69 individuals, and these data were compared with 136 mtDNA CR-I sequences retrieved from GenBank. The analyses aimed to examine the hypothesis that European brown hares migrated into central Europe from a number of late glacial refugia, including some in Asia Minor, and to evaluate the nature of possible maternal gene flow into supposedly indigenous southern and south-eastern European refugium populations from expanding Holocene populations of central Europe.

MATERIALS AND METHODS

Sampling

A total of 926 brown hares were collected from various regions and localities, and operationally grouped into 33 populations (Table 1; Fig. 1). Population assignment was based solely on geographic criteria; for countries subjected to intense sampling, for example Greece and Bulgaria, the high numbers of samples were divided into a larger number of populations, whereas for countries where sampling was scarce, samples were pooled together per country. All hares had the typical brownish coat colour with blackish tinge dorsally and variably greyish thighs. All other coat pattern characteristics (nape, flanks, tail, ear, etc.) conformed to those of typical brown hares. Tissues were stored frozen at −20° or preserved in alcohol until RFLP or sequencing analyses of mtDNA.

Mitochondrial DNA RFLP analysis

Variation in mtDNA was analysed by RFLPs performed on PCR-amplified products of cytochrome *b* (cyt *b*)/control region (CR), cytochrome oxidase I (COI) and 12S–16S rRNA. The amplified segments from each specimen were subsequently screened for polymorphism with the following 20 restriction endonucleases: *Aci*I, *Alu*I, *Ase*I, *Ava*II, *Bam*HI, *Ban*I, *Bst*UI, *Dde*I, *Eco*RI, *Hae*III, *Hha*I, *Hinc*II, *Hinf*I, *Hpa*I, *Mbo*I, *Mse*I, *Msp*I, *Pst*I, *Taq*I, *Xba*I. Details on DNA extraction, the primers used and PCR amplification conditions are given in Mamuris

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Table 1 Sample size (n), total number of haplotypes (T) and of population-specific haplotypes (P) found within each population sample (identified by groups with capital letters as in Fig. 1), and percentages of the total number of haplotypes/number of individuals (V), number of population-specific haplotypes/number of individuals (W), haplotype diversity (h) and nucleotide diversity (π).

	Sampling site	n	T	P	V (%)	W (%)	h	π
1	Crete (Cr-GR)	8	2	–	25.00	–	71.43	0.36
2	South Greece (S-GR)	15	4	3	26.67	20.00	55.24	0.16
3	Zallogo (Za-GR)	43	11	2	25.58	4.65	88.15	0.23
4	Pyrra (Py-GR)	55	17	3	30.90	5.45	89.23	0.25
5	Vradeto (Vr-GR)	81	21	5	25.93	6.17	89.01	0.22
6	Spilia (Sp-GR)	38	14	6	36.84	15.79	90.75	0.31
7	Velestino (Ve-GR)	37	11	4	29.73	10.81	86.04	0.22
8	Elassona (El-GR)	25	16	7	64.00	28.00	94.67	0.34
9	North Greece (N-GR)	32	15	4	48.86	12.50	95.67	0.29
10	Italy (I)	4	1	–	25.00	–	00.00	0.00
11	South Croatia (S-HR)	11	2	–	18.18	–	20.00	0.03
	Total/average of the 'south-eastern European area' (SEEa)	**349**	**114**	**34**	**32.66**	**9.74**	**70.93**	**0.22**
12	North Croatia (N-HR)	5	4	–	80.00	–	73.33	0.06
13	North Serbia (YU)	22	1	–	4.54	–	00.00	0.00
14	Poland (PL)	10	5	2	50.00	20.00	75.56	0.09
15	Switzerland (CH)	126	10	3	7.94	2.38	65.88	0.11
16	Austria (A)	121	7	5	5.76	4.13	65.54	0.10
17	France (F)	26	3	1	11.54	3.85	38.46	0.03
18	North Germany (N-D)	25	5	2	20.00	8.00	67.00	0.06
19	South-west Germany (SW-D)	22	3	–	13.64	–	43.72	0.03
20	The Netherlands (NL)	10	2	1	20.00	10.00	53.33	0.11
21	Spain (E)	2	1	–	50.00	–	00.00	0.00
22	North United Kingdom (N-UK)	16	1	–	6.25	–	00.00	0.00
23	South United Kingdom (S-UK)	17	1	–	5.88	–	00.00	0.00
	Total/average of the 'central European area' (CEUa)	**402**	**43**	**14**	10.70	**3.48**	**40.24**	**0.10**
24	Turkey (TR)	15	10	7	66.67	46.67	94.29	0.37
25	North Israel (N-ISR)	10	2	–	20.00	–	46.67	0.36
	Total/average of the 'Anatolian–Middle East area' (AMa)	**25**	**12**	**7**	**48.00**	**28.00**	**70.48**	**0.37**
26	North-East Greece (NE-GR)	42	8	4	19.05	9.52	86.00	0.62
27	Sandanski (Sa-BL)	2	2	1	100.0	50.00	100.0	0.38
28	Burgas (Bg-BL)	11	6	1	54.55	9.100	77.78	0.49
29	Stara Zagora (Sz-BL)	7	4	1	57.14	14.29	80.95	0.63
30	Vraca (Vc-BL)	41	12	3	29.27	7.32	90.39	0.33
31	Dobritch (Do-BL)	15	12	4	80.00	26.67	96.70	0.51
32	Pleven (Pv-BL)	21	11	1	52.38	4.76	91.23	0.40
33	Vidin (Vi-BL)	11	4	–	36.36	–	76.19	0.18
	Total/average of the 'overlap area' (OVERa)	**150**	**59**	**15**	39.33	**10.00**	**87.41**	**0.44**
	General total/average	**926**	**112**	**70**	**12.10**	**7.56**	**63.73**	**0.22**

et al. (2001). All PCR reactions were performed in an Eppendorf Mastercycler® ep (Hamburg, Germany).

Mitochondrial DNA RFLP data analysis

Haplotype diversity (Nei, 1987) and nucleotide diversity (Nei & Tajima, 1981) values within populations, as well as nucleotide divergence (Nei & Tajima, 1981) values among haplotypes and populations were computed using the statistical package REAP 4.0 (McElroy *et al.*, 1991). Tests were carried out (using 10,000 randomizations) on the statistical significance of pairwise differentiation based on composite haplotype frequencies according to Raymond & Rousset (1995) using the ARLEQUIN 2.0 package (Schneider *et al.*, 2000).

To determine the phylogenetic affinities among haplotypes, parsimony and distance approaches included in PHYLIP 3.6 (Felsenstein, 2004) were used. Another tree was constructed using the Bayesian approach with MrBayes 3.1 (Huelsenbeck & Ronquist, 2001). We used the F81-like model for restriction sites, proposed by the authors, to conduct Bayesian analyses

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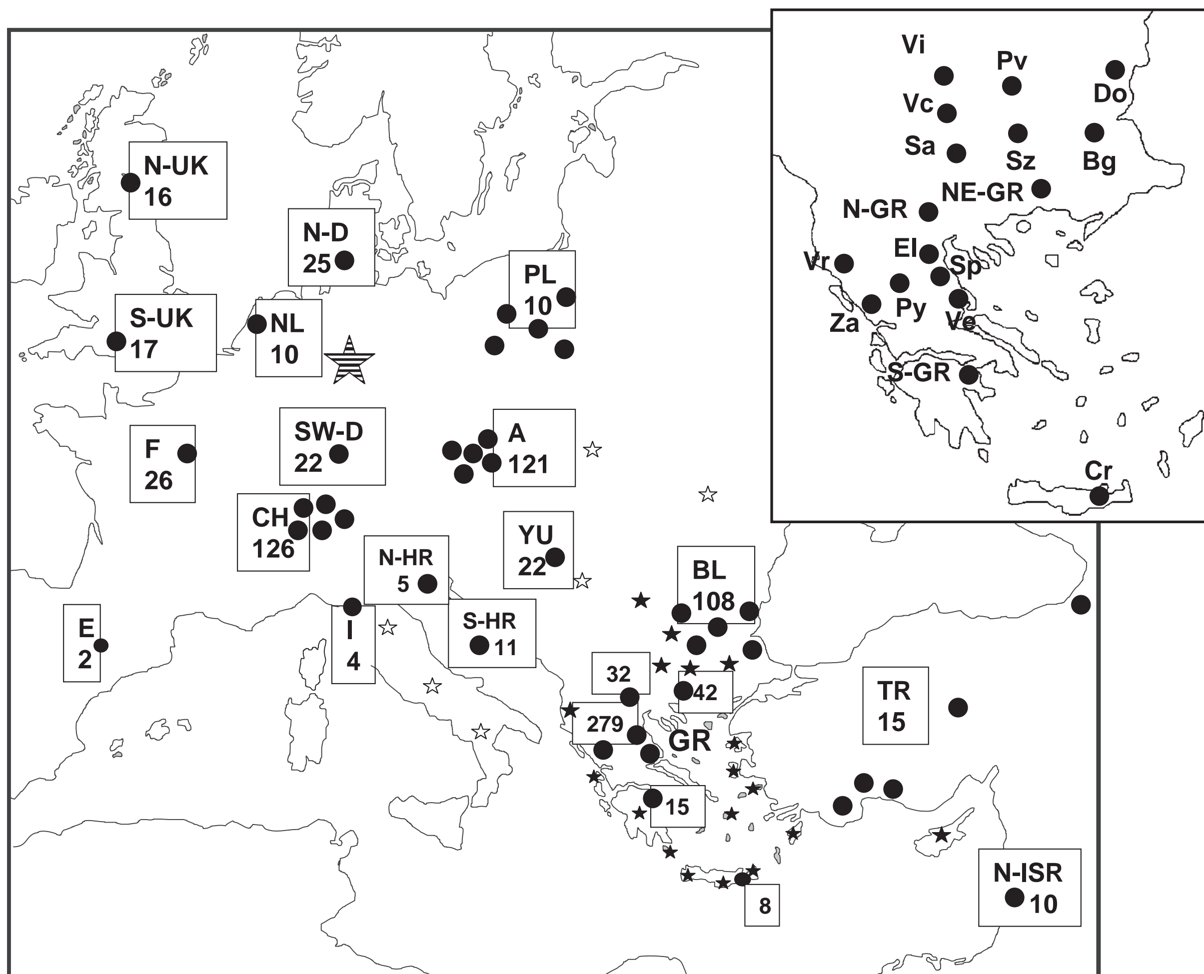

Figure 1 Map showing the distribution of sampling sites (dark circles), with their abbreviations as shown in Table 1, and the number of individuals of *Lepus europaeus* sampled for this study. Stars represent samples for which sequences were retrieved from GenBank (dark stars, Kasapidis *et al.*, 2005; white stars, Pierpaoli *et al.*, 1999; striped star, J. Fickel, A. Schmidt, H. Spittler and C. Pitra, unpublished data). The sample distribution for this study in Greece and Bulgaria is shown in more detail in the inset.

using random starting trees run for 8×10^6 generations and sampled every 100 generations. The burn-in frequency was set to the first 25% of the sampled trees. Direct examination of the sampled log-likelihood values showed that values had reached a stationary equilibrium by this point. All trees preceding this cut-off were discarded when calculating posterior nodal probabilities, mean log-likelihood scores and a summary phylogeny including estimates of branch lengths. Trees were rooted using data obtained from a corresponding mtDNA analysis of mountain hares (*Lepus timidus*).

The software NETWORK 3.1.0.1 (Fluxus technology Ltd, downloaded from http://www.fluxus-technology.com/sharenet.htm) was used to construct a median-joining (MJ) network based on the restriction site data. The neighbour-joining (NJ) algorithm (Saitou & Nei, 1987) was used to cluster populations according to nucleotide divergence.

As an alternative approach to visualizing genetic relationships among populations, multi-dimensional scaling (MDS) was performed on pairwise distance matrices of net nucleotide diversity values among populations using the SPSS 11.0 statistical program package. The resultant MDS-coordinates of populations were subjected to MANOVA (full factorial type III of sum of squares models with population groups as fixed factors), following Kolmogorov–Smirnov tests to prove normality, and to associated Scheffe and least significant difference (LSD) tests, to prove significant variation across population groups.

Inference about the degree of population subdivision based on hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) was implemented using the ARLEQUIN 2.0 package (Schneider *et al.*, 2000). This package was used to compare the component of genetic diversity among the major geographical areas sampled with that observed among populations within each of them. The significance of the resultant *F*-statistics and the variance components were tested with 10,000 permutations.

The historical demography of the populations was examined using 'mismatch distributions', which represent the frequency distribution of pairwise differences among all haplotypes in a

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sample, under the sudden expansion model proposed by Rogers & Harpending (1992) as implemented in ARLEQUIN 2.0 (Schneider *et al.*, 2000). Tajima's *D* test (Tajima, 1989) of the total number of segregating sites was also calculated for each geographical area. Mismatch distributions fitness to Poisson distributions (which reflect the expansion scenario) was assessed by Monte Carlo simulations of 1000 random samples using ARLEQUIN. The sum of squared deviations (SSD) between observed and expected mismatch distributions was used as the test statistic, and its *P*-value represents the probability of obtaining a simulated SSD larger than or equal to the one observed. Estimation and testing (bootstrap resampling with 10,000 replicates) of Tajima's *D* values were also computed using ARLEQUIN.

Mitochondrial DNA sequence analysis

In order to compare our RFLP results with data already published, we chose 69 *L. europaeus* specimens (see Appendix S1 in Supporting Information) and one *L. timidus* specimen for sequence analyses. All *L. europaeus* individuals selected had a different mtDNA RFLP haplotype, representing all haplogroups detected after RFLP analysis. The primer pair LepCyb2L (5′-GAAACTGGCTCCAATAACCC-3′) and LepD2H (5′-ATTTAAGAGGAACGTGTGGG-3′) (Pierpaoli *et al.*, 1999) was used to PCR-amplify one fragment of *c.* 1200 base pairs. Double-strand DNA amplifications were performed in 50-μL volumes containing 2 units of Taq polymerase, 5 μL of 10 × reaction buffer (500 mM KCl, 100 mM Tris, pH 9.0), 5 mM dNTPs, 50 pmoles of each primer, 2.5 mM $MgCl_2$ and *c.* 500 ng of DNA. PCR amplification conditions were as follows: one initial step of denaturation at 95°C for 5 min, followed by 35 cycles of amplification – each cycle being 95°C for 40 s, 52°C for 50 s and 72°C for 1.30 min – and a final extension step at 72°C for 10 min. The PCR products were purified with GFX PCR DNA and a Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) and directly sequenced by Macrogen Inc., using an automated ABI Prism 3730XL DNA sequencer (Perkin Elmer Corporation, Waltham, MA, USA). All products were sequenced in both directions. Sequences were aligned using CLUSTALX (Thompson *et al.*, 1997) with final adjustments by eye.

We aligned 1058 bp, comprising 462 bp of the cyt *b* gene, the tRNA-Thr and tRNA-Pro, and 464 bp of the CR-I region. After analysing our own CR-I sequences, we compared them with three data sets of mtDNA CR-I for *L. europaeus* available in GenBank: 72 sequences described in Kasapidis *et al.* (2005), 22 sequences described in Pierpaoli *et al.* (1999), and 43 sequences described in Fickel *et al.* (direct submission). Haplotype names, sample sizes, sampling localities and accession numbers of all sequences are given in Appendix S1.

For all haplotypes, base composition, nucleotide variation, polymorphic and parsimony-informative sites were assessed using MEGA 3.0 (Kumar *et al.*, 2004). Phylogenetic associations among lineages were assessed with PAUP* 4.0 beta 10 version (Swofford, 1998). To determine the appropriate model of sequence evolution and statistically compare successively nested more parameter-rich models for this data set, the program MODELTEST 3.6 (Posada & Crandall, 1998) was used. With a statistical significance of $P = 0.01$, the HKY85 model (Hasegawa *et al.*, 1985), with γ correction, obtained the best likelihood score and was thus selected for the NJ analysis. Maximum parsimony (MP) trees were also constructed under the heuristic search option with 100 random-taxon-addition replicates and tree bisection–reconnection branch swapping, using PAUP*. Node support was assessed on the basis of 1000 bootstrap replicates. Phylogenetic trees were rooted using the *L. timidus* sequence.

A Bayesian analysis was also performed with MRBAYES 3.1 (Huelsenbeck & Ronquist, 2001), under the HKY85 model of sequence evolution. Depending on the data set, random starting trees run for 2×10^6 to 8×10^6 generations were sampled every 100 generations. The burn-in frequency was set to the first 25% of the sampled trees.

RESULTS

RFLP haplotype diversity

The amplified segments of cyt *b*/CR, COI and 12S–16S rRNA had approximate sizes of 1.8, 1.3 and 2.05 kb, respectively, corresponding to *c.* 30% of the mitochondrial genome of the species (Gissi *et al.*, 1998). The 20 restriction enzymes generated in all of the amplified segments a total of 173 restriction sites corresponding to an estimated average number of 764 bp surveyed. Polymorphism was found in all three segments, but, as expected, at considerably different levels within each region. Cyt *b*/CR was the most polymorphic, and 12S–16S rRNA was the least polymorphic. In total, 112 distinct haplotypes were scored, presented in Appendix S2 with the haplotype frequencies for each sampling site. Pairwise sequence divergence estimates among the 112 haplotypes varied from 0.19% (haplotypes 1–2, 1–20, 1–47, 3–4) to 4.65% (haplotypes 103–111), with an average of 1.84% (see Appendix S3).

With some minor differences, the Bayesian analysis (Fig. 2), the NJ tree (see Appendix S4), and the MJ network (Fig. 3) showed in essence the same topology, with five major haplogroups grouping the 112 haplotypes. One cluster, conventionally named the 'Anatolian/Middle East type haplogroup' (AMh), included all of the haplotypes found in Turkey (TR) and north Israel (N-ISR), as well as a percentage of haplotypes found in brown hares from north-eastern continental Greece (NE-GR, 17.8%) and Bulgaria (BL, 23.8%) (Figs 2, 3 & 4). Haplotypes of the second cluster, the 'south-eastern European type haplogroup' (SEEh), predominantly occurred in Greece and Bulgaria and were also present in all individuals from south Croatia (S-HR) and Italy (I). Three haplotypes of the SEEh were present in three (3.37%) out of 89 hares from southern Switzerland, and another one in three (3.41%) out of 88 hares from eastern Austria (A) (Figs 2, 3 & 4). Haplotypes of the SEEh were totally absent from Anatolian and Israeli hares (Figs 2, 3 & 4). In the

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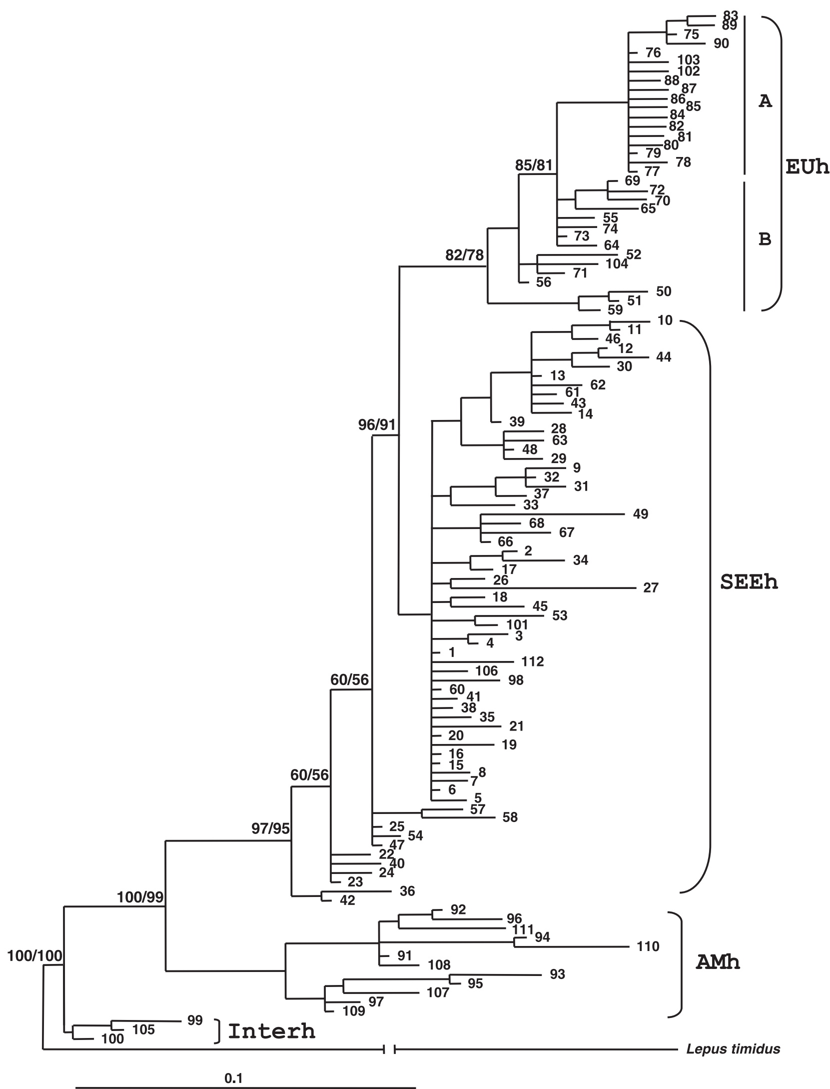

Figure 2 Phylogenetic tree resulting from the Bayesian analysis clustering the 112 restriction fragment length polymorphism haplotypes from *Lepus europaeus*. *Lepus timidus* was used as outgroup. The topology for the major clusters was similar for the neighbour-joining (NJ) tree. Numbers above branches of the major clusters represent percentage posterior probabilities from the Bayesian analysis and NJ bootstrap values (1000 replicates), respectively. EUh, European type haplogroup; SEEh, south-eastern European type haplogroup; AMh, Anatolian/Middle Eastern type haplogroup; Interh, intermediate haplogroup. Each haplotype is identified by its corresponding number, as in Appendix S2.

third cluster, named 'European type haplogroup' (EUh), haplotypes were separated into two, phylogenetically related but phylogeographically distinct, subgroupings: subgroup A (EUh-A) encompassed haplotypes from various regions of central Europe, the British Isles (UK), Spain (E), France (F), the Netherlands (NL), northern Germany (N-D) and Bulgaria (BL), whereas the haplotypes of subgroup B (EUh-B) occurred only in central Greece (C-GR), north-eastern Greece (NE-GR), southern Greece (S-GR), Crete (Cr-GR) and in various regions of Bulgaria (Figs 2, 3 & 4). Haplotypes of the EUh were also totally absent from Anatolian and Israeli hares (Figs 2, 3 & 4). Finally, three haplotypes (99, 100, 105), from Bulgaria and north-eastern Greece, with an intermediate position between the AMh and the SEEh, as revealed by the network MJ analysis (Figs 2, 3 & 4), formed a fifth intermediate haplogroup (INTERh).

Population diversity and structure

To conduct a comprehensive population genetic analysis we pooled the sampling sites with only one individual. The presence of different haplogroups in Greece and Bulgaria led us to split these two regions into several populations (Figs 1 & 4), in order to display more detail in the distribution of the haplogroups within each region. However, given the sole presence of the EUh-A in the other European countries, there was no need for further separation.

Of the 112 haplotypes recorded, 70 (62.5%) were population-specific (i.e. observed only in one population) and 44 (39.28%) were found only in one individual per population (Table 1, see Appendix S2). The sixteen haplotypes 5, 6, 12, 13, 15, 16, 23, 28, 73, 75, 76, 77, 79, 83, 87, 91 were the most common, grouping 542 (58.53%) of all examined hares (see Appendix S2). The number of observed haplotypes within populations ranged from one (E, YU, N-HR, I, NE-TR, N-UK and S-UK) to 21 (Vr-GR) (Table 1).

Haplotype diversity values for all populations ranged from 0.00% to 100.00% with an average of 63.73%, and nucleotide diversity values ranged from 0.00% to 0.63% with an average of 0.22% (Table 1). A statistically significant difference in haplotype frequencies among all populations was observed ($P < 0.00001$), whereas of the 561 pairwise F_{ST} comparisons among populations only 52 were statistically significant. The

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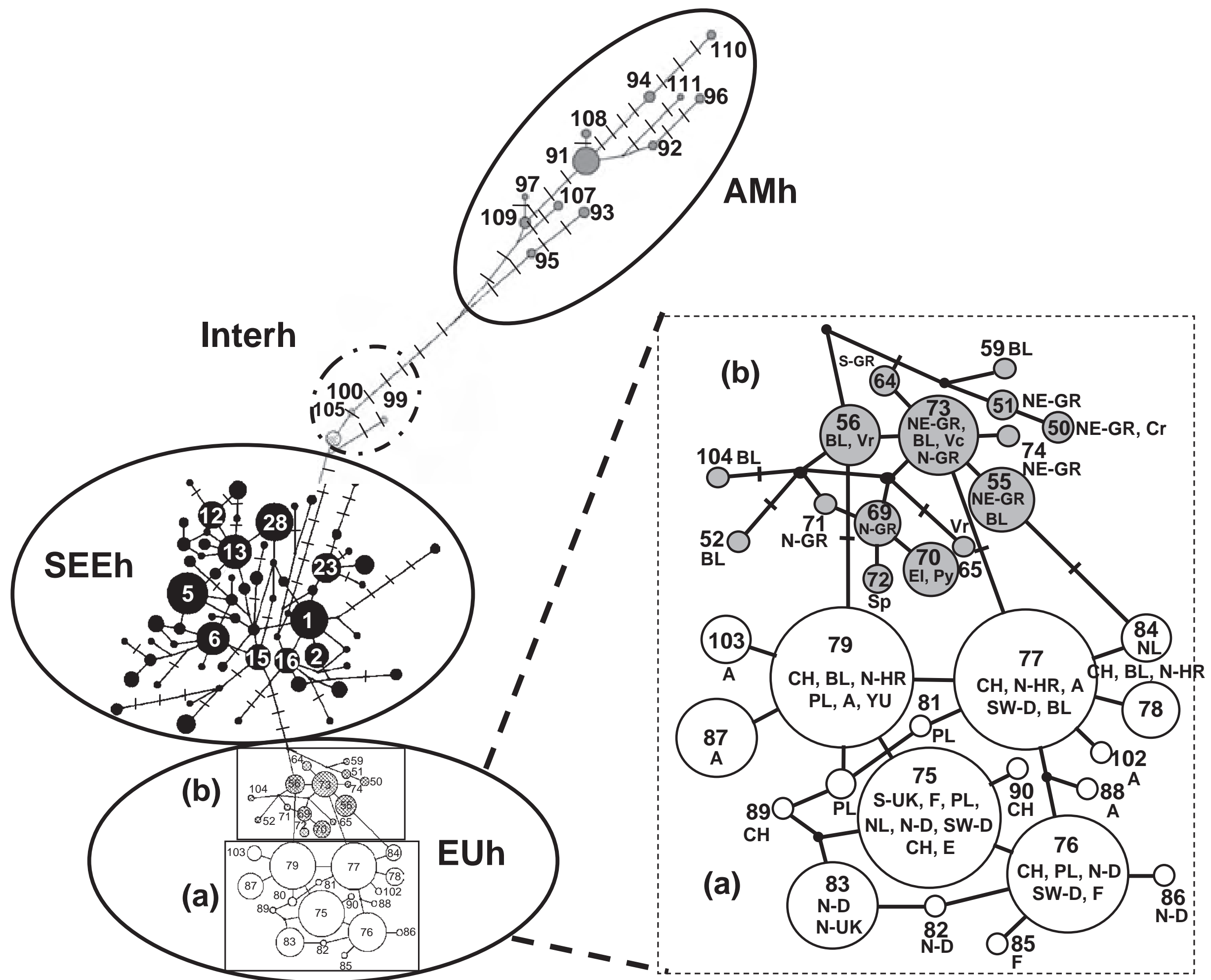

Figure 3 Median-joining (MJ) network showing the mutational relationships among 112 *Lepus europaeus* haplotypes detected in a sample of 926 individuals. A circle represents a haplotype. Circle sizes are proportional to the number of individuals with specific haplotypes in the total sample. Each segment on connecting lines between circles as indicated by vertical bars represents a single mutational change. AMh, Anatolian/Middle Eastern type haplogroup; Interh, intermediate haplogroup; SEEh, south-eastern European type haplogroup; EUh, European type haplogroup. Haplotypes of EUh subgroup A and EUh subgroup B are shown in more detail in the inset. Each haplotype is identified by its corresponding number, as in Appendix S2.

mean nucleotide divergence among all populations was 0.30%, ranging from 0.00% to 1.14%. The MDS analysis (Fig. 5), as well as the NJ phylogenetic tree, both based on the mean nucleotide divergence matrix, showed the same, strong geographic structure. Four major areas were formed (Fig. 5), reflecting the partitioning of mtDNA haplogroups and the levels of intra-population diversity: the ‘central European area’ (CEUa) comprised the populations from central Europe, Spain, the United Kingdom, Serbia and north Croatia, dominated by the EUh-A; the ‘south-eastern European area’ (SEEa) comprised the populations from Greece (with the exception of NE-GR), Italy and south Croatia, reflecting the presence of the SEEh and EUh-B; the ‘Anatolian/Middle East area’ (AMa) included the populations from Anatolia and north Israel (i.e. the AMh); the ‘overlap area’ (OVERa), covering the populations from Bulgaria and north-eastern Greece, with an admixture of haplotypes from all five haplogroups, clustered in a connecting position between the other three areas (Fig. 5). The MANOVA test for the MDS-coordinate values indicated highly significant ($P < 0.000005$) variation across population groups.

The OVERa had the highest mean haplotype and nucleotide diversity (0.874 and 0.0044, respectively) followed by the SEEa (0.709 and 0.0022, respectively) and the AMa (0.705 and 0.0037, respectively). The CEUa had the lowest values (0.402 and 0.001, respectively) (Table 1). All pairwise F_{ST} comparisons among the four areas were highly significant ($P < 0.000005$ for all). According to the AMOVA, 10.59% of the mtDNA variation was distributed among populations within major areas, 38.99% was distributed within populations, and 50.43% was distributed among areas. All these values were highly significant (Table 2). If no structure was assumed, the overall F_{ST} value was 53.5%.

Table 3 summarizes estimates of the mismatch distributions for the four pooled major sampling areas. According to the

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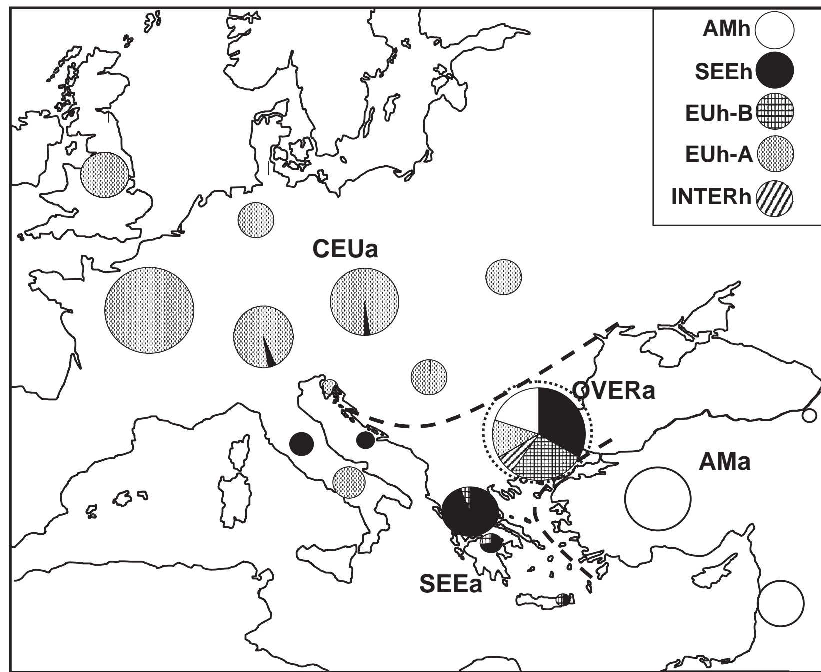

Figure 4 Map showing the distribution of the five haplogroups of *Lepus europaeus* identified in the four major geographical areas. The representations of the five haplogroups are shown at the top right. The 'central European area' (CEUa) comprises mainly the 'European type haplogroup, subgroup A' (EUh-A) and a few individuals belonging to the 'south-eastern European type haplotype' (SEEh). The 'south-eastern European area' (SEEa) comprises mainly the SEEh and a few individuals of the 'European type haplogroup, subgroup B' (EUh-B). The 'Anatolian/Middle East area' (AMa) contains only the 'Anatolian/Middle Eastern type haplogroup' (AMh). The 'overlap area' (OVERa) contains all five haplogroups.

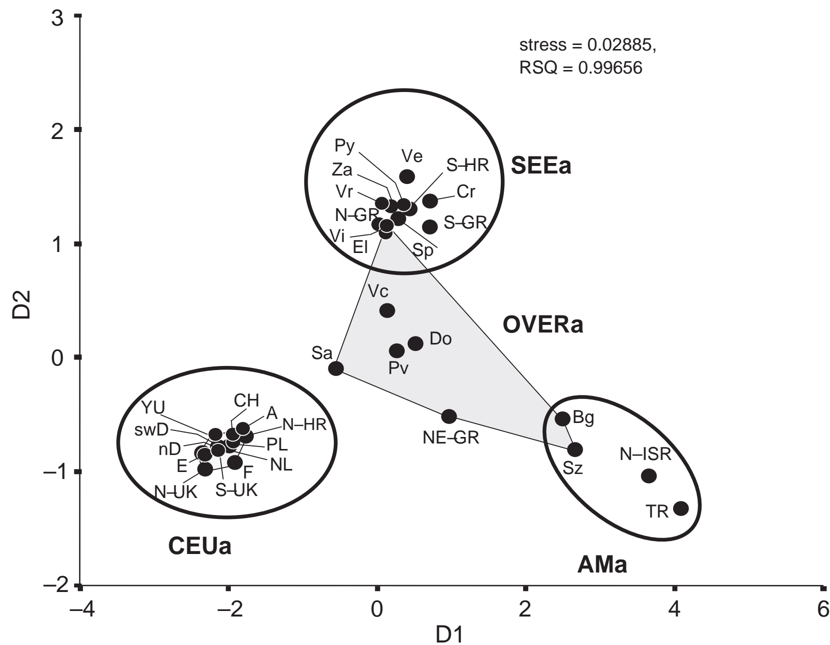

Figure 5 Scatter plot of the first- and second-dimension coordinates of populations of *Lepus europaeus* as obtained from multidimensional scaling of the matrix of pairwise distances based on nucleotide divergence (Nei & Tajima, 1981) from mtDNA restriction fragment length polymorphism analysis. SEEa, south-eastern European area; OVERa, overlap area; CEUa, central European area; AMa, Anatolian/Middle East area.

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Table 2 Results of the hierarchical analysis of molecular variance for *Lepus europaeus* using the four major geographical areas of sampling as the levels of grouping, or without grouping.

Structure	Source of variation	Total variance (%)	Fixation indices (F_{ST})	*P*-value
No structure	Among populations	53.51	0.53507	0.00001
	Within populations	46.49		
Four major areas	Among regions	50.43	0.61011	0.00001
	Among populations within groups	10.59	0.21352	0.00001
	Within populations	38.99	0.50425	0.00001

The percentage of total variance, the *F* statistics, and the probability (*P*) estimated from permutation tests are given at each hierarchical level (Excoffier *et al.*, 1992).

Table 3 Mismatch distribution results of the pooled populations from the four major areas in which *Lepus europaeus* is found.

	Populations from			
	SEEa	AMa	CEUa	OVERa
Parameters				
S	144	58	36	103
θ_0	6.764	0.01	0.000	8.432
θ_1	5265.00	94.453	3838.75	39.385
τ	12.752	13.346	5.580	22.041
Goodness-of-fit test				
SSD	0.004	0.005	0.0013	0.007
P	0.610	0.270	0.280	0.450
Tajima's *D*	−0.794	−0.181	−2.082	−0.593
P	0.227	0.446	0.0077	0.296

The parameters of the model of sudden expansion (Rogers & Harpending, 1992) are presented as well as a goodness-of-fit test to the model; Tajima's (1989) *D*-test value and its statistical significance are also given.

S, number of polymorphic sites; θ_0, pre-expansion and θ_1, post-expansion population sizes; τ, time in number of generations elapsed since the sudden expansion episode; SSD, sum of squared deviations. SEEa, south-eastern European area; AMa, Anatolian/Middle East area; CEUa, central European area; OVERa, overlap area.

parameters estimated for each area, only the CEUa was close to an expected Poisson model, indicating recent population expansion.

Sequence analysis and comparison with other studies

Of the 464 CR-I sites examined, 104 were variable, 76 of which were parsimony-informative (data not shown, available on request). Sequence divergence for the CR-I region ranged from 0.22% to 10.54% with an average of 4.65%. According to the NJ tree (Fig. 6), MP and Bayesian analyses (not shown), 68 out of the 69 sequences clustered, with high bootstrap values, within the main four haplogroups they came from. The sequence AM30 from an individual defined as INTERMEDIATE based on the RFLP analysis (the individual with the haplotype 105; Fig. 2) failed to cluster separately from the AMh, very probably as a result of the much smaller mtDNA CR-I region surveyed by sequence analysis compared with the region surveyed by means of RFLP. Therefore, the INTERh was not represented in the sequence NJ tree. Sequence divergence within haplogroups was 1.29% (0.22–2.42%), 3.38% (0.22–7.12%), 0.70% (0.22–1.31%) and 0.77% (0.22–1.10%) for the SEEh, AMh, EUh-A and EUh-B, respectively. Mean and net sequence divergences between haplogroups ranged from 1.47% and 0.73% (EUh-A/EUh-B) to 7.16% and 4.83% (AMh/EUh-A). All 43 *L. europaeus* sequences reported to GenBank by J. Fickel, A. Schmidt, H. Spittler and C. Pitra (unpublished data) clustered within the EUh-A (Fig. 6). Nineteen of the 22 sequences described by Pierpaoli *et al.* (1999), mainly from Italy but also from Austria, Hungary, Romania and Serbia, corresponding to 38 individuals, also clustered within the EUh-A (Fig. 6). One sequence (Leu3) from southern Italy clustered within the EUh-B, and the remaining two sequences (Leu1 and Leu2 from central and northern Italy, respectively) clustered within the SEEh (Fig. 6). On the other hand, none of the 72 sequences reported in Kasapidis *et al.* (2005) clustered within the EUh-A (Fig. 6). Fifty-six sequences corresponded either to the SEEh or to the EUh-B. The other 16 sequences clustered within the AMh. The majority of them (nine) came from Cyprus and Greek islands off the Anatolian coast (Samos, Lesvos, Rhodes, Chios), six from north-eastern Greece and one from northern Israel.

DISCUSSION

Phylogeography of European brown hares

MtDNA data indicated five major haplogroups (AMh, SEEh, EUh-A, EUh-B, INTERh) with a distinct geographic distribution pattern very well supported by the *F*-statistics. In fact, 59.8% of the overall mtDNA variability was as a result of partitioning into these five haplogroups. The corresponding geographical partitioning of the mtDNA variability was also evident from the highly significant *F*-statistics and from the highly significant differences of the MDS-coordinates of pairwise net nucleotide divergence values among populations across the four areas (AMa, SEEa, OVERa, CEUa). Grouping inferred by mtDNA analysis was further confirmed by sequence analysis, which provided a higher resolution of the four haplogroups. Moreover, the three sequence data sets retrieved from GenBank fitted phylogeographically to the haplogroups identified in this study, providing further unambiguous support to this partitioning pattern.

Interestingly, our data indicate the presence of haplotypes of all five haplogroups in Bulgaria and north-eastern Greece, an area that represents a large overlap zone (OVERa), which was also implied by Kasapidis *et al.* (2005) in their analysis of the brown hare. In that zone we recovered 31.3% of all haplotypes traced here, identifying this part of the south-eastern Balkans as the region with the highest mtDNA nucleotide diversity.

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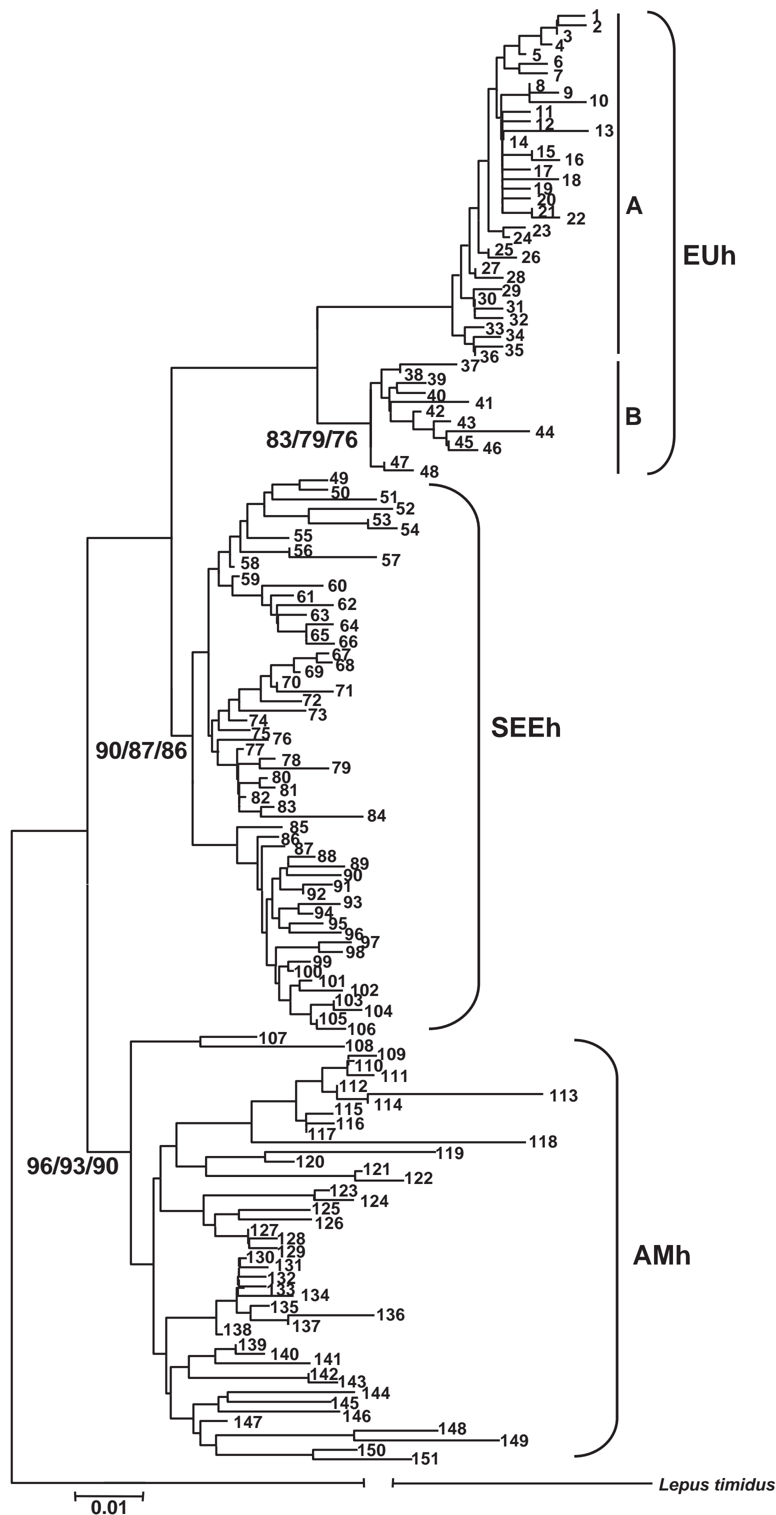

Figure 6 Neighbour-joining (NJ) phylogenetic tree for *Lepus europaeus*, using the HKY85 model with γ correction among the aligned nucleotides of the mtDNA control region (CR) for the sequences combining our data set with data from GenBank (contributed by J. Fickel, A. Schmidt, H. Spittler and C. Pitra, unpublished data), from Kasapidis *et al.* (2005) and from Pierpaoli *et al.* (1999). *Lepus timidus* was used as outgroup. Numbers at the internodes of the major clusters indicate percentage posterior probabilities from the Bayesian analysis and bootstrap, and reliability percentage values computed in NJ and maximum parsimony analyses (1000 replicates), respectively. EUh, European type haplogroup; SEEh, south-eastern European type haplogroup; AMh, Anatolian/Middle Eastern type haplogroup. Details on the numbering of haplotypes in the phylogenetic tree resulting from the NJ analysis are given in Appendix S1.

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Our data so far suggest that OVERa does not extend into Anatolia, as speculated by Kasapidis *et al.* (2005). However, samples from north-western Anatolia must be studied to confirm this preliminary finding. Among the haplotypes found in OVERa, three (99, 100, 105 in haplogroup INTERh) (Figs 2 & 3) hold an intermediate position between the AMh and the SEEh, as revealed by the network MJ analysis. The connection of the SEEh and the AMh through these three haplotypes (Bayesian and network MJ analyses), their restricted occurrence only in OVERa, and the simultaneous presence of both the EUh-A and the EUh-B in OVERa suggest that these three haplotypes might be the remainders of an ancient gene pool from which the current AMh and SEEh have both evolved. Missing data from some parts of the central Balkans, Romania, and areas north of the Black Sea, as well as from Turkish areas neighbouring Greece, prohibit further speculation. The network analysis indicates that only three haplotypes from the SEEh (15, 16 and 19) were ancestral to the EUh-B. Three haplotypes of the EUh-B (55, 56, 73) showed close relationships with three haplotypes of the EUh-A (77, 79, 84) (Fig. 3). Haplotype 56 of the EUh-B is connected by only one, so far undetected, haplotype, to haplotype 79 of the EUh-A, which occurs in Bulgaria, northern Serbia and in large parts of central, north-central, north-western and south-western Europe. Similarly, haplotype 73 of the EUh-B, which occurs only in Bulgaria and northern and north-eastern Greece, is connected to haplotype 77 of the EUh-A by only one, so far undetected, haplotype. A third haplotype from Bulgaria, north and north-eastern Greece (55, EUh-B) is also connected by only one, so far unidentified, haplotype to a haplotype that was found only in the Netherlands (84, EUh-A). Thus, all presently recovered haplotypes of central, north-western and south-western Europe are descendants of two haplotypes (77 and 79, EUh-A), which were also found in Bulgaria. The close phylogenetic relationships between all these haplotypes, occurring in large parts of central, north-western and south-western Europe, support the idea of rapid colonization of these areas by brown hares with the amelioration of the climate following the LGM (e.g. Corbet, 1986).

The divergence pattern of mtDNA haplotypes within the EUh-A indicates only shallow genetic differentiation across central, north-western and south-western Europe. This does not support the existence of a late glacial refugium for brown hares in the Iberian Peninsula (Corbet, 1986; Suchentrunk *et al.*, 2000), nor does it indicate post-glacial gene flow from eastern European or western Siberian regions (Suchentrunk *et al.*, 2000). Thus, our data do not provide support for Bilton *et al.*'s (1998) hypothesis of post-glacial migration of several mammalian species from eastern European or western Siberian regions into central Europe. Rather, they suggest that the colonization of large parts of Europe by brown hares started from a late glacial or early Holocene source population in the central or south-central Balkans. This scenario fits with the general post-glacial expansion pattern of populations from southern (Mediterranean) refugia, as described by Taberlet *et al.* (1998) and Hewitt (2000). This northward expansion of the colonizing populations was relatively rapid and probably reached as far as the Iberian Peninsula. The mismatch distribution, indicating a recent expansion, and the star-like connection pattern of haplotypes from the CEUa after the network analysis provide further support for the expansion hypothesis (Slatkin & Hudson, 1991).

Brown hares from the British Isles, which are considered as a separate subspecies (*L. e. occidentalis*), are phylogenetically closely linked to hares from northern continental Europe. All hares from the northern UK population have one haplotype (83, EUh-A) that has so far been identified only in the northern German population close to the Danish border, whereas the population from the southern UK has exclusively haplotype 75, which is the most common and widespread haplotype of the EUh-A and which occurs also in the northern German population. These findings identify at least northern Germany as a possible source region for the origin of the current British brown hare populations. They might have reached the British Isles either by natural migration across the land-bridge that existed during the early Holocene between the British Isles and continental Europe, or they might have been introduced by the ancient Romans or even earlier by Neolithic or Mesolithic settlers (Corbet, 1986; Suchentrunk *et al.*, 2006). As expected, mtDNA variability in the British brown hares is very low.

Five brown hares from the southern Swiss Alps exhibited three phylogenetically closely related haplotypes (66, 67, 68) of the SEEh. One of these (67) was the only one found in the four Italian brown hares and it was also present in a population from central-western Greece (Vradeto). This distributional pattern suggests natural gene flow from north-western Greek populations into central Italy via the region of the northern Adriatic that formed a land-bridge between the Italian peninsula and the Balkans during the late Pleistocene and the early Holocene. This is also congruent with the combined analysis of our data and those of Pierpaoli *et al.* (1999), showing that the two haplotypes (Leu1 and Leu2) from Italy are phylogenetically very closely related to the SEEh (Fig. 6), and it does not support the existence of the subspecies *L. e. meridiei* in the Italian peninsula (Pierpaoli *et al.*, 1999). Under such a scenario, the few Swiss hares belonging to the SEEh could be the descendants of an ancient Italian gene pool that expanded northwards after the withdrawal of the south Alpine glaciers. Alternatively, the presence of the SEEh in Italy, Switzerland and Austria could be the result of recent translocations.

According to the combined analysis of our data and those of Pierpaoli *et al.* (1999), haplotypes belonging to the EUh-A are predominant in Italy. A very plausible phylogenetic scenario is that those haplotypes could have reached the Italian peninsula during a phase of dispersion of brown hares after the LGM (Pierpaoli *et al.*, 1999). An alternative or complementary scenario, based on the information that over-hunting and releases of brown hares in Italy began early in 1900 and continued on a large scale up until, at least, the 1990s (Pierpaoli *et al.*, 1999), could explain the massive presence of the EUh-A in central and northern Italy as the result of recent translocations of allochthonous brown hares. This practice

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might have replaced possibly native original haplotypes of the SEEh in large parts of Italy.

The presence of AM haplotypes in the south-eastern Balkans indicates ancient gene flow from Anatolia to Europe across the late Pleistocene Bosporus land-bridge, which disappeared only *c.* 8000 years ago with the rising sea level (Gökaşan *et al.*, 1997). This interpretation fits with the hypothesis that hares managed to migrate from Anatolia into parts of the south-eastern Balkans and they even colonized some Greek islands off the coast of Anatolia in the late glacial period and/or early Holocene when these islands were still connected to Anatolia (Kasapidis *et al.*, 2005). Hares from the OVERa with haplotypes of the AMh had similar levels of nucleotide diversity (0.0097) to hares from Turkey and Israel (0.012), an indication that there has not been a particular loss of mtDNA diversity in the course of the migration from Anatolia to south-eastern Europe. This sustained genetic diversity is probably the result of the maintenance of large enough effective population sizes through long-term migration into the OVERa, or several colonization waves, or at least one short period of massive migration. In contrast to the case for the EUh-A, population genetic analysis of both RFLP and sequence data showed that the AM haplogroup/area harboured the highest nucleotide diversity except for the OVERa, very probably reflecting an unbroken history of the species in Anatolia. Anatolia has been considered a biogeographical crossroads for many mammalian species, with continuous gene flow from the Euro-Siberian, Irano-Turanian and Saharo-Sindian regions to Anatolia during the Pleistocene and the Holocene (Cheylan, 1991; Sert *et al.*, 2005).

Considering the high observed F_{ST}-values within autochthonous Greek hares, indicating generally little mtDNA gene flow and high philopatry of females (see also Mamuris *et al.*, 2001), the OVERa appears to be quite extensive. Unintentional anthropogenic translocations of introgressed hares from south-eastern parts of Bulgaria to western and northern Bulgaria might have artificially extended the OVERa within Bulgaria. Moreover, the existence of the relatively large OVERa and the limited presence of the INTERh only in OVERa indicates that admixture of hare genomes with considerably divergent mtDNA might not be an occasional phenomenon with little evolutionary consequence; rather, such divergent lineages might be passed on successfully over several generations and thus might considerably bias phylogenetic and taxonomic inferences, if only a few samples from restricted geographical provenances are studied. A pronounced example of introgressive hybridization was reported for the Iberian hare (*L. granatensis*), which is considerably introgressed by ancient mountain hare-type mtDNA (Melo-Ferreira *et al.*, 2005; Alves *et al.*, 2006), and for various *Lepus* species from central and Far East Asia (Ben Slimen *et al.*, 2007).

Anthropogenic transfers/breeding and introductions

Apart from the historical ancient translocations of brown hares that could have influenced the shaping of mtDNA phylogeographic patterns in the studied area (e.g. Kasapidis *et al.*, 2005; Suchentrunk *et al.*, 2006), the restocking programmes with the introduction of allochthonous individuals that have been carried out in several European countries in the past decades might have seriously influenced the historical distribution and genetic integrity of indigenous hare populations and species (Flux, 1983; Pierpaoli *et al.*, 1999). Breeding stations in Bulgaria, Slovakia, Hungary and Poland traditionally functioned as source populations for such restocking operations during the 1970s and 1980s, and still do so, particularly for hunting societies in diverse parts of central and western Europe.

The impact of uncontrolled introductions and releases of brown hares in Greece has been discussed in previous studies (Mamuris *et al.*, 2001; Kasapidis *et al.*, 2005; Stamatis *et al.*, 2007). Some of the released hares could have harboured haplotypes of the SEEh. This might be the case for the hares from Austria and Switzerland belonging to the SEEh. On the other hand, the absence of hares of the SEEh in other parts of central and north-western Europe could be the result either of the absence of massive releases of hares from south-eastern Europe, or of the lack of an effect of massive releases on the mtDNA composition of the studied populations. Although massive restocking operations in central Europe could also be partly responsible for the wide expansion of certain haplotypes of the EUh-A (i.e. 75, 76), we nevertheless trace a phylogeographic signal in this part of Europe, as evidenced by the phylogenetic relationships between the post-glacial haplotypes and their geographic distribution. In contrast to the case for central Europe, the dispersion of the haplotypes of the SEEh in Greece is geographically very restricted. In fact, in the latter area the number of common haplotypes between populations decreased with geographic distance in general, whereas the percentage of unique haplotypes within each region remained high.

To conclude, all our results concordantly indicate that post-glacial colonization of large parts of Europe started from only one late glacial/early Holocene source region in the central or south-central Balkans. The presence of numerous haplotypes of all five haplogroups in Bulgaria and north-eastern Greece, including Anatolian/Middle Eastern ones, indicates a large overlap zone. Anatolian/Middle Eastern haplotypes in this overlap zone reveal gene flow from Anatolia to Europe across the late Pleistocene Bosporus land-bridge. Although various restocking operations could be partly responsible for the presence of unexpected haplotypes in certain areas, we nevertheless trace a strong phylogeographic signal throughout all the regions under study.

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REFERENCES

Alves, P.C., Harris, D.J., Melo-Ferreira, J., Branco, M., Suchentrunk, F., Boursot, P. & Ferrand, N. (2006) Hares on thin ice: introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. *Molecular Phylogenetics and Evolution*, **16,** 605–618.

Angermann, R. (1983) The taxonomy of Old World *Lepus*. *Acta Zoologica Fennica*, **174,** 17–21.

Ben Slimen, H., Suchentrunk, F., Memmi, A. & Ben Ammar Elgaaied, A. (2005) Biochemical genetic relationships among Tunisian hares (*Lepus* sp.), South African cape hares (*L. capensis*), and European brown hares (*L. europaeus*). *Biochemical Genetics*, **43,** 577–596.

Ben Slimen, H., Suchentrunk, F., Shahin, A.B. & Ben Ammar Elgaaied, A. (2007) Phylogenetic analysis of mtCR-1 sequences of Tunisian and Egyptian hares (*Lepus* sp. or spp., Lagomorpha) with different coat colours. *Mammalian Biology*, **72,** 224–239.

Bennett, K., Tzedakis, P.C. & Willis, K.J. (1991) Quaternary refugia of north European trees. *Journal of Biogeography*, **18,** 103–115.

Bilton, D.T., Mirol, P.M., Mascheretti, S., Fregda, K., Zima, J. & Searle, J.B. (1998) Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. *Proceedings of the Royal Society B: Biological Sciences*, **265,** 1219–1226.

Cheylan, G. (1991) Patterns of Pleistocene turnover, current distribution and speciation among Mediterranean mammals. *Biogeography of Mediterranean invasions* (ed. by R.H. Groves and F. Di Castri), pp. 227–262. Cambridge University Press, Cambridge.

Corbet, G.B. (1986) Relationships and origins of the European lagomorphs. *Mammal Review*, **16,** 105–110.

De Beaufort, F. (1991) La faune des mammifères de Grèce: caractéristiques, endèmisme, particularismes. *Biologia Gallo-Hellenica*, **18,** 99–106.

Excoffier, L., Smouse, P. & Quattro, J. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131,** 479–491.

Felsenstein, J. (2004) *PHYLIP (phylogeny inference package) version 3.6*. Distributed by the author, Department of Genome Sciences, University of Washington, Seattle.

Fickel, J., Lieckfeld, D. & Pitra, C. (1999) Analyze der genetischen Diversität und Struktur in benachbarten Populationen des Feldhasen (*Lepus europaeus*, Pallas 1778). *Zeitschrift fur Jagdwissenschaft*, **45,** 230–237.

Fickel, J., Schmidt, A., Putze, M., Spittler, H., Ludwig, A., Streich, W.J. & Pitra, C. (2005) Genetic structure of populations of European brown hare: implications for management. *Journal of Wildlife Management*, **69,** 760–770.

Flux, J.E.C. (1983) Introduction to taxonomic problems in hares. *Acta Zoologica Fennica*, **174,** 7–10.

Flux, J.E.C. & Angermann, R. (1990) The hares and jackrabbits. *Rabbits, hares and pikas. Status survey and conservation action plan* (ed. by J.A. Chapman and J.E.C. Flux), pp. 61–94. IUCN/SSC Lagomorph Specialist Group, Gland, Switzerland, and Cambridge, UK.

Frenzel, B., Pécsi, M. & Velichko, A.A. (1992) *Atlas of paleoclimates and paleoenvironments of the Northern Hemisphere, Late Pleistocene–Holocene*. Geographical Research Institute, Hungarian Academy of Sciences, Budapest, G. Fischer Verlag, Stuttgart.

Gissi, C., Gullberg, A. & Arnason, U. (1998) The complete mitochondrial DNA sequence of the rabbit, *Oryctolagus cuniculus*. *Genomics*, **50,** 161–169.

Gökaşan, E., Demirbağ, E., Oktay, F.Y., Ecevitoğlu, B., Şimşek, M. & Yüce, H. (1997) On the origin of the Bosporus. *Marine Geology*, **140,** 183–199.

Hartl, G.B., Suchentrunk, F., Nadlinger, K. & Willing, R. (1993) An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. *Acta Theriologica*, **38,** 33–57.

Hasegawa, M., Kishino, M. & Yano, T. (1985) Dating the human–ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22,** 160–174.

Hewitt, G.M. (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405,** 907–913.

Huelsenbeck, J.P. & Ronquist, F. (2001) MrBayes: Bayesian inference of phylogeny. *Bioinformatics*, **17,** 754–755.

Kasapidis, P., Suchentrunk, F., Magoulas, A. & Kotoulas, G. (2005) The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuactions and anthropogenic translocations. *Molecular Phylogenetics and Evolution*, **34,** 55–66.

Kumar, S., Tamura, K. & Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5,** 150–163.

Mamuris, Z., Sfougaris, A.I. & Stamatis, C. (2001) Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDBNA–PCR analysis: Implications for conserving genetic diversity. *Biological Conservation*, **101,** 187–196.

Mamuris, Z., Sfougaris, A.I., Stamatis, C. & Suchentrunk, F. (2002) Genetic structure of Greek brown hare (*Lepus europaeus*) populations based on the random amplified polymorphic DNA (RAPD) method. *Biochemical Genetics*, **40,** 323–338.

McElroy, D., Moran, P., Bermingham, E. & Kornfield, J. (1991) *The restriction enzyme analysis package, version 4.0*. University of Maine, Orono, ME.

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Melo-Ferreira, J., Boursot, P., Suchentrunk, F., Ferrand, N. & Alves, P.C. (2005) Invasion from the cold: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. *Molecular Ecology*, **14,** 2459–2464.

Nei, M. (1987) *Molecular evolutionary genetics*. Columbia University Press, New York.

Nei, M. & Tajima, F. (1981) DNA polymorphism detectable by restriction endonucleases. *Genetics*, **97,** 145–163.

Pierpaoli, M., Riga, F., Trocchi, V. & Randi, E. (1999) Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. *Molecular Ecology*, **8,** 1805–1817.

Posada, D. & Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14,** 817–818.

Raymond, M. & Rousset, F. (1995) An exact test for population differentiation. *Evolution*, **49,** 1280–1283.

Rogers, A.R. & Harpending, H. (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9,** 552–569.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4,** 406–425.

Schneider, S., Roessli, D. & Excoffier, L. (2000) *ARLEQUIN, version 2.0. A software for population genetic data analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva.

Sert, H., Suchentrunk, F. & Erdoğăn, A. (2005) Genetic diversity within Anatolian brown hares (*Lepus europaeus* Pallas, 1778) and differentiation among Anatolian and European populations. *Mammalian Biology*, **70,** 171–186.

Slatkin, M. & Hudson, R.R. (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, **129,** 555–562.

Stamatis, C., Suchentrunk, F., Sert, H., Triantaphyllidis, C. & Mamuris, Z. (2007) Genetic evidence for survival of released captive-bred brown hares (*Lepus europaeus*) during restocking operations in Greece. *Oryx*, **41,** 548–551.

Suchentrunk, F., Michailov, C., Markov, G. & Haiden, A. (2000) Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoogeographical crossroads. *Acta Theriologica*, **45,** 1–12.

Suchentrunk, F., Mamuris, Z., Sfougaris, A.I. & Stamatis, C. (2003) Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. *Biochemical Genetics*, **41,** 127–140.

Suchentrunk, F., Ben Slimen, H., Stamatis, C., Sert, H., Scandura, M., Apollonio, M. & Mamuris, Z. (2006) Molecular approaches revealing prehistoric, historic, or recent translocations and introductions of hares (genus *Lepus*) by humans. *Human Evolution*, **21,** 151–165.

Swofford, D.L. (1998) *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4*. Sinauer Associates, Sunderland, MA.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G. & Cosson, J.F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7,** 453–464.

Tajima, F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123,** 585–595.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24,** 4876–4882.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Haplotype names and corresponding identification numbers appearing in Fig. 6.
Appendix S2 Composite haplotypes and respective absolute frequencies found in the *Lepus europaeus* populations studied.
Appendix S3 Pairwise sequence divergence estimates among the 112 haplotypes of *Lepus europaeus* and the one of *Lepus timidus* assessed by the restriction fragment length polymorphism analysis.
Appendix S4 Phylogenetic tree resulting from the neighbour-joining (NJ) analysis clustering the 112 restriction fragment length polymorphism haplotypes from *Lepus europaeus.*

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BIOSKETCH

Costas Stamatis has had substantial experience in the study of the population genetics of wildlife and farmed animals. Currently, he is a doctoral student of population genetics working on the phylogeography of the brown hare, and a research technician working on several ongoing projects.

Editor: Brett Riddle

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Appendix S1. Haplotype names and corresponding identification numbers at the tips of branches in the dendrogram of Fig. 6 in parentheses, sample sizes, sampling localities and accession numbers of the brown hare (*Lepus europaeus*) mtDNA CR-I sequences

This study			
EUA01 (14)	1	Aberdeenshire, Scotland (N-UK)	***DQ469656***
EUA02 (8)	1	Offenburg, South-West Germany (SW-D)	***DQ469661***
EUA03 (14)	1	Offenburg, South-West Germany (SW-D)	***DQ469660***
EUA04 (14)	1	Eastern Austria (A)	***DQ469667***
EUA05 (5)	1	Membrolles, France (F)	***DQ469665***
EUA06 (14)	1	Wiltshire, South England (S-UK)	***DQ469654***
EUA07 (8)	1	Airport Schipol, The Netherlands (NL)	***DQ469657***
EUA08 (14)	1	Niebüll, North Germany (N-D)	***DQ469655***
EUA09 (14)	1	Switzerland (CH)	***DQ469663***
EUA10 (8)	1	Poland (PL)	***DQ469669***
EUA11 (11)	1	Membrolles, France (F)	***DQ469659***
EUA12 (14)	1	Switzerland (CH)	***DQ469662***
EUA13 (4)	1	Switzerland (CH)	***DQ469664***
EUA14 (25)	1	Eastern Austria (A)	***DQ469666***
EUA15 (30)	1	Eastern Austria (A)	***DQ469658***
EUA16 (30)	1	Poland (PL)	***DQ469668***
EUB01 (42)	1	Southern Greece (S-GR)	***DQ469671***
EUB02 (47)	1	Crete (Cr-GR)	***DQ469670***
EUB03 (40)	1	Crete (Cr-GR)	***DQ469675***
EUB04 (39)	1	North-Eastern Greece (NE-GR)	***DQ469672***
EUB05 (39)	1	Spilia (Sp-GR)	***DQ469676***
EUB06 (47)	1	Bulgaria (BL)	***DQ469673***
EUB07 (40)	1	Spilia (Sp-GR)	***DQ469674***
SEE01 (91)	1	North-Eastern Greece (NE-GR)	***DQ469648***
SEE02 (60)	1	Western Greece, Vradeto (Vr-GR)	***DQ469683***
SEE03 (98)	1	Elassona (El-GR)	***DQ469680***
SEE04 (103)	1	Velestino (Ve-GR)	***DQ469651***
SEE05 (94)	1	Western Greece, Vradeto (Vr-GR)	***DQ469684***
SEE06 (94)	1	Central Greece, Pyrra (Py-GR)	***DQ469685***
SEE07 (88)	1	Western Greece, Vradeto (Vr-GR)	***DQ469652***
SEE08 (93)	1	Spilia (Sp-GR)	***DQ469650***
SEE09 (86)	1	Velestino (Ve-GR)	***DQ469677***
SEE10 (97)	1	Elassona (El-GR)	***DQ469679***
SEE11 (100)	1	Velestino (Ve-GR)	***DQ469681***
SEE12 (99)	1	Western Greece, Vradeto (Vr-GR)	***DQ469653***
SEE13 (63)	1	Western Greece, Vradeto (Vr-GR)	***DQ469649***
SEE14 (95)	1	Central Greece, Pyrra (Py-GR)	***DQ469682***
SEE15 (61)	1	Southern Greece (S-GR)	***DQ469678***
SEE16 (92)	1	Elassona (El-GR)	***DQ469686***
AM01 (118)	1	North Israel (N-ISR)	***DQ469698***
AM02 (112)	1	North Israel (N-ISR)	***DQ469645***

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AM03 (111)	1	North Israel (N-ISR)	***DQ469646***
AM04 (117)	1	North Israel (N-ISR)	***DQ469707***
AM05 (115)	1	North Israel (N-ISR)	***DQ469708***
AM06 (114)	1	North Israel (N-ISR)	***DQ469706***
AM07 (116)	1	North Israel (N-ISR)	***DQ469699***
AM08 (109)	1	North Israel (N-ISR)	***DQ469643***
AM09 (110)	1	North Israel (N-ISR)	***DQ469647***
AM10 (113)	1	North Israel (N-ISR)	***DQ469701***
AM11 (148)	1	Turkey (TR)	***DQ469691***
AM12 (149)	1	Turkey (TR)	***DQ469697***
AM13 (108)	1	Turkey (TR)	***DQ469704***
AM14 (119)	1	Turkey (TR)	***DQ469694***
AM15 (120)	1	Turkey (TR)	***DQ469709***
AM16 (123)	1	Turkey (TR)	***DQ469695***
AM17 (124)	1	Turkey (TR)	***DQ469696***
AM18 (144)	1	Turkey (TR)	***DQ469693***
AM19 (141)	1	Turkey (TR)	***DQ469702***
AM20 (107)	1	Turkey (TR)	***DQ469644***
AM21 (145)	1	Turkey (TR)	***DQ469705***
AM22 (146)	1	Turkey (TR)	***DQ469642***
AM23 (151)	1	Turkey (TR)	***DQ469700***
AM24 (147)	1	Turkey (TR)	***DQ469703***
AM25 (150)	1	Turkey (TR)	***DQ469692***
AM26 (136)	1	Thrace, North-Eastern Greece (NE-GR)	***DQ469687***
AM27 (137)	1	Thrace, North-Eastern Greece (NE-GR)	***DQ469690***
AM28 (132)	1	Thrace, North-Eastern Greece (NE-GR)	***DQ469688***
AM29 (135)	1	North-Eastern Greece (NE-GR)	***DQ469689***
AM30 (125)	1	Bulgaria (BL)	***DQ469710***
Pierpaoli *et al.* (1999)			
Leu 1 (50)	1	Central Italy	***AF157453***
Leu 2 (49)	1	Northern Italy	***AF157454***
Leu 3 (43)	1	Southern Italy	***AF157452***
Leu 4 (14)	4	Central and southern Italy; Uruguay	***AF157447***
Leu 5 (20)	1	Northern Italy	***AF157449***
Leu 6 (13)	1	Central Italy	***AF157448***
Leu 7 (14)	2	Southern Italy	***AF157446***
Leu 8 (25)	1	Northern Italy	***AF157450***
Leu 9 (14)	1	Central Italy	***AF157451***
Leu 10 (25)	2	Northern and southern Italy	***AF157433***
Leu 11 (26)	1	Southern Italy	***AF157434***
Leu 12 (1)	2	Hungary; southern Italy	***AF157435***
Leu 13 (3)	1	Southern Italy	***AF157436***
Leu 14 (3)	11	Central and northern Italy; Serbia; Romania	***AF157437***
Leu 15 (4)	2	Hungary; Serbia	***AF157438***
Leu 16 (6)	1	Austria	***AF157439***
Leu 17 (33)	1	Northern Italy	***AF157445***
Leu 18 (31)	1	Northern Italy	***AF157442***
Leu 19 (32)	1	Northern Italy	***AF157441***

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Leu 20 (30)	3	Hungary; Serbia; northern Italy	***AF157440***
Leu 21 (28)	1	Hungary	***AF157443***
Leu 22 (27)	1	Northern Italy	***AF157444***
Kasapidis *et al.* (2005)			
A1 (130)	5	Thrace; Central Macedonia	***AY466782***
A2 (131)	1	Thrace	***AY466783***
A3 (133)	1	Thrace	***AY466784***
A4 (134)	1	Eastern Macedonia	***AY466785***
A5 (138)	1	Thrace	***AY466786***
A6 (139)	3	Samos island	***AY466787***
A7 (140)	3	Lesvos island	***AY466788***
A8 (142)	1	Rhodes island	***AY466789***
A9 (143)	1	Rhodes island	***AY466790***
A10 (122)	1	Chios island	***AY466791***
A11 (121)	2	Chios island	***AY466792***
A12 (129)	1	Cyprus	***AY466793***
A13 (128)	1	Cyprus	***AY466794***
A14 (127)	1	Cyprus	***AY466795***
A15 (126)	1	Eastern Macedonia	***AY466796***
A16 (117)	1	Northern Israel	***AY466797***
B1 (77)	1	Crete: Iraklio	***AY466798***
B2 (77)	1	Crete: Hania	***AY466799***
B3 (84)	1	Crete: Iraklio	***AY466800***
B4 (83)	1	Crete: Iraklio	***AY466801***
B5 (81)	1	Crete: Hania	***AY466802***
B6 (80)	1	Crete: Iraklio	***AY466803***
B7 (82)	1	Crete: Hania	***AY466804***
B8 (78)	2	Kythira island	***AY466805***
B9 (78)	1	Kythira island	***AY466806***
B10 (56)	3	Central Peloponnese	***AY466807***
B11 (57)	1	Central Peloponnese	***AY466808***
B12 (79)	1	Naxos island	***AY466809***
B13 (44)	2	Eastern Macedonia	***AY466810***
B14 (46)	1	Central Macedonia	***AY466811***
B15 (45)	1	Bulgaria	***AY466812***
B16 (58)	1	Central Macedonia	***AY466813***
B17 (41)	1	Thrace	***AY466814***
B18 (59)	2	Eastern Macedonia	***AY466815***
B19 (38)	3	Crete: Ierapetra	***AY466816***
B20 (37)	1	Central Greece	***AY466817***
B21 (104)	1	Central Macedonia	***AY466818***
B22 (105)	1	Western Macedonia	***AY466819***
B23 (106)	1	Western Macedonia	***AY466820***
B24 (101)	1	Central Macedonia	***AY466821***
B25 (102)	1	Western Macedonia	***AY466822***
B26 (87)	1	Epirus	***AY466823***
B27 (86)	1	Western Macedonia	***AY466824***
B28 (96)	1	Western Macedonia	***AY466825***

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B29 (86)	1	Central Greece	***AY466826***
B30 (86)	1	Epirus	***AY466827***
B31 (89)	1	Bulgaria	***AY466828***
B32 (90)	1	Bulgaria	***AY466829***
B33 (64)	1	Western Macedonia	***AY466830***
B34 (65)	1	Central Greece	***AY466831***
B35 (66)	3	Central Greece	***AY466832***
B36 (65)	1	Central Macedonia	***AY466833***
B37 (62)	2	Central Macedonia	***AY466834***
B38 (85)	1	Western Macedonia	***AY466835***
B39 (38)	1	Thrace	***AY466836***
B40 (68)	1	Bulgaria	***AY466837***
B41 (67)	1	Bulgaria	***AY466838***
B42 (69)	3	E. Macedonia; Thrace	***AY466839***
B43 (70)	1	Thrace	***AY466840***
B44 (71)	2	Central Macedonia	***AY466841***
B45 (72)	1	Eastern Macedonia	***AY466842***
B46 (73)	1	Thrace	***AY466843***
B47 (74)	3	Thrace	***AY466844***
B48 (59)	1	Central Macedonia	***AY466845***
B49 (75)	1	Eastern Macedonia	***AY466846***
B50 (76)	1	Eastern Macedonia	***AY466847***
B51 (48)	2	Western Macedonia	***AY466848***
B52 (54)	1	Eastern Macedonia	***AY466849***
B53 (53)	1	Eastern Macedonia	***AY466850***
B54 (52)	1	Eastern Macedonia	***AY466851***
B55 (51)	2	Lefkada island	***AY466852***
B56 (55)	1	Thrace	***AY466853***
Fickel *et al.* (unpublished)			
01 (14)	1	Germany	***AY103494***
02 (30)	1	Germany	***AY103495***
05 (14)	1	Germany	***AY103498***
06 (8)	1	Germany	***AY103499***
07 (14)	1	Germany	***AY103500***
08 (3)	1	Germany	***AY103501***
12 (5)	1	Germany	***AY103505***
13 (15)	1	Germany	***AY103506***
14 (18)	1	Germany	***AY103507***
16 (23)	1	Germany	***AY103509***
17 (25)	1	Germany	***AY103510***
18 (22)	1	Germany	***AY103511***
20 (14)	1	Germany	***AY103513***
25 (21)	1	Germany	***AY103518***
26 (14)	1	Germany	***AY103519***
29 (24)	1	Germany	***AY103522***
30 (30)	1	Germany	***AY103523***
32 (25)	1	Germany	***AY103525***
33 (27)	1	Germany	***AY103526***

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34 (35)	1	Germany	***AY103527***
36 (12)	1	Germany	***AY103529***
38 (16)	1	Germany	***AY103531***
39 (2)	1	Germany	***AY154661***
40 (29)	1	Germany	***AY154662***
45 (19)	1	Germany	***AY163356***
47 (25)	1	Germany	***AY163358***
48 (14)	1	Germany	***AY163359***
49 (14)	1	Germany	***AY163360***
50 (14)	1	Germany	***AY163361***
52 (14)	1	Germany	***AY163363***
56 (25)	1	Germany	***AY163367***
57 (7)	1	Germany	***AY163368***
58 (34)	1	Germany	***AY163369***
59 (7)	1	Germany	***AY163370***
60 (17)	1	Germany	***AY163371***
63 (11)	1	Germany	***AY163374***
64 (10)	1	Germany	***AY163375***
65 (31)	1	Germany	***AY163376***
66 (11)	1	Germany	***AY300032***
67 (14)	1	Germany	***AY300033***
68 (36)	1	Germany	***AY300034***
69 (4)	1	Germany	***AY300035***
70 (9)	1	Germany	***AY300036***

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Appendix S2

Composite haplotypes and respective absolute frequencies found in the *Lepus europaeus* populations studied. Order of enzymes is: cytochrome-b (cyt *b*)/control region (CR): *Alu*I, *Ase*I, *Ava*II, *Dde*I, *Hae*III, *Hinf*I, *Mbo*I, *Mse*I, *Msp*I, *Taq*I, *Xba*I; cytochrome oxidase I (COI): *Alu*I, *Hae*III, *Hha*I, *Hinf*I, *Bst*UI, *Mbo*I, *Ava*II; 12S-16S rRNA: *Alu*I. For the purposes of presentation nonpolymorphic enzymes were omitted.

		Cr-GR	S-GR	Za-GR	Py-GR	Vr-GR	Sp-GR	Ve-GR	El-GR	N-GR	NE-GR	Sa-BL	Bg-BL	Sz-BL	Vc-BL	Do-BL	Pv-BL	Vi-BL	I	N-HR	S-HR	YU	PL	CH	A	F	N-D	SW-D	NL	E	N-UK	S-UK	NE-TR	C-TR	SW-TR	N-ISR
1	ABAAAABAAAAAAAAAAAA				4	6		2		2			1		4		3	3			9															
2	ABAAAABAAAACAAAAAAA				1			3							7	2	3	4																		
3	ABAAAAACAAACAAAAAAA								1																											
4	ABAAAAACAAAAAAAAAAA				1				1																											
5	BBAAAAACAAAAAAAAAAA			5	9	22			2	2								2																		
6	BAAAAAAAAAAAAAAAAAA			2	4	11		2		5																										
7	BAAAAAACACAAAAAAAAA					6																														
8	BAAAAAAAAAACAAAAAAA									1																										
9	BAAAAADFAAADAAAAAAA						5																													
10	CAAAAAAABAACAABAAAA									2																										
11	CAAAAAAABAAAAABAAAA				1	3																														
12	CBAAAAAAAAABAABAAAA			11	2	4																														
13	CBAAAAAAAAAAAABAAAA	2	10	4	5	3		10	2																											
14	CBADAAAAAAAAAABAAAA			3																																
15	AAAAAAAAAAAAAAAAAAA			6		7				1											1															
16	AAAAAABAAAAAAAAAAAA			6		2	2			1						3	1																			
17	AAAAAABAAAACAAAAAAA			1			3									1																				
18	AAAAAABAAAAABAAAAAA						6																													
19	AAABAADAAAAAAAAAAAA								1																											
20	ABAAAAAAAAAAAAAAAAA			1	1				1																											
21	ABBAEAAAAAAAAAAAAAA			2																																
22	ABAABAAAABAAAAAAAAA						8																													
23	ABAABABAABAAAAAAAAA	2			4		1	2																												
24	ABAABABACBAAAAAAAAA								1																											
25	AAAAAABAABAAAAAAAAA						1																													
26	ABABAABAAAAAAAAAAAA				1																															
27	EBACCABDAAAACAAAAAA					1				1																										
28	FBAAAAAAAAAAAABAAAA				14	2	4	7	3	2																										
29	FAAAAAAAAAAAAAAAAAA					1																														
30	CBAAADAAAAABAABAAAA							7																												
31	BAAAAADFAAAAAABAAAA								1																											
32	BAAAAADFAAAAAAAAAAA						2		1																											
33	BAAAAAEEAAAAAAAAAAA							1																												
34	ACAAAABEAAACAAAAAAA				3																															
35	BBAAAAAAAAADAAAAAAA								1																											
36	DBCABABAABADAAAAAAA								1																											
37	BAAAAAFFAAAAAAAAAAA								1																											
38	BAAAAAACAAAAAAAAAAA						1	1		5																										

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Appendix S2 Continued

		Cr-GR	S-GR	Za-GR	Py-GR	Vr-GR	Sp-GR	Ve-GR	El-GR	N-GR	NE-GR	Sa-BL	Bg-BL	Sz-BL	Vt-BL	Do-BL	Pv-BL	Vi-BL	I	N-HR	S-HR	YU	PL	CH	A	F	N-D	SW-D	NL	E	N-UK	S-UK	NE-TR	C-TR	SW-TR	N-ISR
39	CBAAAAAAAAAAAAAAAAA				1				2																											
40	ABAABABAACAAAAAAAAA						1																													
41	BAAAAAAAACAAAAAAAAA					1																														
42	ABCABABAABAAAAAAAAA				1		1																													
43	CBAAADAAAAAAAABAAAA							1																												
44	ABAAAAAAAAABAABAAAA				1																															
45	AAAAAABAAAAADABAAAA									1																										
46	CAAAAAAAAAAAAABAAAA		1							1																										
47	ABAAAABAABAAAAAAAAA		2																																	
48	FBAAAAAAAAAAAAAAAAA							1																												
49	HAAAAEADADAAAAAAAAA										3																									
50	ABAEAHCBAAAAAAAAAAA										2																									
51	AAAEAHCBAAAAAAAAAAA										2																									
52	BAAAACCHAAAAAAAAAAA														1																					
53	AAAAAAAIAAADAAAAAAA																1																			
54	AACAAABAABAAAAAAAAA												1																							
55	ABAAACCBAAACAAAAAAA									1	5	1		1	3	1																				
56	AAAAACCBAAAAAAAAAAA					1				2	2		1		6		5																			
57	ACAAAJBAABAAAAAAAAA														1																					
58	ABAAAJAFABAAAAAAAAA										1		1	1																						
59	AAAEAKCBAAAAAAAAAAA											1																								
60	BBAAAAAAAAAAAAAAAAA					2				1																										
61	CBAAAAAABAAAAABAAAA					1																														
62	CBAAAAAAAAAADABAAAA					3																														
63	FBAAAABAAAAAAAAAAAA					2																														
64	ABAADCCBAAAAAAAAAAA		2																																	
65	BBAAACCBADAAAAAAAAA					1																														
66	AAAAAAADAAAAAAAAAAA																							1												
67	AAAGAAAGAAAAAAAAAAA					1													4					3												
68	AAAHAAADAAAAAAAAAAA																							1												
69	DBAAACCBAAAAAAAAAAA			2			1		1	1																										
70	DBAAACCBAAAABAAAAAA				2				5																											
71	DAAAACCBAAAAAAAAAAA									1																										
72	IBAAACCBAAAAAAAAAAA						2																													
73	ABAAACCBAAAAAAAAAAA					1				2	8				8	1	3	2																		
74	ABAACCCBAAAAAAAAAAA										1																									
75	AAAAABCBAAAAABABAAA																						5	80		5	13	16	4	2		17				
76	ABAAABCBAAAAABABAAA																						1	4		20	6	5								
77	ABAAACCBAAAAABABAAA																1			3				21	76			1								
78	ABABACCBAAAAABABAAA															1	1			1				5												
79	AAAAACCBAAAAABABAAA														1					2		22	1	9	19											
80	AAAAACHBAAAAABABAAA																						2													
81	ABAAACHBAAAAABABAAA																						1													
82	ABAAABIBAAAAABABAAA																										1									
83	AAAAABIBAAAAABABAAA																										4				16					
84	ABAAACCBAAACABABAAA																												6							

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Appendix S2 Continued

		Cr-GR	S-GR	Za-GR	Py-GR	Vr-GR	Sp-GR	Ve-GR	El-GR	N-GR	NE-GR	Sa-BL	Bg-BL	Sz-BL	Vt-BL	Do-BL	Pv-BL	Vi-BL	I	N-HR	S-HR	YU	PL	CH	A	F	N-D	SW-D	NL	E	N-UK	S-UK	NE-TR	C-TR	SW-TR	N-ISR
85	ABAAABJBAAAAABABAAA																									1										
86	ABAAABCJAAAAABABAAA																										1									
87	MAAAACCBAAAAABABAAA																								16											
88	ABAAAMCBAAAAABABAAA																								1											
89	AAAAABHBAAAAABABAAA																							1												
90	AAAAAQCBAAAAABABAAA																							1												
91	GBCDDFGAABBAAAAAAAB										16		6	2	1	1	1																		2	
92	GBCDDFMAABBAAAAAAAB												1				1																			
93	JBCAFIKKABBAAAAAAAB										1			3																						
94	GBCDAGGAABBAAAAABBB																																1			7
95	JBCAFINKABBAAAAAAAB										1																								1	3
96	LBCDAFMAABBAAAAAAAB																																		1	
97	JACADFNAABBAAAAAAAB																																	1		
98	NAAAANBAAAAAAAAAAAA															1																				
99	AACAFLAAAEAAAAAAAAB															1																				
100	ABCAFPAAABAAAAAAAAB															1																				
101	ABAAAAAIAAAAAAAAAAA														5																					
102	ABAAACCBAAAAFBABAAA																								1											
103	AAAAACCBAAAEABABAAA																								5											
104	PAAAACCBAAACAAAAAAA															1																				
105	AACAFPAAABAAAAAAAAB														3	1	1																			
106	ABAAANBAAAAAAAAAAAA														1																					
107	JBCAGFNAABBAAAAAAAB																																		2	
108	GBCDDFGAABBAAADAAAB																																		1	
109	JBCADFNAABBAAAAAAAB																																		3	
110	QACDAGGKABBAAAAABBB																																		2	
111	GBCDDRPAABBGAAAAAAB																																		1	
112	BBAAAJAAAAAFAAAAAAA																								3											

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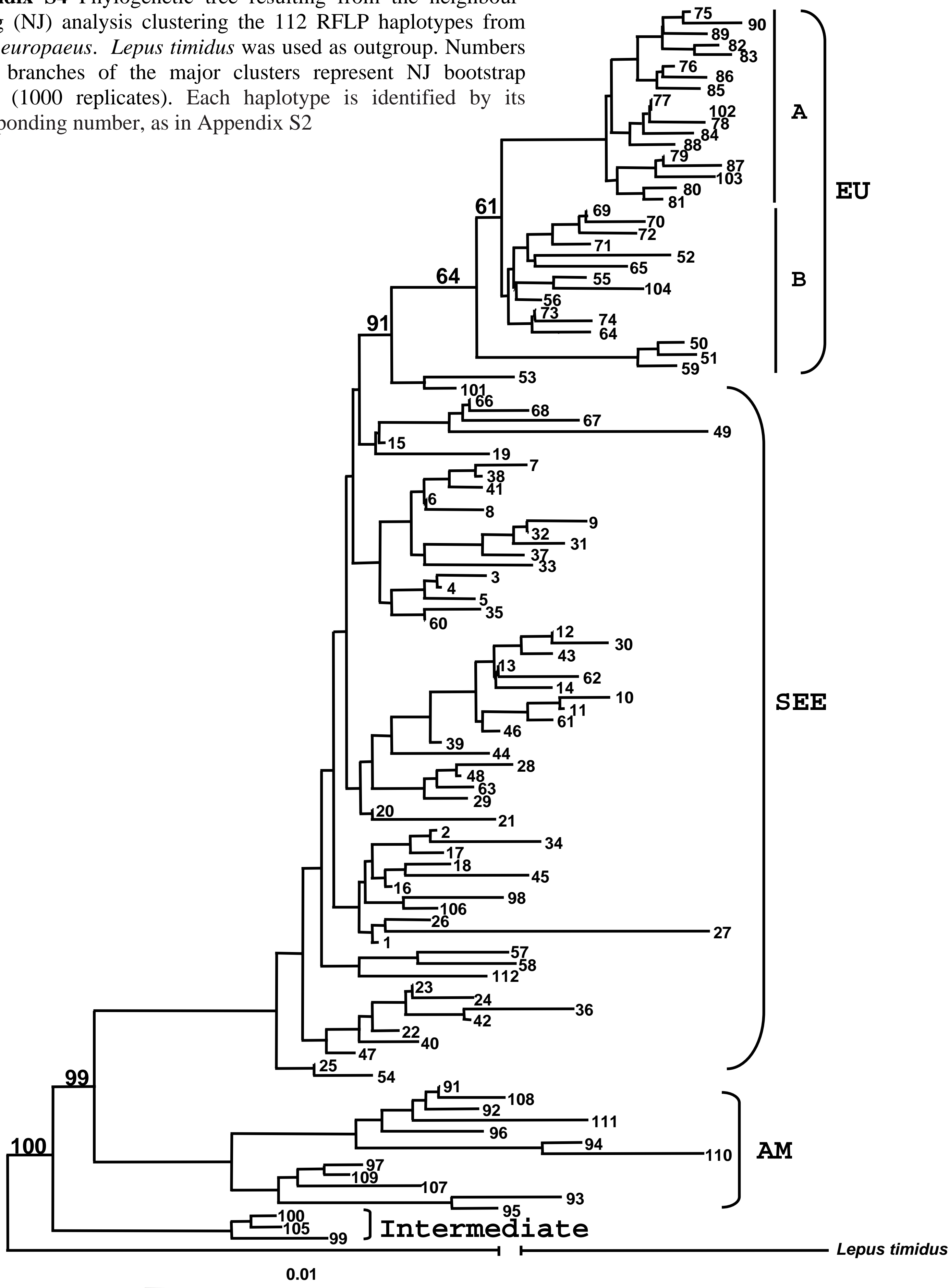
Appendix S4 Phylogenetic tree resulting from the neighbour-joining (NJ) analysis clustering the 112 RFLP haplotypes from *Lepus europaeus*. *Lepus timidus* was used as outgroup. Numbers above branches of the major clusters represent NJ bootstrap values (1000 replicates). Each haplotype is identified by its corresponding number, as in Appendix S2



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ORIGINAL INVESTIGATION

Y DNA and mitochondrial lineages in European and Asian populations of the brown hare (*Lepus europaeus*)

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Abstract

Both the Cyt*b* gene of mtDNA and Y chromosome markers were studied in a relatively large sample of brown hares (*L. europaeus*) from Europe and Anatolia (Turkey and Israel), together with other seven *Lepus* species, in order to enable comparative analysis of possible sex-specific gene flow. Furthermore, Y chromosome markers were compared with data from biparentally inherited markers in an attempt to understand whether or not their pattern of distribution was congruent with that of allozymes or whether they rather matched mtDNA phylogenies, with which they share uniparental inheritance. Consistent with the general observation, levels of interspecific genetic variability were very low for the Y chromosome markers compared with mtDNA. Moreover, lack of interspecific variation for the Y-DNA studied within *Lepus* genus rendered these markers improper for any further phylogenetic analysis. With the highest nucleotide diversity in Anatolia compared with Europe, both marker systems confirmed an unbroken species history in Anatolia, corroborated the hypothesis of continuous gene flow from Anatolia's neighbouring regions, and supported the idea of a quick postglacial colonization followed by expansion of the species in large parts of Europe. Phylogenetic analysis under mtDNA revealed the existence of four different haplogroups with a well defined distribution across Europe and Anatolia. Both genetic systems supported the deep separation of Anatolian and European lineages of *L. europaeus*. Nevertheless, Anatolian Y-DNA lineages extended across a longer geographic distance in south-eastern Europe than Anatolian mtDNA haplotypes, probably as a result of higher female philopatry that makes mtDNA introgression more difficult in brown hares.
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Keywords: Mitochondrial DNA; SRY; DBY; Brown hare; *Lepus europaeus*

Introduction

Many biological aspects of a species, such as dispersal rate and pattern, and the mating systems, combined with environmental and landscape characteristics (e.g. geographical barriers) in the recent and remote past (e.g. glaciations) seriously affect levels and patterns of genetic

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variation. However, several other factors, particularly anthropogenic, could have additional impacts in shaping species' population genetic status, especially when game species, such as brown hare (*Lepus europaeus* Pallas, 1778) are studied. The quaternary climatic oscillations, and in particular the last glacial maximum (LGM, ca. 20.000 ybp) and the subsequent warmer period, produced remarkable consequences on the population genetic variation in several species (e.g., Taberlet et al. 1998; Hewitt 2000; Hofreiter et al. 2004). Nevertheless, concerning the brown hare, several other parameters, mostly related to human activities, such as changes in agricultural management, heavy hunting pressure, and more or less uncontrolled introductions of individuals, could have had a particular influence on the presently observed pattern of genetic variation. It is likely that European populations of brown hare have been affected by uncontrolled and rarely documented restocking operations over the last decades that could have modified the genetic status both by contributing to the recent demographic expansion and by mixing different genetic pools (Flux and Angermann 1990; Suchentrunk et al. 2006; Stamatis et al. 2007).

During the past decade, several studies, using exclusively mtDNA data have attempted to portray phylogenetic relationships among diverse populations within *Lepus europaeus* species. Under mtDNA analysis (Thulin et al. 1997; Pierpaoli et al. 1999; Mamuris et al. 2001; Kasapidis et al. 2005; Ben Slimen et al. 2007; Fickel et al. 2008; Stamatis et al. 2007, 2008, in press; Sert et al. in press), brown hare populations from Europe, Asia Minor, and other parts of the Middle East, showed a phylogeographic pattern, which likely corresponds to late-Pleistocene refugia in the central/southern Balkans and in Anatolia and reflects the post-glacial colonization history of brown hares in Europe. Furthermore, a high degree of spatial partitioning of mtDNA was observed since four haplogroups predominately or exclusively occurred in well-defined geographical areas (Kasapidis et al. 2005; Stamatis et al. 2008, in press).

On the other hand, all available population genetic data on brown hares from several regions in Europe indicate fairly high gene flow at the nuclear DNA level across large ranges (e.g. Hartl et al. 1993; Fickel et al. 1999, 2005; Suchentrunk et al. 2000, 2001, 2003; Mamuris et al. 2002; Ben Slimen et al. 2005; Sert et al. 2005). Moreover, different classifications based on morphological parameters such as fur coloration and patterns, body size, external body measurements, as well as skull and tooth characteristics (overview in De Beaufort 1991), have defined various subspecies, namely *Lepus europaeus carpathous*, *L. e. creticus*, *L. e. cyrensis*, *L. e. ghigii*, *L. e. meridiei*, *L. e. niethammeri*, *L. e. parnassius*, *L. e. rhodius*, *L. e. transsylvanicus*. However, genetic analyses do not support all these classifications, which are perhaps largely due to the large intra- and interspecific morphological variation in the genus *Lepus* (e.g. Flux and Angermann 1990), but possibly also to discordance of ecogenetic and phylogenetic causes of morphological variation.

Obviously, significant discrepancy exists between mtDNA-based evolutionary hypotheses and those derived from proteins and morphology, which can be considered indirect reflections of the nuclear genome (Suchentrunk et al. in press). Differences in transmission between the two genomes (Hoelzer 1997; Seielstad et al. 1998) combined with the fact that natal dispersal might be sex biased, i.e. males disperse whilst females tend to remain within their breeding group (Hulbert et al. 1996; Reitz and Leonard 1994), are the likely causes of their incongruent topologies. Given these contradictions and in order to obtain a more accurate picture of the species' evolutionary history, comparative investigation of other genomic regions characterized by different inheritance patterns and mutation rates is necessary.

To gain further insight into this issue, we examined sequences of the mtDNA Cytochrome *b* (Cyt*b*) gene in comparison with exonic sequence of SRY (sex determination region) and intronic sequence of DBY (DEAD box Y-linked) genes of the Y chromosome, two male-specific molecular markers, showing no recombination (Gubbay et al. 1990; Sinclair et al. 1990; Hellborg and Ellegren 2004). Since the early 1990s mtDNA has been extensively used in phylogeography due to its transmission without recombination (but see Rokas et al. 2003), high mutation rate, and the availability of universal primers for PCR amplification (Kocher et al. 1989). However, mtDNA provides information only about the female germ line and its rapid evolution makes it prone to mutational saturation (homoplasy) over long evolutionary timescales. Furthermore, it is well known that evolutionary patterns of single genes or sequences are not necessarily paralleled by organism evolution (e.g. Avise 2004). On the other hand, the mammalian Y chromosome has strict paternal inheritance and a slow mutation rate relative to mtDNA (Schaffner 2004). Although mtDNA and Y chromosome loci are both uniparentally inherited haploid systems, the sex-biased dispersal patterns of brown hare could result in significant geographic structure of mtDNA haplotypes, but a single species-wide gene pool of Y chromosome types (Melnick and Hoelzer 1992).

Therefore, studying both mtDNA and the Y chromosome should enable comparative analysis (a) to follow populations' gene flow across species' distribution in Europe and Anatolia; and (b) to understand whether or not Y chromosome markers, having a dispersal pattern linked to male-mediated nuclear gene flow, yield topologies congruent with allozymes and morphology or they match mtDNA phylogenies, with which they share uniparental inheritance.

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Material and methods

Sampling

Nine hundreds twenty six brown hares were sampled in thirty-six localities from Europe, Turkey, and Israel. All hares had the typical brownish coat color with blackish tinge dorsally and variably grayish thighs. All other coat pattern characteristics (nape, flanks, tail, ear, etc.) conformed to those of typical brown hares. Tissues were stored frozen at −20 °C or preserved in alcohol until further molecular analysis. Our previous RFLP mtDNA study (Stamatis et al. in press) on these 926 brown hares showed that the haplotypes detected were partitioned into four phylogeographically well defined major haplogroups, namely a ''south-eastern European type haplogroup'' (SEEh), an ''Anatolian/Middle Eastern type haplogroup'' (AMh), a ''European type haplogroup, a subgroup A'' (EUh-A), a ''European type haplogroup, and a subgroup B'' (EUh-B).

mtDNA sequence analysis

In accordance with the above-mentioned study (Stamatis et al. in press) we chose 75 *L. europaeus* and one *L. timidus* specimens for sequence analyses. Specimens were chosen to represent all countries and all haplogroups. All *L. europaeus* individuals selected had a different mtDNA RFLP haplotype. The primer pair LepCyb2L (5′-GAAACTGGCTCCAA-TAACCC-3′) and LepD2H (5′-ATTTAAGAGGAACGT GTGGG-3′; Pierpaoli et al. 1999) was used to PCR-amplify a fragment of ca. 1200 bp of the Cyt*b* gene. Double strand DNA amplifications were performed in 50 μL volumes, containing 2 units of Taq polymerase (Invitrogen), 5 μL of 10x reaction buffer (500 mM KCl, 100 mM Tris pH 9.0), 0.8 mM dNTPs, 50 pmoles of each primer, 2.5 mM $MgCl_2$, and 500 ng of DNA. PCR amplification conditions were as follows: one initial step of denaturation at 95 °C for 5 min, followed, by 35 cycles of amplification; each cycle being 95 °C for 40 s, 52 °C for 50 s, and 72 °C for 1.5 min and a final extension step at 72 °C for 10 min. The PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosiences) and directly sequenced by Macrogen Inc., using an automated ABI Prism 3730XL DNA sequencer (Perkin Elmer Corporation). All products were sequenced in both directions. A part of 462 bp of the Cyt*b* gene was aligned for all sequences using ClustalX (Thompson et al. 1997) and controlled by eye.

For all haplotypes, base composition, nucleotide variation, polymorphic and parsimony informative sites were assessed using MEGA version 3.0 (Kumar et al. 2004). Phylogenetic analysis was performed with PAUP* 4.0 beta 10 version (Swofford 1998). In order to determine the appropriate model of sequence evolution and to statistically compare successively nested more parameter-rich models for this data set, the program MODELTEST Version 3.6 (Posada and Crandall 1998) was used. With a statistical significance of $P = 0.01$ the HKY85 model (Hasegawa et al. 1985), with γ correction, obtained the best likelihood score and was thus selected for the Neighbour-Joining analysis. Maximum parsimony (MP) trees were also constructed under the heuristic search option with 100 random-taxon-addition replicates and tree bisection–reconnection branch swapping, using PAUP*. Node support was assessed on the basis of 1000 bootstrap replicates. Phylogenetic trees were rooted using a *L. timidus* sequence.

A Bayesian analysis was also performed with MrBayes version 3.1 (Huelsenbeck and Ronquist 2001), under the HKY85 model of sequence evolution. Depending on the data set, random starting trees run for 3×10^6 generations were user sampled every 100 generations. Burn-in frequency was set to the first 25% of the sampled trees.

The software Network 3.1.0.1 (Fluxus technology Ltd, downloaded from http://www.fluxus-technology.com/share-net.htm) was used to construct a median joining (MJ) network.

Y DNA sequence analysis

Detection of haplotypes by PCR–SSCP

To identify genetic variation of the Y DNA between populations, we used two different pairs of PCR primers: The first pair, SRY-Fw (5′-CGGCCAGGAACGGGT-CAAGCG) and SRY-Rv (5′- CCTTCCGGCGAGGTCTG-TAC), was designed according to the sequence with Accession number EF437194 (nts 598–838; Putze et al. 2007) and amplified a fragment of 239 bp of the SRY gene coding region. The second pair, DBY8-Fw (5′-CCCCAACAAGA-GAATTGGCT) and DBY8-Rv (5′-CAGCACCACCATA KACTACA, Hellborg and Ellegren 2004) amplified the intron 8 of DBY gene (179 bp). PCR conditions were: 5 min at 95 °C and 35 cycles of 40 s at 95 °C, 40 s at 52 °C and 30 s at 72 °C, followed by a 10 min extension at 72 °C, for both fragments. The amplification reactions were carried out on 200–300 ng of genomic DNA in a 50 μl final volume containing 1X PCR buffer, 1 unit of *Taq* DNA polymerase (Invitrogen), 50 pmoles of each primer, 2 mM $MgCl_2$ and 0.2 mm of each dNTP. For PCR checking prior to SSCP analysis, amplifications were electrophoresed in 2% agarose gels. Prior to routine PCR application both sets of primers and PCR conditions were checked in 22 brown hares of known gender. We proceeded to further analysis after all the 926 brown hares were correctly gender assigned based on the presence or absence of the two Y regions by PCR analysis. To test the two Y DNA segments reliability in resolving phylogenetic relationships on the species level, another eight *Lepus* species [*L. nigricollis* ($n = 3$), *L. timidus* ($n = 7$), *L. corsicanus* ($n = 2$), *L. granatensis* ($n = 7$), *L. capensis* ($n = 8$), *L. brachyurus* ($n = 6$), *L. saxatilis* ($n = 2$), *L. castroviejoi* ($n = 2$)] together with *Oryctolagus cunniculus* ($n = 3$) were included in the analysis.

PCR amplifications were performed in all sampled individuals of *L. europaeus* but, for only 445 of them we obtained a PCR product (Fig. 1A). Discrimination of conformers using the SSCP technique was accomplished as follows: 4 μL of amplified DNA was mixed with 10 μL of denaturing loading buffer (95% deionized formamide, 10 mm NaOH, 0.01% bromophenol blue and 0.01% xylene cyanol). Samples were denatured for 6 min at 95 °C and kept on ice until gel loading. Total volume of samples were run in a 12% polyacrylamide gel (37.5:1 acrylamide: methylbisacrylamide) with 0.5X TBE buffer on a vertical electrophoresis system. The electrophoresis was performed at a constant voltage of 200 V and temperature (4 °C) for 20 h. Routine SSCP separations always included

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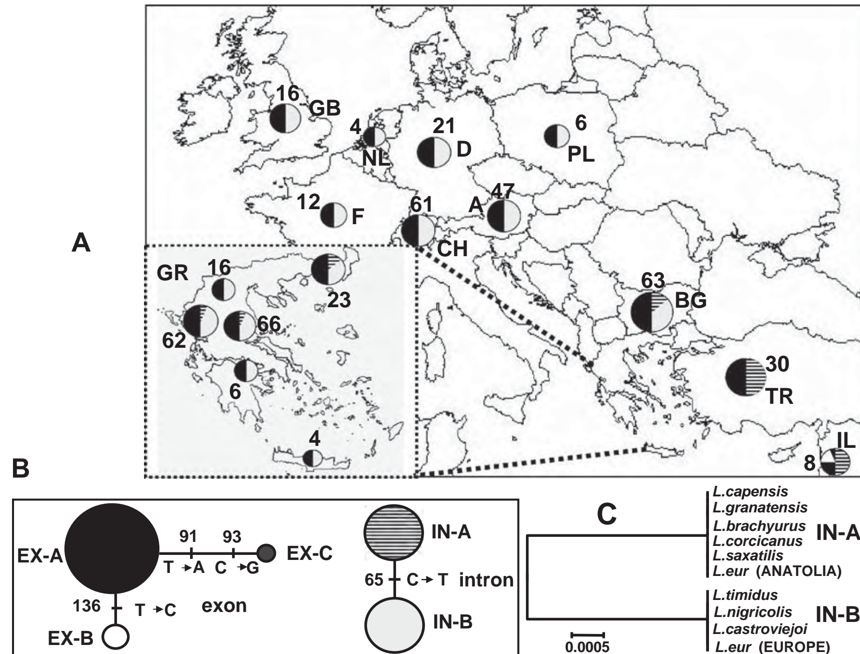

Fig. 1. (A) Map showing the distribution of sampling sites (circles), for the 445 male brown hares, with their abbreviations as in Table 1, the number of individuals per site, and the composition of each population for Y DNA. Sample distribution in Greece is shown in detail in the insert. For each circle, one half represents the composition of the population for the DBY-intron and the other half the composition of the population for the SRY-exon. The different colors and patterns for each half are as in B. (B). Two networks showing the mutational relationships for Y DNA, in both the exon and the intron, for *L. europaeus* and *Lepus* species. (C) Representation of *Lepus* species separation according to their DBY-intron composition.

previously typed samples that served as standards to ensure correct genotype scoring. The results were visualized by silver staining. Sequencing of each different haplotype SSCP profile found in different populations was performed as described for the Cyt*b* section.

Results

Cytochrome *b* (Cyt*b*) analysis

Of the 462 nucleotide sites examined, there were 66 variable, 49 of which were parsimony informative. Fifty-seven haplotypes (Table 1) were assessed with a sequence divergence ranging from 0.00217 to 0.06359 with an average of 0.02517. The topology for the major clusters was identical for Bayesian, Maximum Parsimony (MP), and Neighbour-Joining (NJ) analyses (Fig. 2A), as well as for the median joining (MJ) network (Fig. 2B). The 57 Cyt*b* sequences clustered into four haplogroups, with bootstrap values higher than 70%. CBEU-A haplogroup was found throughout Europe except Greece. CBSEE haplogroup together with CBEU-B haplogroup were found in Greece and Bulgaria. CBAM haplogroup was detected in Turkey, Israel, Bulgaria, and north-eastern Greece.

Sequence divergence within haplogroups was 0.0024, 0.0031, 0.0069, and 0.0199 for CBEU-A, CBEU-B, CBSEE, and CBAM, respectively. Mean and net sequence divergences between haplogroups ranged from 0.0115 and 0.0087 (CBEU-A/CBEU-B) to 0.0383 and 0.0268 (CBAM/CBEU-B).

Y DNA analysis

The SSCP technique resolved two different profiles for the DBY intron and three different profiles for the SRY exon that were easily scored. Analysis of sequences showed that each profile corresponded to a particular haplotype, two for the DBY intron (IN-A; Accession number: EU939380 and IN-B; Accession number: EU939381) and three for the SRY exon (EX-A; Accession number: EU939383, EX-B; Accession number: EU939384, and EX-C; Accession number: EU939385). The high resolving power of the SSCP technique was confirmed by randomly sequencing 20–30 individuals for each profile (and all individuals from southern and northern Israel) for both intron and exon, which did not reveal additional variable haplotypes. The phylogenetic relationships of the alleles were easily determined by constructing two simple networks (Fig. 1B). For the exon, the vast majority of the hares tested had the haplotype EX-A, three hares from southern Israel had the haplotype EX-B and just one hare from northern Israel had the haplotype EX-C. All individuals from different species analysed harboured the EX-A

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Table 1. Haplotype names, sampling localities, and accession numbers of the brown hare mtDNA Cyt*b* sequences.

Haplotype names	Sampling localities	Accession numbers
CBEUA01	England (GB), (2) Poland (PL), (2) Austria (A), (2) France (F), (2) Switzerland (CH), The Netherlands (NL)	EU939323
CBEUA02	England (GB)	EU939324
CBEUA03	Austria (A)	EU939325
CBEUA04	Germany (D)	EU939326
CBEUA05	Germany (D)	EU939327
CBEUA06	Switzerland (CH)	EU939328
CBEUA07	Germany (D)	EU939329
CEEUB01	Southern Greece (GR)	EU939330
CEEUB02	North-Eastern Greece (GR)	EU939331
CBEUB03	Bulgaria (BL), Crete (GR), Crete (GR)	EU939332
CBEUB04	Central Greece (GR)	EU939333
CBEUB05	Central Greece (GR)	EU939334
CBSEE01	(3) Central Greece (GR)	EU939335
CBSEE02	North-Eastern Greece (GR), (2) Western Greece GR), Central Greece (GR)	EU939336
CBSEE03	Southern Greece (GR)	EU939337
CBSEE04	Central Greece (GR)	EU939338
CBSEE05	Western Greece (GR)	EU939339
CBSEE06	Central Greece (GR)	EU939340
CBSEE07	Western Greece (GR)	EU939341
CBSEE08	Western Greece (GR)	EU939342
CBSEE09	Central Greece (GR)	EU939343
CBSEE10	Central Greece (GR)	EU939344
CBSEE11	Central Greece (GR)	EU939345
CBAM01	Turkey (TR)	EU939346
CBAM02	Israel (IL)	EU939347
CBAM03	North-Eastern Greece (GR)	EU939348
CBAM04	North-Eastern Greece (GR)	EU939349
CBAM05	North-Eastern Greece (GR)	EU939350
CBAM06	North-Eastern Greece (GR)	EU939351
CBAM07	Turkey (TR)	EU939352
CBAM08	Turkey (TR)	EU939353
CBAM09	Turkey (TR)	EU939354
CBAM10	Turkey (TR)	EU939355
CBAM11	Turkey (TR)	EU939356
CBAM12	Israel (IL)	EU939357
CBAM13	Israel (IL)	EU939358
CBAM14	Turkey (TR)	EU939359
CBAM15	Israel (IL)	EU939360
CBAM16	Israel (IL)	EU939361
CBAM17	Israel (IL)	EU939362
CBAM18	Turkey (TR)	EU939363
CBAM19	Turkey (TR)	EU939364
CBAM20	Israel (IL)	EU939365
CBAM21	Turkey (TR)	EU939366
CBAM22	Turkey (TR)	EU939367
CBAM23	Turkey (TR)	EU939368
CBAM24	Turkey (TR)	EU939369
CBAM25	Israel (IL)	EU939370
CBAM26	Bulgaria (BG)	EU939371
CBAM27	Turkey (TR), Israel (IL)	EU939372
CBAM28	Israel (IL)	EU939373
CBAM29	Israel (IL)	EU939374
CBAM30	Turkey (TR)	EU939375
CBAM31	(2) Israel (IL)	EU939376
CBAM32	Israel (IL)	EU939377
CBAM33	Israel (IL)	EU939378
CBAM34	Israel (IL)	EU939379

haplotype. As for the intron the haplotype IN-A was found in all hares from Anatolia ($n = 30$) and Israel ($n = 8$), in 27% of hares from Bulgaria ($n = 17$), and in 6.8% of hares from Greece ($n = 12$), while the haplotype IN-B was found in the vast majority of the hares from all the sampling sites in Europe, but never traced in Anatolia and Israel (Fig. 1A). Of the 12 hares bearing the IN-B haplotype from Greece, eight were detected in north-eastern Greece and four in central Greece. For the intron a separation was also observed at the species level, since *L. nigricollis*, *L. castroviejoi*, and *L. timidus* had the IN-B haplotype whereas *L. corsicanus*, *L. granatensis*, *L. capensis*, *L. brachyurus*, and *L. saxatilis* had the IN-A haplotypes (Fig. 1C).

Potential linkage between the two Y-chromosomal markers could have affected haplotype analysis within and between populations. Data, however, do not support this hypothesis.

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Discussion

Although Cyt*b* could be characterized as an evolutionary relative conservative gene, levels of interspecific genetic variability were extremely lower for the Y chromosome markers. Absence of polymorphism in Y-chromosomal markers was also reported in a recent study on the European brown hare (Putze et al. 2007) concerning the same segment of SRY gene examined here, yet in a considerably smaller sample ($n = 77$). These findings are consistent with the general observation of a low level of intraspecific sequence variability on the mammalian Y chromosome (Shen et al. 2000). In fact different studies showed low divergence to completely monomorphic Y chromosome sequences in several species even for much longer DNA segments (Wallner et al. 2003; Hellborg and Ellegren 2004). Absence of genetic variability for these specific Y chromosome segments was observed also at the interspecific level within genus *Lepus*, rendering these markers improper for any further phylogenetic analysis. In a survey of nucleotide diversity within five mammalian species targeting Y chromosome-specific gene introns, Hellborg and Ellegren (2004) found generally low levels of intra-species Y chromosome variation and for three of them [lynx (*Lynx lynx*), reindeer (*Rangifer tarandus*), cattle (*Bos taurus*)], the surveyed Y chromosome sequence was completely monomorphic. On the other hand, in assessing divergence among cetacean species using 750 base pairs surrounding the Y-specific sex-determining region (SRY), Nishida et al. (2003) found no polymorphism within species, but moderately high divergence among species.

An advantage of this study was that all individuals analysed for SRY were previously genotyped at the mtDNA level (Stamatis et al. 2008, in press) and assigned to different haplogroups, enabling several direct comparisons between the two sets of uniparental markers. Moreover, different sets of the same individuals were studied for nuclear genetic variation, using microsatellites, RAPDs, and allozymes (Mamuris et al. 2002; Suchentrunk et al. 2003; Sert et al. 2005; Ben Slimen et al. 2008). Therefore, several conclusions could be drawn based on the combined analysis of both uniparental and biparental genetic systems.

Consistent with previous data from different mtDNA regions (Stamatis et al. in press) phylogenetic analysis of Cyt*b* gene in this study corroborates the existence of four different haplogroups with a well-defined distribution across Europe and Anatolia. Furthermore, according to genetic distances, our analysis consistently supports the deep separation of Middle Eastern (Anatolia, Israel) and European lineages of *L. europaeus* at the mtDNA level (Stamatis et al. 2008, in press). In contrast, microsatellites do not suggest such a marked differentiation (Ben Slimen et al. 2008).

All studies based on nuclear biparental markers showed an overall pattern of population variability and differentiation with a relatively high amount of private alleles but indications of gene flow between neighbouring populations and an overall fit to an "isolation-by-distance model" that can be interpreted as reflecting regionally differentiated gene pools that are still connected by gene flow (Mamuris et al. 2002; Suchentrunk et al. 2003; Sert et al. 2005; Ben Slimen et al. 2008). On the contrary, Y-DNA data underline the existence of two major phylogenetic clades within *L. europaeus* between Anatolia and Europe. Nucleotide divergence among "Anatolian" and "European" SRY haplotypes was very shallow, with only one nucleotide substitution. The highly conservative nature, however, of this segment, and the fact that the same nucleotide substitution was the only one separated the genus *Lepus* at the species level, probably indicate a substantial degree of genetic divergence.

Up till now we had never traced a European mtDNA haplotype in Turkey and Israel. However, this could be due to the sampling opportunities in Anatolia, i.e. relatively small number of individuals in a considerably extensive sampling area as compared with very dense sampling in Europe and particularly in Greece (see also Sert et al. in press). Similarly, Anatolian mtDNA haplotypes were absent from north-western, central and south Greece and from the rest of Europe. Different studies (Kasapidis et al. 2005; Stamatis et al. in press) indicate the presence of a large introgression zone with numerous haplotypes of all haplogroups in Bulgaria and north-eastern Greece. Within that zone an admixture of both types of Y-DNA was recorded, with different combinations between mitochondrial and Y DNA lineages (individuals with either Anatolian or European haplotypes for both markers or Anatolian mtDNA and European Y-DNA or *vice versa*). The presence of Anatolian Y-DNA in the south-eastern Balkans supports the occurrence of ancient gene flow from Anatolia to Europe across the late Pleistocene Bosporus land bridge that disappeared only ca. 8000 years ago with the rising sea level (Geoffrey and Hosey 1982; Gökaşan et al. 1997). Interestingly, unlike mtDNA, Anatolian Y-DNA extended into central Greece, where it was detected in a few individuals. It is very likely that the tendency towards philopatry of female brown hares makes mtDNA introgression more difficult in comparison with Y-DNA, resulting in different distributional patterns. It is also possible that Anatolian Y-DNA in central Greece is the remnant of past releases of individuals imported from Bulgaria (Stamatis et al. 2007). Our data so far suggested that the European Y-DNA does not extend into Anatolia, but particularly samples from north-western Anatolia must be studied to confirm this finding. In any case, our findings suggest that male-mediated gene flow was much lower than

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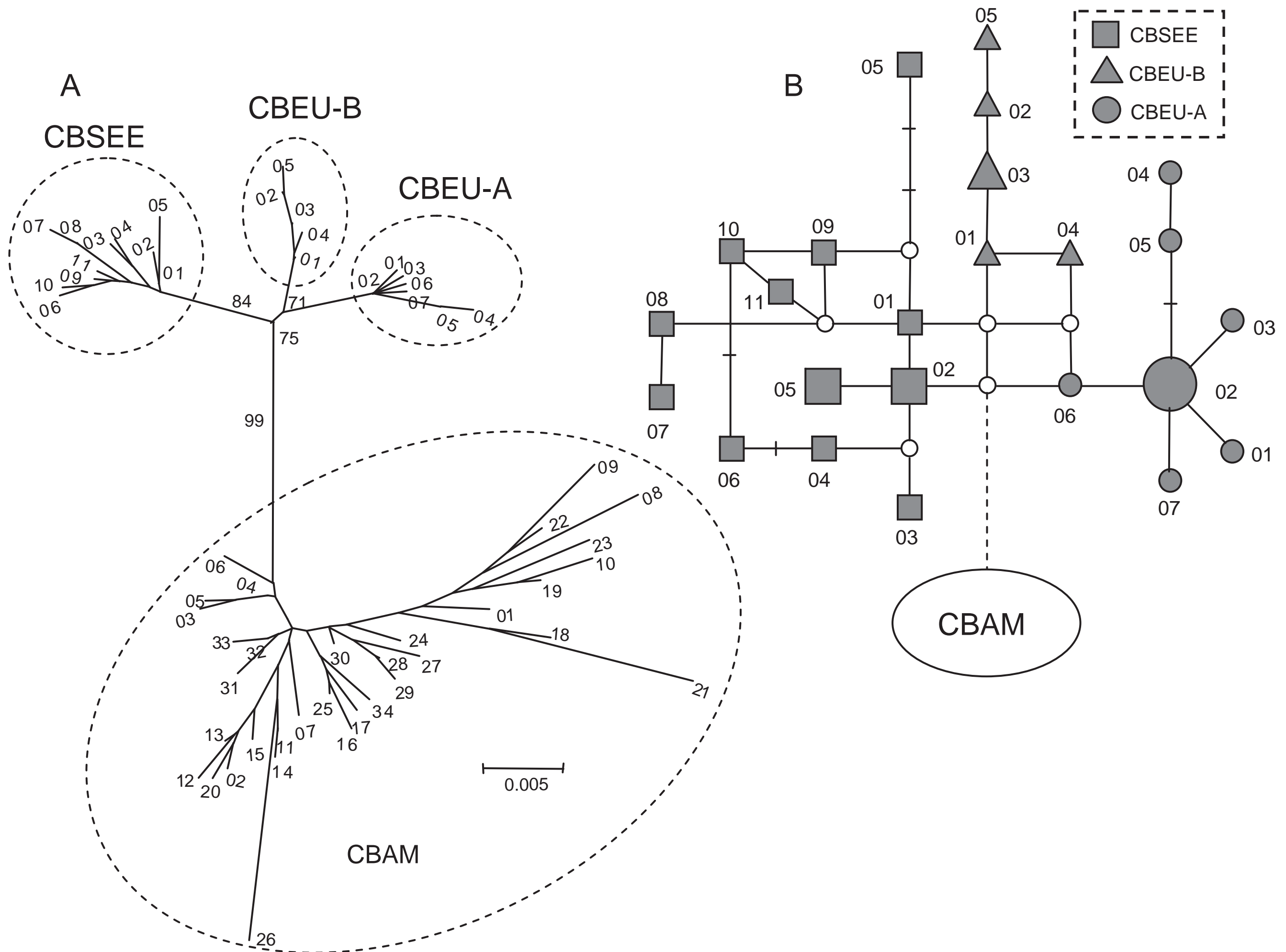

Fig. 2. (A) Phylogenetic tree resulting from the Neighbour-Joining analysis clustering the 57 Cyt*b* haplotypes from *L. europaeus*. The topology for the major clusters was similar also for all other analyses. Numbers above branches of the major clusters represent percentages of bootstrap values (1000 replicates). Each haplotype is identified by its corresponding number, as in Table 1. (B) Median joining (MJ) network showing the mutational relationships among *L. europaeus* Cyt*b* haplotypes from Europe. Each haplotype is represented by circles (CBEU-A haplogroup), triangles (CBEU-B haplogroup), and squares (CBSEE haplogroup). Sizes of circles, triangles, and squares are proportional to numbers of individuals bearing this specific haplotypes in the total sample. Each segment on connecting lines between circles, triangles, and squares as indicated by vertical bars represents a single mutational change. Each haplotype is identified by its corresponding number, as in Table 1.

autosomal gene flow. This could be explained upon the assumption that variability in Y-chromosomal markers is not completely neutral. Within the introgression zone in Bulgaria and north-eastern Greece, endogenous counter-selection of progenies between hares from European and Anatolian clades could be expressed as reduced fertility or viability in progenies of the heterogametic sex, a mechanism known as Haldane's rule in hybrids zones. This phenomenon often leads to a differential of gene flow between sex-linked markers.

As expected, according to other genetic markers, Y-DNA indicated that brown hares from Greece as well as from large parts of Europe, are not separated into discernible phyletic groups, although its high conservative status renders this Y-DNA region rather improper for sub-species analyses.

With the highest Cyt*b* nucleotide diversity of the four haplogroup, our results confirmed the unbroken history of brown hares in Anatolia and corroborated the hypothesis of continuous gene flow from the Euro-Siberian, Irano-Turanian, and Saharo-Sindian regions to this area during Pleistocene and Holocene (Ceylan 1991; Sert et al. 2005, in press). At the very opposite, the lowest nucleotide diversity, and the star-like pattern of the MJ network for the CBEU-A haplogroup, strongly supported the idea of a relatively quick colonization of large parts of Europe and postglacial expansion of the species (Kasapidis et al. 2005; Fickel et al. 2008;

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Stamatis et al. in press). An allozyme analysis yielded the same pattern of genetic variability: overall genetic diversity was the highest in Anatolian hares, intermediate in brown hares from the southern and south-eastern Balkans, and the lowest in central European populations (Sert et al. 2005). Even the low variability of our Y-DNA segments pointed toward this direction. All detected polymorphism was accumulated within Anatolia, regardless of the relatively much lower sample analysed in this area ($n = 38$) compared with Europe ($n = 407$). Surprisingly, three Israeli brown hares disposed the only nucleotide substitutions found for the exon, which separated them from the rest of the Anatolian hares. This divergence is unlikely to be a PCR artefact since multiple PCR reactions performed with different DNA polymerases and gave the same result for these three specimens. It is more likely that this polymorphism in Israel was generated by a continuous gene flow from the neighbouring areas and subsequent introgression, keeping in mind the extensive interspecies hybridization within *Lepus* reported in several occasions (e.g., Thulin et al. 1997; Melo-Ferreira et al. 2005, 2007; Alves et al. 2006; Ben Slimen et al. 2007). On the other hand, the observed genetic variation did not necessarily result only from the retention of ancestral polymorphism, but could have arisen through mutation and complete lineage sorting over a relatively small number of generations. Yet, a more careful approach is needed to clarify this issue, since Y-DNA analysis failed to detect any such polymorphism within the other *Lepus* species.

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References

Alves, P.C., Harris, D.J., Melo-Ferreira, J., Branco, M., Suchentrunk, F., Boursot, P., Ferrand, N., 2006. Hares on thin ice: introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. Mol. Phylogenet. Evol. 16, 605–618.

Avise, J.C., 2004. Molecular Markers, Natural History, and Evolution. Sinauer Associates, Sunderland, MA.

Ben Slimen, H., Suchentrunk, F., Memmi, A., Ben Ammar Elgaaied, A., 2005. Biochemical genetic relationships among Tunisian hares (*Lepus* sp.), South African cape hares (*L. capensis*), and European brown hares (*L. europaeus*). Biochem. Genet. 43, 577–596.

Ben Slimen, H., Suchentrunk, F., Shahin, A.B., Ben Ammar Elgaaied, A., 2007. Phylogenetic analysis of mtCR-1 sequences of Tunisian and Egyptian hares (*Lepus* sp. or spp., Lagomorpha) with different coat colours. Mamm. Biol. 72, 224–239.

Ben Slimen, H., Suchentrunk, F., Stamatis, C., Mamuris, Z., Sert, H., Alves, P.C., Kryger, U., Shahin, A.B., Ben Ammar Elgaaied, A., 2008. Population genetics of cape and brown hares (*Lepus capensis* and *L. europaeus*): a test of Petter's hypothesis of conspecificity. Biochem. Syst. Ecol. 36, 22–39.

Ceylan, G., 1991. Patterns of Pleistocene turnover, current distribution and speciation among Mediterranean mammals. In: Groves, R.H., Di Castri, F. (Eds.), Biogeography of Mediterranean Invasions. Chambridge University Press, Chambridge, pp. 227–262.

De Beaufort, F., 1991. La faune des mammifères de Grèce: Caractéristiques, endèmisme, particularismes. Biol. Gallo-Hell. 18, 99–106.

Fickel, J., Lieckfeld, D., Pitra, C., 1999. Analyze der genetischen Diversität und Struktur in benachbarten Populationen des Feldhasen (*Lepus europaeus*, Pallas, 1778). Z. Jagdwiss. 45, 230–237.

Fickel, J., Schmidt, A., Putze, M., Spittler, H., Ludwig, A., Streich, W.J., Pitra, C., 2005. Genetic structure of populations of European brown hare: implications for management. J. Wildl. Manage. 69, 760–770.

Fickel, J., Hauffe, H.C., Pecchioli, E., Soriguer, R., Vapa, L., Pitra, C., 2008. Cladogenesis of the European brown hare (*Lepus europaeus* Pallas, 1778). Eur. J. Wildl. Res. 54, 495–510.

Flux, J.E.C., Angermann, R., 1990. The hares and jackrabbits. In: Chapman, J.A., Flux, J.E.C. (Eds.), Rabbits, Hares and Pikas. Status Survey and Conservation Action Plan, 4. IUCN/SSC Lagomorph Specialist Group, Gland, Switzerland, pp. 61–94.

Geoffrey, R., Hosey, B., 1982. The Bosporus land-bridge and mammal distributions in Asia Minor and the Balkans. Säugetierkd. Mitt. 30, 53–62.

Gökaşan, E., Demirbağ, E., Oktay, F.Y., Ecevitoğlu, B., Şimşek, M., Yüce, H., 1997. On the origin of the Bosphorus. Mar. Geol. 140, 183–199.

Gubbay, J., Collington, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 346, 245–250.

Hartl, G.B., Suchentrunk, F., Nadlinger, K., Willing, R., 1993. An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. Acta Theriol. 38, 33–57.

Hasegawa, M., Kishino, M., Yano, T., 1985. Dating the human–ape split by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Hellborg, L., Ellegren, H., 2004. Low levels of nucleotide diversity in mammalian Y chromosomes. Mol. Biol. Evol. 21, 158–163.

Hewitt, G.M., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913.

Hoelzer, G.A., 1997. Inferring phylogenies from mtDNAvariation: mitochondrial-gene trees versus nuclear-gene trees revisited. Evolution 51, 622–626.

Hofreiter, M., Serre, D., Rohland, N., Rabeder, G., Nagel, D., Conard, N., Münzel, S., Pääbo, S., 2004. Lack of phylogeography in European mammals before the last glaciation. Proc. Natl. Acad. Sci. USA 101, 12963–12968.

Huelsenbeck, J.P., Ronquist, F., 2001. Mrbayes: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Hulbert, I.A.R., Iason, G.R., Elston, D.A., Racey, P.A., 1996. Homerange sizes in a stratified upland landscape of two lagomorphs with different feeding strategies. J. Appl. Ecol. 33, 1479–1488.

Kasapidis, P., Suchentrunk, F., Magoulas, A., Kotoulas, G., 2005. The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuactions and antrhopogenic translocations. Mol. Phylogenet. Evol. 34, 55–66.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5, 150–163.

Mamuris, Z., Sfougaris, A.I., Stamatis, C., 2001. Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA–PCR analysis: implications for conserving genetic diversity. Biol. Conserv. 101, 187–196.

Mamuris, Z., Sfougaris, A.I., Stamatis, C., Suchentrunk, F., 2002. Genetic structure of Greek brown hare (*Lepus europaeus*) populations based on the Random Amplified Polymorphic DNA (RAPD) method. Biochem. Genet. 40, 323–338.

Melnick, D.J., Hoelzer, G.A., 1992. Differences in male and female macaque dispersal lead to contrasting distributions of nuclear and mitochondrial DNA variation. Int. J. Primatol. 13, 379–393.

Melo-Ferreira, J., Boursot, P., Suchentrunk, F., Ferrand, N., Alves, P.C., 2005. Invasion from the cold: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. Mol. Ecol. 14, 2459–2464.

Melo-Ferreira, J., Boursot, P., Randi, E., Kryukov, A., Suchentrunk, F., Ferrand, N., Alves, P.C., 2007. The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. Mol. Ecol. 16, 605–618.

Nishida, S., Pastene, L.A., Goto, M., Hiroko, K., 2003. SRY gene structure and phylogeny in the cetacean species. Mammal Study 28, 57–66.

Pierpaoli, M., Riga, F., Trocchi, V., Randi, E., 1999. Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. Mol. Ecol. 8, 1805–1817.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Putze, M., Nürnberg, S., Fickel, J., 2007. Y-chromosomal markers for the European brown hare (*Lepus europaeus*, Pallas, 1778). Eur. J. Wildl. Res. 53, 257–264.

Reitz, F., Leonard, Y., 1994. Characteristics of European hare *Lepus europaeus* use of space in a French agricultural region of intensive farming. Acta Theriol. 39, 143–157.

Rokas, A., Ladoukakis, E., Zouros, E., 2003. Animal mitochondrial DNA recombination revisited. Trends Ecol. Evol. 18, 411–417.

Schaffner, S.F., 2004. The X chromosome in population genetics. Nat. Rev. Genet. 5, 43–51.

Seielstad, M., Minch, E., Cavalli-Sforza, L.L., 1998. Genetic evidence for a higher female migration rate in humans. Nat. Genet. 20, 278–280.

Sert, H., Suchentrunk, F., Erdogăn, A., 2005. Genetic diversity within Anatolian brown hares (*Lepus europaeu*s Pallas, 1778) and differentiation among Anatolian and European populations. Mamm. Biol. 70, 171–186.

Sert, H., Ben Slimen, H., Erdoğan, A., Suchentrunk, F., 2009. Mitochondrial HVI sequence variation in Anatolian hares (*Lepus europaeus* Pallas, 1778). Mamm. Biol., in press, doi:10.1016/j.mambio.2008.05.008.

Shen, P., Wang, F., Underhill, P.A., Franco, C., Yang, W.H., Roxas, A., Sung, R., Lin, A.A., Hyman, R.W., Vollrath, D., Davis, R.W., Cavalli-Sforza, L.L., Oefner, P.J., 2000. Population genetic implications from sequence variation in four Y chromosome genes. Proc. Natl. Acad. Sci. USA 97, 7354–7359.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., Goodfellow, P.N., 1990. A gene from the human sex-determining region encodes a protein with a homology to a conserved DNA-binding motif. Nature 346, 240–244.

Stamatis, C., Suchentrunk, F., Sert, H., Triantaphyllidis, C., Mamuris, Z., 2007. Genetic evidence for survival of released captive-bred brown hares (*Lepus europaeus*) during restocking operations in Greece. Oryx 41, 548–551.

Stamatis, C., Giannouli, S., Suchentrunk, F., Sert, H., Stathopoulos, C., Mamuris, Z., 2008. Recruitment of mitochondrial tRNA genes as auxiliary variability markers for both intra- and inter-species analysis: the paradigm of brown hare (*Lepus europaeus*). Gene 410, 154–164.

Stamatis, C., Suchentrunk, F., Moutou, K.A., Giacometti, M., Haerer, G., Djan, M., Vapa, L., Vukovic, M., Tvrtković, N., Sert, H., Alves, P.C., Mamuris, Z., 2009. Phylogeography of the brown hare (*Lepus europaeus*) in Europe: a legacy of south-eastern Mediterranean refugia? J. Biogeogr., in press.

Suchentrunk, F., Michailov, C., Markov, G., Haiden, A., 2000. Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoogeographical crossroads. Acta Theriol. 45, 1–12.

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Suchentrunk, F., Jaschke, C., Haiden, A., 2001. Little allozyme and mtDNA variability in brown hares (*Lepus europaeus*) from New Zealand and Britain—a legacy of bottlenecks? Mamm. Biol. 66, 48–59.

Suchentrunk, F., Mamuris, Z., Sfougaris, A.I., Stamatis, C., 2003. Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. Biochem. Genet. 41, 127–140.

Suchentrunk, F., Ben Slimen, H., Stamatis, C., Sert, H., Scandura, M., Apollonio, M., Mamuris, Z., 2006. Molecular approaches revealing prehistoric, historic, or recent translocations and introductions of Hares (genus *Lepus*) by humans. Hum. Evol. 21, 151–165.

Suchentrunk, F., Kryger, U., Ben Slimen, H. 2009. Molecular evidence of conspecificity of South African hares conventionally considered *Lepus capensis* Linnaeus, 1758. Mamm. Biol., submitted.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G., Cosson, J.F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453–464.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Thulin, C.G., Jaarola, M., Tegelstrom, H., 1997. The occurrence of mountain hare mitochondrial DNA in wild brown hares. Mol. Ecol. 6, 463–467.

Wallner, B., Brem, G., Müller, M., Achmann, R., 2003. Fixed nucleotide differences on the Y chromosome indicate clear divergence between *Equus przewalskii* and *Equus caballus*. Anim. Genet. 34, 453–456.

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Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDBNA RFLP-PCR analysis: implications for conserving genetic diversity

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Abstract

The genetic differentiation and the phylogenetic status of brown hare (*Lepus europaeus*) populations from central Greece as well as the impact of the releases of reared individuals on the native populations genetic structure was assessed, using mtDNA RFLP-RCR analysis. Data analysis revealed extensive haplotype diversity (42 out of 56 haplotypes were unique) within and among wild populations. Haplotype diversity was equally distributed within and between geographical regions, while significant genetic structuring was evident from heterogeneity of haplotype frequencies among sampling sites. Specific mtDNA profiles clearly differentiated reared from wild individuals and proved highly indicative for reared hares from past releases caught within wild populations. MtDNA analysis suggests the introgression of allochthonous gene pools into the native populations. To conserve indigenous genotypes and to prevent loss of genetic diversity, restocking operations should be stopped and an appropriate management adjusted to the local population dynamics should be developed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Lepus europaeus; Genetic structure; mtDNA

1. Introduction

The brown hare (*Lepus europaeus* Pallas, 1778) is widely distributed throughout Europe, including the mainland and the islands of Greece, where it constitutes an important game species present in open woodland, farmland with pasture and grassland up to 1500 m (Mitchell-Jones et al., 1999). In Europe, a marked decline in brown hare populations has been recorded since the 1960s (Marboutin and Peroux, 1995). During the past decades, restocking programs with the introduction of allochthonous individuals have been carried out in several European countries that might have seriously influenced the historical distribution and genetic integrity of indigenous hare species (Flux, 1983; Thulin et al., 1997; Fickel et al., 1999; Pierpaoli et al., 1999). In Greece, releases of reared individuals imported mainly from Italy, Yugoslavia and Bulgaria have been made during the past decade, according to the records of the Ministry of Agriculture and of the hunting associations. Thus, the local population genetic structure might be seriously "polluted" by the introduction of foreign introduced genomes. In the long run, this may lead to a loss of the local and/or regional genetic diversity. Conservation of genetic diversity is a major task in conservation and evolutionary biology, since genetic variation is the raw material for evolutionary changes within populations (Frankel and Soulé, 1981). Consequently, the World Conservation Union (IUCN) has recognized genetic diversity as one of three levels of diversity requiring conservation (McNeely et al., 1990). From this aspect, genetic information, always combined with other ecological and biological factors of the species, can be used as the basis of recommendations for conservation.

Significant genetic differentiation among populations implies demographic differentiation, and demographically separated populations should be managed and conserved as separate units (Moritz, 1994). Brown hare population genetic studies, based on allozyme analysis, have not revealed essential biochemical genetic differences

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among samples collected from various parts of Europe (Hartl et al., 1993; Suchentrunk et al., 2000a, b). On the other hand, studies on mtDNA using restriction fragment length polymorphisms (RFLPs) or direct sequence analysis have shown different levels of genetic variation among populations across Europe (Hartl et al., 1993; Perez-Suarez et al., 1994; Thulin et al., 1997; Pierpaoli et al., 1999; Suchentrunk et al., 2000b). To date, although several aspects of brown hare populations in Greece remain uncertain there have been no genetic studies that could provide information relevant to the management and conservation of brown hares. Furthermore, the real phylogenetic status of the introduced animals and the extent of the impact of these releases to the indigenous populations are not clear yet.

To create the basis for the development of management and conservation actions, this study investigates the genetic structure of brown hare populations in Greece, using RFLP analysis of PCR-amplified mitochondrial DNA. Maternal inheritance and the absence of recombination make mitochondrial DNA an appropriate tool for reconstructing the recent history of populations (Avise, 1994). The study aimed to examine (1) the phylogenetic status of indigenous brown hares, (2) the present level of genetic diversity within and among populations and (3) the genetic impact of releases on wild populations.

2. Materials and methods

2.1. Samples

A total of 210 brown hares, 24 reared and 186 wild were examined. Reared specimens came from two different farms (20 and four, respectively) and their tissue samples were collected before they were released. Of the wild individuals, 16 came from north (nine) and south (seven) mainland Greece and the remaining 168 belonged to six sampling areas (operationally called populations) from central Greece (Epirus and Thessaly; Fig. 1). Samples from reared individuals were collected in 1999, whereas wild samples were collected during two consecutive hunting seasons (1998 and 1999) and tissues were brought in ice to the laboratory immediately after hunting. The main studied area covers an area of 23 100 km^2 and was chosen for the following reasons. It comprises the whole range of brown hare habitats. There is evidence that in the last 10 years reared brown hares, imported from abroad (mainly from Italy), have been released by the hunting associations within the whole area, except from two controlled hunting areas (PY and SP; Fig. 1) in which releases have never been performed. Finally, besides the present genetic study, there is an ongoing parallel survey within the same area concerning hare ecology and biology.

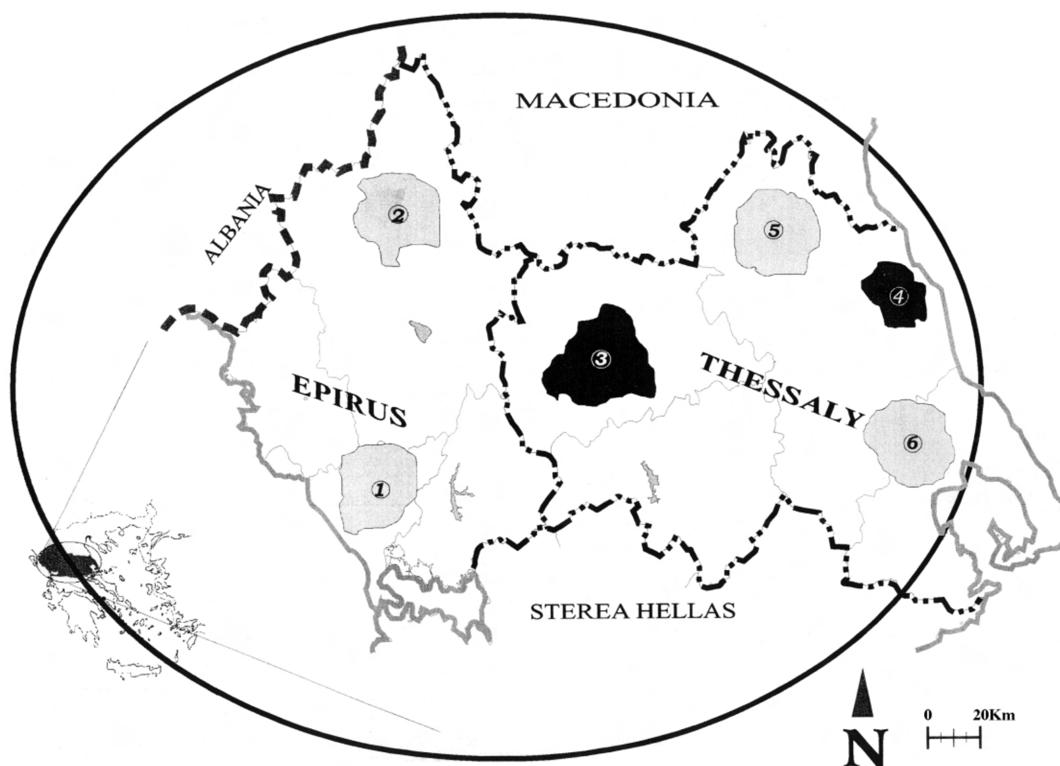

Fig. 1. Brown hare sampling sites: (1) Zallogo (ZA); (2) Vradeto (VR); (3) Pyrra (PY); (4) Spilia (SP); (5) Elassona (EL); (6) Velestino (VE). (Sampling sites from south and north Greece is not shown).

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2.2. mtDNA RFLP analysis

Mitochondrial DNA (mtDNA) variation was analyzed by RFLPs performed on PCR-amplified products. DNA was extracted from each specimen according to Bernatchez et al. (1988). Three primer pairs were used to amplify the mtDNA: L14841 and H16498 for Control Region; L5950 and H7196 for COI; L1091 and H3080 for 12S-16S rRNA (Palumbi et al., 1991 and literature cited within).

Double strand DNA amplifications were performed in 100 μl volumes, containing 4 units of Taq polymerase, 10 μl of 10× reaction buffer (500 mM KCl, 100 mM Tris pH 9.0), 10 mM dNTPs, 100 ng of each primer, 5 mM $MgCl_2$ and ca. 100 ng of DNA. PCR amplification conditions were as follows: one preliminary denaturation at 95°C for 5 min, followed by strand denaturation at 94°C for 1 min, annealing at 53°C for 30 s and primer extension at 72°C for 2 min (Control Region, 12S-16S rRNA) or 1 min (COI).

The amplified segments from each specimen were subsequently screened for polymorphism with the following 20 restriction endonucleases: *Aci*I, *Alu*I, *Ase*I, *Ava*II, *Bam*HI, *Ban*I, *Bst*UI, *Dde*I, *Eco*RI, *Hae*III, *Hha*I, *Hinc*II, *Hinf*I, *Hpa*I, *Mbo*I, *Mse*I, *Msp*I, *Pst*I, *Taq*I, *Xba*I. The digested samples were electrophoretically separated on 6% polyacrylamide gels (PAGE).

2.3. Data analysis

Distinct single endonuclease patterns (restriction morphs) were identified by specific letter in order of appearance. Each specimen was assigned a multiletter code that described its composite mtDNA genotype (haplotype). The raw data were fragment profiles, but we inferred site differences among haplotypes from changes in fragment profiles as the gain or loss of particular restriction sites that these could account for. The restriction site pattern data were analyzed using the REAP (McElroy et al., 1991) and the PHYLIP 3.5 (Felsenstein, 1993) computer packages. Both distance and character-based analyses were used to define genetic groups and phylogenetic relationships. Trees were rooted, when needed, using the data obtained from the same mtDNA analysis of a rabbit (*Oryctolagus cuniculus*). However, given that *Oryctolagus* is sufficiently genetically divergent to destabilize ingroup topologies, the most divergent brown hare haplotype (No. 30) was also used to root trees (Halanych et al., 1999; Pierpaoli et al., 1999). For restriction site data, Monte Carlo randomization tests were performed to determine the significance of haplotype frequency distributions among sampling sites using the program MONTE in REAP (McElroy et al., 1991). Chi-squared tests were conducted over a number of geographical scales in a hierarchical manner and the significance level was obtained by 10000 randomizations. To test for isolation by distance, a Mantel test was carried out using NTSYS software (Rohlf, 1993) using the log of geographical distance (km) and the nucleotide divergence as input matrices. N_{ST} (Lynch and Crease, 1990) was used to estimate the degree of population subdivision at the nucleotide level. The resulting index gives the ratio of the average genetic distance between genes from different populations relative to that among genes in the population.

3. Results

3.1. Haplotype diversity

The amplified segments of Control Region, COI and 12S/16S rRNA had an approximate size of 1.8 kb, 1.3 kb and 2.05 kb, respectively, corresponding to about 30% of the mitochondrial genome of lagomorphs (Gissi et al., 1998). However, a length heteroplasmy was observed in some specimens. This phenomenon appears to be common in lagomorphs (Biju-Duval et al., 1991; Casane et al., 1997) and is due to variations in the number of copies of short tandemly repeated sequences in the major non-coding regions of mtDNA. Nine individuals from different sampling sites, exhibited such heteroplasmy and were excluded from the analysis.

Twelve, nine and 13 of the 20 restriction enzymes used to screen the brown hare populations had a recognition site in Control Region, COI and 12S/16S rRNA, respectively. These enzymes generated in all of the amplified segments a total of 156 restriction sites corresponding to an estimated average number of 650 bp surveyed. Polymorphism was found in all three segments, but as expected, in considerably different levels within each region. The Control Region appeared the most polymorphic with 10 of the 12 restriction enzymes having a recognition site (Table 1), generating 37 different haplotypes (composite genotypes), whereas 12S/16S rRNA region was the least polymorphic with just three haplotypes generated by two restriction enzymes of the 13 having a recognition site (Table 1). The COI region exhibited slightly higher polymorphism than the 12S/16S rRNA region with nine haplotypes generated by four polymorphic enzymes of the nine having a recognition site (Table 1). In total, 56 different haplotypes were scored and they are presented in Table 1 with the haplotype frequencies within each sampling site.

The vast majority (42 of 56) of the recorded haplotypes were unique, i.e. observed only in one population, and only 14 were common between populations (Table 1). The 16 individuals sampled from northern and southern Greece had seven unique haplotypes, closely related to haplotypes No. 7, No. 20, No. 23 (Table 1 and Fig. 2) and nine haplotypes common with the individuals from Central Greece. These 16 brown hares

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Table 1
Composite genotypes (haplotypes) and haplotype numbers and frequencies within the studied populations of brown hare[a]

Haplotype (composite genotype)	Sample locality							
	ZA	VR	PY	SP	EL	VE	'R-like'	Reared
Type1 ABAAAABAAABAAAAA	2(0.077)		3(0.088)					
Type2 ABAAAABAAACAAAAA			1(0.029)			3(0.136)		
Type3 ABAAAAACAACAAAAA					1(0.050)			
Type4 ABAAAAACAABAAAAA					1(0.050)			
Type5 BBAAAAACAABAAAAA	3(0.115)	5(0.238)	6(0.176)		4(0.200)			
Type6 BAAAAAAAAABAAAAA		3(0.143)	2(0.059)		1(0.050)	1(0.045)		
Type7 BAAAAAACACBAAAAA		4(0.190)						
Type8 BAAAAAAAADBAAAAA		1(0.048)						
Type9 BAAADAAAAABAAAAA		1(0.048)						
Type10 BAAAAADGAADAAAAA				5(0.208)				
Type11 CAAAAAAABACAABAA	1(0.038)	1(0.048)						
Type12 CAAAAAAABABAABAA			1(0.029)					
Type13 CBAAAAAAAAAAABAA	3(0.115)	1(0.048)	2(0.059)					
Type14 CBAAAAAAAABAABAA	4(0.154)	1(0.048)	3(0.088)		1(0.050)	8(0.364)		
Type15 CBADAEAAAABAABAA	2(0.077)							
Type16 AAAAAAAAAABAAAAA	4(0.154)	1(0.048)						
Type17 AAAAAABAAABAAAAA	2(0.077)							
Type18 AAAAAABAAACAAAAA	1(0.038)			1(0.041)				
Type19 AAAAAABAAABBAAAA				3(0.125)				
Type20 AAAAAAAAADBAAAAA	1(0.038)							
Type21 AAABAADAAABAAAAA					1(0.050)			
Type22 AAAEAABAAABAAAAA				1(0.041)				
Type23 ABAAAAAAAABAAAAA	1(0.038)		1(0.029)		1(0.050)			
Type24 ABBAEAAAAABAAAAA	1(0.038)							
Type25 ABAABAAAABBAAAAA				6(0.250)				
Type26 ABAABABAABBAAAAA			3(0.088)	1(0.041)				
Type27 ABAABABACBBAAAAA					1(0.050)			
Type28 AAAAAABAABBAAAAA				1(0.041)				
Type29 ABABAABAAABAAAAA			1(0.029)					
Type30 EBACCDBFAABCAABA		1(0.048)						
Type31 FBAAAAAAAABAABAA			9(0.265)	3(0.125)	2(0.100)			
Type32 FAAAAAAAAABAAAAA		1(0.048)						
Type33 CBAAAFAAAAAAABAA						6(0.273)		
Type34 BAAAAADGAABAABBA					1(0.050)			
Type35 BAAAAADGAABAAABA				1(0.041)	1(0.050)			
Type36 BAAAAAEEAABAAAAB						1(0.045)		
Type37 ACAAAABEAACAAAAA			2(0.059)					
Type38 BBAAAAAAAADAAAAA					1(0.050)			
Type39 DBCABABHAEDAAAAA					1(0.050)			
Type40 BAAAAAFGAABAAABA					1(0.050)			
Type41 BAAAAAACAABAAAAA					1(0.050)	1(0.045)		
Type42 CBAAAAAAADBAAAAA					1(0.050)			
Type43 ABAABABAACBAAAAA				1(0.041)				
Type44 BAAAAAAAACBAAAAA		1(0.048)						
Type45 ABCABABAABBAAAAA				1(0.041)				
Type46 CBAAAFAAAABAABAA						1(0.045)		
Type47 ABAAAAAAAAAAABAA	1(0.038)							
Type48 CBAAAAAGAABAABAA						1(0.045)		
Type49 DBAAACCBAABAAAAA							5(0.357)	
Type50 DBAAACCBAABBAAAA							6(0.428)	
Type51 DAAADCCBAABAAAAA							1(0.071)	
Type52 IBAAACCBAABAAAAA							2(0.143)	
Type53 AAAAABCBAABABAAA								10(0.416)
Type54 ABAAABCBAABABAAA								4(0.166)
Type55 ABAAACCBAABABAAA								5(0.208)
Type56 ABABACCBAABABAAA								3(0.125)

[a] Composite genotypes are denoted by capital letters in the following order. Control Region: *Alu*I, *Ase*I, *Ava*II, *Dde*I, *Hae*III, *Hinf*I, *Mbo*I, *Mse*I, *Msp*I, *Taq*I; COI/COII: *Alu*I, *Hae*III, *Hha*I, *Hinf*I; 12S/16S RNA: *Aci*I, *Alu*I. For the purposes of presentation non polymorphic enzymes were omitted.

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were not included in further analyses. Of the unique haplotypes, four were observed within the reared population and they grouped all reared specimens together (Table 1). These four haplotypes showed the same specific pattern in all individuals for three enzymes [patterns B or C (H*inf*I), C (M*bo*I) and B (M*se*I)] on the Control Region segment and for one enzyme [pattern B (H*ha*I)] on the COI segment (Table 1). The same specific patterns for the Control Region segment were also observed in 14 wild individuals, but not the pattern B on the COI segment (Table 1). On the other hand, these individuals exhibited two specific patterns on the Control Region segment [pattern I or F (A*lu*I)] (Table 1). Therefore, these 14 hares were separated from the wild samples and were assigned to a new group, called 'reared-like' population. The distribution and the number of 'reared-like' hares varied considerably between sampling areas (Table 2). No 'reared-like' individuals were found in the two controlled hunting areas (PY and SP), or in the VEL region. Most of the 'reared-like' individuals (10/14) were sampled in the EL area, constituting 33.3% of the total sample of this area, while two individuals were sampled in both ZA and VR areas, corresponding to 7.14 and 8.73% of the total area sample, respectively (Table 2).

The number of observed haplotypes within populations ranged from four (reared and 'reared-like') to 16 (EL; Table 2), but their distribution varied between populations. Reared and 'reared-like' hares showed approximately one different composite haplotype per six and three individuals, respectively (Table 2). This ratio in wild hares ranged from approximately one (EL region) to three (VEL region; Table 2). Percentages of unique haplotypes (unique haplotypes/sample) within each population also varied between sampling areas from 8.82% (PY region) to 45% (EL region; Table 2). All haplotypes detected within reared and 'reared-like' populations were unique. The most common haplotypes, grouping 34.5% (50/145) of the wild hares were

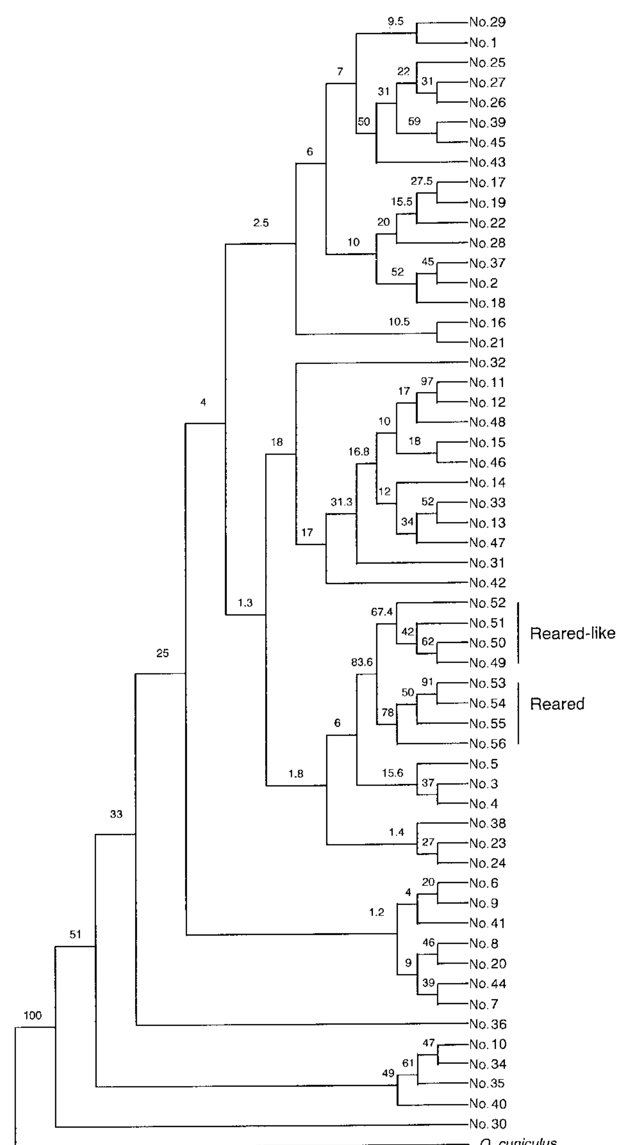

Fig. 2. Majority rule consensus tree clustering the 56 haplotypes described in Table 1. Bootstrap estimates (as a percentage) are indicated above branches.

Table 2
Number of total haplotypes and of unique haplotypes found within each population, percentages of number of unique haplotypes/total number of individuals (*S*) and of number of the unique haplotypes/total number of haplotypes (*R*), ratio of number individuals/total number of haplotypes (*T*) and percentages of haplotype and nucleotide diversity (numbers in parentheses indicate 'reared-like' individuals found in each population)

Population	Sample size	Total haplotypes	Unique haplotypes	*S*	*R*	*T*	% Haplotype diversity	% Nucleotide diversity
ZA	26 (2)	13	5	19.23	38.46	2.00	93.54	1.37
VR	21 (2)	12	6	28.57	50.00	1.75	90.95	1.53
PY	34	12	3	8.82	25.00	2.83	88.77	1.40
SP	23	11	7	30.43	63.63	2.09	88.77	1.79
EL	20 (10)	16	9	45.00	56.25	1.25	96.32	1.72
VE	23	8	4	17.34	50.00	2.85	80.09	1.21
Partial total	147	48	34					
Partial average				23.12	70.83	3.06	89.74±0.09	1.50±0.000
Reared-like	14	4	4	28.57	100	3.50	71.43	0.45
Reared	24	4	4	16.66	100	6.00	72.29	0.49
Total	185	56	42					
Average				22.70	75.00	3.30	85.27±0.11	1.25±0.000

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haplotype No. 5 with 18 individuals from four sampling sites (PY, ZA, VR, EL), haplotype No. 14 with 17 individuals from five sites (PY, VEL, ZA, VR, EL) and haplotype No. 31 with 14 individuals from three sites (PY, SP, EL; Table 1 and Fig. 2).

Pairwise sequence divergence estimates among the 56 haplotypes varied from 0.35% (haplotypes No. 11–No. 12) to 5.3% (haplotypes No. 11–No. 30) with an average of 2.16% (data not shown). The average pairwise sequence divergences among the haplotypes observed within wild, reared and 'reared-like' hares were 2.0, 0.8 and 0.6%, respectively. The average sequence divergence between wild and reared hares was 2.8%, almost the same as that observed between wild and 'reared-like' hares (2.7%). Finally, this average was 1.3% for all pairwise comparisons between reared and 'reared-like' individuals.

With some minor differences phenograms based on pairwise haplotype divergence (UPGMA, neighbour-joining, Fitch-Margoliash) and the majority rule consensus tree showed the same topology (Fig. 2), no matter if the outgroup was the *O. cuniculus* or the haplotype No. 30. Apart from the reared and 'reared-like' haplotypes that always grouped in the same cluster (Fig. 2), the other haplotypes clustered without any obvious correlation with the sampling sites. All major clusters were separated with bootstrap values < 50% (Fig. 2).

3.2. Population diversity

The distance matrix of net interpopulation nucleotide divergence (Table 3) was used to construct a UPGMA tree relating the eight populations studied (Fig. 3). The populations clustered into three distinct clades (net nucleotide divergence between clades ranging from 0.8 to 1.6%), reflecting the partitioning of mtDNA haplotypes and the levels of intrapopulation diversity rather than isolation by distance. These clusters included (Fig. 3): (A) the reared and the 'reared-like' population, with average intrapopulation nucleotide diversity of 0.47%; (B) the SP, EL and VR populations, with average intrapopulation nucleotide diversity of 1.7% and (C) the PY, ZA and VEL populations with average intrapopulation nucleotide diversity of 1.3%.

Statistically significant differences in haplotype frequencies among all populations were observed ($\chi^2=887.26$, $P<0.0001$). Yet, significant substructuring was found for the two populations within the cluster A ($\chi^2=36.00$, $P<0.0001$) and for the three populations within the clusters B ($\chi^2=97.84$, $P<0.0001$) and C

Table 3
Pairwise estimates of nucleotide divergence ($\times 10^2$) among the brown hares populations

Population	ZA	VR	PY	SP	EL	VEL	Reared-like	Reared
ZA	–							
VR	0.246	–						
PY	0.043	0.293	–					
SP	0.267	0.338	0.242	–				
EL	0.112	0.045	0.078	0.132	–			
VE	0.116	0.601	0.188	0.631	0.406	–		
Reared-like	1.529	1.544	1.500	1.555	1.346	1.915	–	
Reared	1.588	1.716	1.680	1.657	1.569	2.023	0.895	–

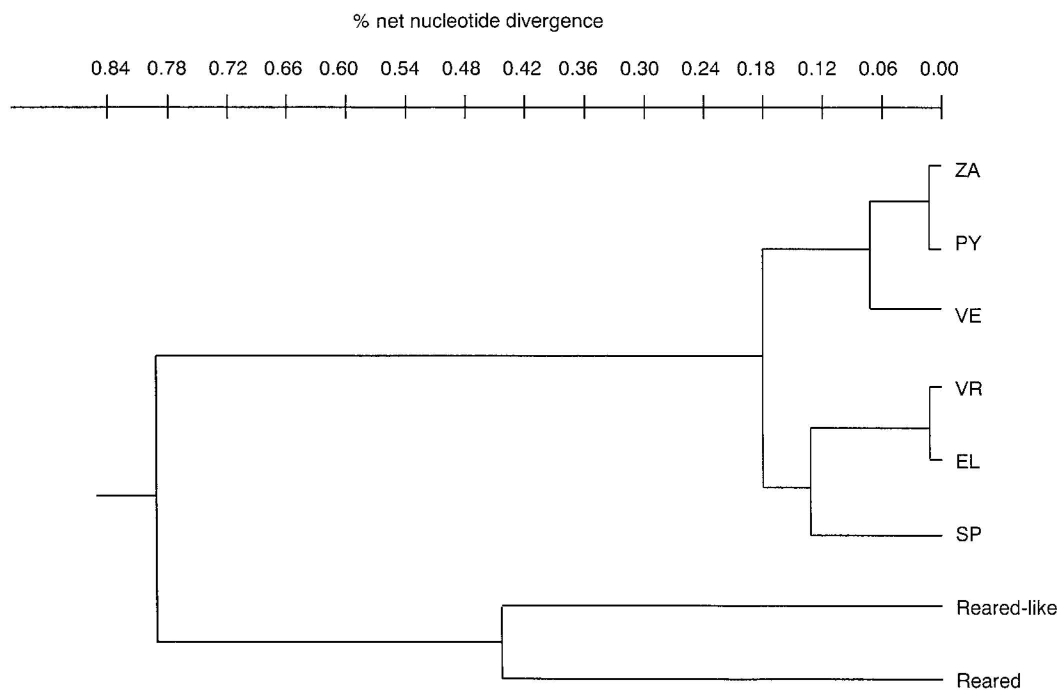

Fig. 3. UPGMA phenogram clustering the eight brown hare populations according to the distance matrix resulting from the estimation of the net average number of nucleotide substitutions per site between population (nucleotide divergence).

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($\chi^2 = 99.08$, $P < 0.0001$) (Fig. 3). As expected, pairwise tests among populations demonstrated significant differences between all (data not shown). Nucleotide divergence among populations showed no apparent correlation with geographical distance (Mantel test, $P > 0.05$).

Mean intrapopulation nucleotide diversity observed was 1.25%, ranging from 0.45% ('reared-like') to 1.79% (SP) and mean interpopulation diversity was 2.11% (Table 2). Thus, based on the N_{ST} estimate of 0.63, 37% of the overall genetic diversity observed was within populations as opposed to 63% account for the interpopulation genetic diversity. However, excluding the reared and the 'reared-like' populations, the N_{ST} estimate decreased to 0.54, suggesting that, for the six wild populations, intrapopulation diversity was almost as much as interpopulation diversity.

4. Discussion

The average pairwise haplotype divergence of 2.0% for the sampled Greek brown hares was approximately five-fold higher than the highest value (0.38%) recorded in Scandinavian brown hares (Thulin et al., 1997), almost two-fold higher than the average (1.3%) of Italian hares (Pierpaoli et al., 1999), three-fold lower than the average (6.2%) of Iberian brown hares (Perez-Suarez et al., 1994) and within the range observed for other widely distributed mammals, such as ghost bats (*Macroderma gigas*) (2.56–7.37%) (Worthington-Wilmer et al., 1994). Therefore, mtDNA analysis indicates that brown hares, at least from continental Greece, are not separated into discernible phyletic groups.

4.1. Genetic variability and population diversity

With an average of approximately one haplotype per two individuals and 0.015 of intrapopulation nucleotide diversity (Table 2) the Greek brown hares appeared highly polymorphic, exhibiting a high degree of population differentiation with limited maternal gene flow. This observation is in agreement with previous studies on Iberian (Perez-Suarez et al., 1994), Scandinavian (Thulin et al., 1997) and Italian (Pierpaoli et al., 1999) brown hares, but contrasts with the study of Hartl et al. (1993), in which a survey of 131 brown hares from 18 different sampling sites in Austria revealed only six haplotypes. However, in the latter study, only six-base cutters were used with limited polymorphism detection ability when compared with a combination of four-, five- and six-base cutters.

Despite the statistically significant differences in haplotype frequencies observed among all populations, their genetic divergence appeared to be unrelated to geographic distances. According to N_{ST} estimate, the relative genetic variability was equally distributed across large geographic distances and within regional samples. In fact, although the number of common haplotypes between populations decreased, in general, with geographical distance (i.e. no common haplotype between VR-SP, one between ZA-EL, ZA-VEL, ZA-EL and five between ZA-VR, ZA-PY, PY-EL), the percentage of unique haplotypes within each region remained high (Tables 1 and 2), indicating that the populations are substantially isolated. However, this isolation is probably due rather to socio-ecological behavior of brown hares than to physical barriers that could prevent gene dispersal, since no such barriers exist in the surveyed area. Using radio-tracking it has been established that the home range of brown hares had a size of 200 to 340 ha (Homolka, 1985). In the present study, except of SP region (3140 ha), each sampling area covered an area ranging from 6400 ha (PY) to ca. 8000 ha (EL, VEL, VR, ZA). The detection of unique haplotypes in the centre as well as at the edges of the spatial distribution of each population may be indicative of the adaptation of the population in each biotope and the lack of migration across long distances. The latter, is further strengthened by the observation that no reared individuals were detected within the two controlled areas (PY and SP), although reared hares have been released in neighboring areas to these sites (Fig. 1 and Table 2). Females almost exclusively inherit mtDNA (Avise, 1994). Therefore, the absence of migration combined with the fact that natal dispersal might be sex biased, i.e. males disperse whilst females tend to remain within their breeding group (Reitz and Leonard, 1994; Hulbert et al., 1996) may result to an overall reduction in gene flow. That may lead to genetic structuring of the population, with breeding groups constituting genetically isolated units. However, because of sex-specific dispersal, it is possible that the interpopulation diversity assessed by mtDNA markers is overestimated. Fickel et al. (1999) found a distinct mtDNA differentiation across short geographic distances in German brown hare populations but this differentiation was less evident when microsatellites were used to screen the same populations.

4.2. The impact of the releases on wild populations

Within the past decade, according to the records of hunting associations, more than 2000 reared brown hares have been released in the studied area, while 826 of them have been released the last 2 years (1998 and 1999). An interesting outcome of this study was the detection of specific haplotypes that clearly differentiated the reared individuals from wild brown hares (Table 1). In the present study, these markers permitted the identification of the occurrence of 'reared-like' mtDNA haplotypes, yet from a slightly different breeding line

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than the reared one examined here, in a total of 14 hares ('reared-like' population) sampled in the wild. In fact, in absence of wild population genetic data prior to releases, it is difficult to assign the real indigenous haplotypes. Nevertheless, it is highly probable that haplotypes No. 5, No. 14 and No. 31 found in all sampling sites, grouping 34.5% of the wild animals, as well as the genetically close related haplotypes (i.e. No. 3, No. 4, No. 13, No. 33, No. 38, No. 46, No. 48) represent native phyletic groups (Table 1 and Fig. 2). Although, there is no further data to confirm the source of the 'reared-like' haplotypes, apart from the fact that they were observed only in areas where releases have been performed the last 2 years, molecular genetic evidence supports the idea that either they come directly from past releases, or they have been transmitted to wild hares via mating in the wild. Except for one difference, the same mtDNA markers were detected in both reared and 'reared-like' individuals, while mean genetic divergence was two-fold lower between reared and 'reared-like' populations (1.3%) than between 'reared-like' and natural populations (2.7%).

Theoretically, the high mtDNA diversity revealed by the present study in Greek brown hare populations could be maintained if the populations are large enough and stable for a long period of time. However, ongoing studies in the sampling areas indicate that population densities do not exceed five individuals per 100 ha (VR area) with an average of two individuals per 100 ha (Sfougaris et al., unpublished data). These values are very low compared with other European countries such as Poland (24–31 individuals per 100 ha; Wasiliewski, 1991), France (23–71 individuals per 100 ha; Pepin, 1987) and North Italy (38–53 individuals per 100 ha; Meriggi and Verri, 1990). It is often assumed that, when a population goes through a severe bottleneck, random genetic drift will induce a massive loss of genetic variability. According to the records of the Ministry of Agriculture, during the years 1986–1990, a serious decline occurred in the brown hare populations due to the viral haemorrhagic disease. Although hunting activity was stopped in some sites within the sampled area for one hunting season in 1990, it is unlikely that populations fully recovered from this bottleneck phenomenon. Despite the large mortality induced by the disease (40–90%), much mtDNA variation is still observed within brown hare populations. However, there is no doubt that such diseases, resulting in massive death, combined with low population densities must have a significant effect on the population genetic structure, leading to a complete loss of several indigenous haplotypes.

Loss of genetic variability is a major threat to the survival of any species, decreasing the species' potential for adaptation to environmental changes (Lesica and Allendorf, 1995). However, the introduction of captive-bred animals does not seem to have increased or even maintained the genetic variability. In contrast to natural populations, both reared and 'reared-like' populations showed relatively little genetic differentiation, with nucleotide diversity of 0.49 and 0.45%, respectively (Table 2) and average haplotype divergence of 0.8 and 0.6%, respectively. Moreover, the occurrence of the same haplotypes within two different farms indicates that the same imported breeding lines may be recycled in Greece.

A crucial issue that remains to be checked, dealing also with the efficacy of restocking programs, is the survival of the released animals. High rates of virus infections due to different life conditions in captivity (mode of nutrition, administration of antibiotics) and/ or unsuccessful adaptation in nature could seriously affect the survival of released animals. Whatever the fate of released individuals, our data indicate that a percentage survives at least between two hunting periods, enough to have one reproductive cycle and to transmit their genome. Therefore, uncontrolled introduction and releases of brown hares, at least at periods when natural populations go through a bottleneck, could have a serious impact on their present genetic structure, reducing local/regional genetic diversity. That was the case in France where there is indication that indigenous hares have been completely replaced by the introduction of hares from eastern Europe (Flux, 1983). In Italy, the historical distribution and genetic integrity of indigenous hare species may be seriously influenced by the introduction of allocthonous brown hare populations (Pierpaoli et al., 1999).

4.3. Suggestions for management and conservation

Although *L. europaeus* is not an endangered species, our data indicate that regional gene pools are endangered, since there is a detectable change in genetic structure of Greek brown hare populations, resulting from restocking operations. Overall percentage of the 'reared-like' haplotypes detected in the areas where releases are performed, are approximately 14%, whereas within the EL area this percentage was as high as 33%. Nevertheless, it is questionable whether the percentages of 'reared-like' individuals found in each area reflect the real impact of releases, given that in VE region, where releases are also performed, no 'reared-like' individuals were found.

Under these circumstances the first conservation action in order to preserve the genetic integrity of indigenous brown hares is immediately to stop restocking programs all over Greece. If a dramatic decline in population densities occurs in a regional scale (due to overhunting and/or to diseases), the first step in preventing further decrease and allowing the recovery of population size will be to call off hunting activity for a period of time. Should this action prove inadequate or genetic monitoring reveal a serious decline of genetic

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diversity, translocation of wild specimens among populations could be considered as further action. In this case, the units of management must be defined.

Some authors suggest the species as the appropriate unit of conservation (Caughley and Gunn, 1996), whereas some others consider evolutionarily significant units (ESUs) as the unit of conservation. These units could be populations, stocks, subspecies, or species (Moritz, 1994; Waples, 1995), whichever show phylogeographic differentiation at the mtDNA level and significant divergence of allele frequencies at nuclear loci (Moritz, 1994). Although genetic studies, using RAPDs and VNTRs, are in progress, mtDNA data already suggest that the magnitude of the genetic divergence among the Greek *L. europaeus* geographical populations is significant. Certainly, the populations studied could not be considered to follow independent evolutionary trajectories. However, from the perspective of the molecular genetic variation the six wild populations and in particular the populations from the two controlled hunting areas (PY and SP; Fig. 1), in which releases have never been performed, could be qualifying as management units. These two populations are significant for conservation in that they probably bear only native genotypes with sufficient high level of genetic variation and could serve as pools for translocations of individuals to other target endangered populations. Therefore, within these areas hunting controls must be intensified.

In any case, in order to determine native genotypes and to record the present level of polymorphism, a detailed study of brown hare genetic stocks for the whole of Greece must be undertaken, in particular within areas where no releases are performed. In addition, the study of the level of genetic divergence and variability between the Greek and European hares can be a useful tool in determining the interaction between the released and indigenous individuals throughout Europe, wherever restocking programs have become a common practice for the enhancement of natural populations. Given that mtDNA harbors only a very limited amount of genetic variability compared with the nuclear genome, different molecular methods must be used.

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References

Avise, J.C., 1994. Molecular Markers, Natural History and Evolution. Chapman and Hall, New York.

Bernatchez, L., Savard, L., Dodson, J., Pallota, D., 1988. Mitochondrial DNA sequence heterogeneity among James-Hudson Bay anadromous coregonines. Finnish Fish Research 9, 17–26.

Biju-Duval, C., Ennafaa, H., Dennebouy, N., Monnerot, M., Mignotte, F., Soriguer, R.C., El Gaaied, A., El Hili, A., Mounolou, J.-C., 1991. Mitochondrial DNA evolution in lagomorphs: origin of systematic heteroplasmy and organization of diversity in European rabbits. Journal of Molecular Evolution 33, 92–102.

Casane, D., Dennebouy, N., De Rochambeau, H., Mounolou, J.C., Monnerot, M., 1997. Nonneutral evolution of tandem repeats in the mitochondrial DNA control region of lagomorphs. Molecular Biology and Evolution 14, 779–789.

Caughley, G., Gunn, A., 1996. Conservation Biology in Theory and Practice. Blackwell Science, Cambridge, Massachusetts.

Felsenstein, J., PHYLIP (Phylogeny inference package), version 3.5c. Seattle: Department of Genetics, University of Washington, 1993

Fickel, J., Lieckfeld, D., Pitra, C., 1999. Analyse der genetischen Diversität und Struktur in benachbarten Populationen des Feldhasen (*Lepus europaeus*, Pallas 1778). Z. Jagdwiss. 45, 230–237.

Flux, J.E.C., 1983. Introduction to taxonomic problems in hares. Acta Zoologica. Fennica 174, 7–10.

Frankel, O.H., Soulé, M.E., 1981. Conservation and Evolution. Cambridge University Press, Cambridge.

Gissi, C., Gullberg, A., Arnason, U., 1998. The complete mitochondrial DNA sequence of the rabbit, *Oryctolagus cuniculus*. Genomics 50, 161–169.

Halanych, K.M., Demboski, J.R., Jansen van Vuuren, B., Klein, D.R., Cook, J.A., 1999. Cytochrome b phylogeny of North American hares and jackrabbits (*Lepus*, Lagomorpha) and the effects of saturation in outgroup taxa. Molecular Phylogenetics Evolution 11, 213–221.

Hartl, G.B., Suchentrunk, F., Nadlinger, K., Willing, R., 1993. An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. Acta Theriologica 38, 33–57.

Homolka, M., 1985. Spatial activity of hares (*Lepus europaeus*). Folia Zoologica 34, 217–226.

Hulbert, I.A.R., Iason, G.R., Elston, D.A., Racey, P.A., 1996. Home-range sizes in a stratified upland landscape of two lagomorphs with different feeding strategies. Journal of Applied Ecology 33, 1479–1488.

Lesica, P., Allendorf, F.W., 1995. When are peripheral populations valuable for conservation? Conservation Biology 9, 753–760.

Lynch, M., Crease, T.J., 1990. The analysis of population survey data on DNA sequence variation. Molecular Biology and Evolution 7, 377–394.

Marboutin, E., Peroux, R., 1995. Survival pattern of European hare in a decreasing population. Journal of Applied Ecology 32, 809–816.

McElroy, D., Moran, P., Bermingham, E., Kornfield, J. REAP: the restriction enzyme analysis package, Version, 4.0.Department of Zoology, Orono, ME: University of Maine, 1991.

McNeely, J.A., Miller, K.R., Reid, W.V., Mittermeier, R.A., Werner, T.B., 1990. Conserving the world's biological diversity. Washington, DC: IUCN, World Resources Institute, Conservation International, WWF-US and the World Bank.

Meriggi, A., Verri, A., 1990. Population dynamics and habitat selection of the European hare on poplar monocultures in northern Italy. Acta Theriologica 35, 69–76.

Mitchell-Jones, A.J., Amori, G., Bogdanowicz, W., Krystufek, B., Reijnders, P.J.H., Spitzenberger, F., Stubbe, M., Thissen, J.B.M., Vohralik, V., Zima, J., 1999. The atlas of European mammals. Poyser Natural History. T & A. D. Poyser for the Societas Europaea Mammalogica. Academic Press, London.

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Moritz, C., 1994. Defining 'evolutionary significant units' for conservation. Trends in Ecology and Evolution 9, 363–373.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The Simple Fool's Guide to PCR. Department of Zoology, University of Hawaii, Honolulu, Hawaii.

Pepin, D., 1987. Dynamics of a heavily exploited population of brown hare in a large-scale farming area. Journal of Applied Ecology 24, 725–734.

Perez-Suarez, G., Palacios, F., Boursot, P., 1994. Speciation and paraphyly in Western Mediterranean hares (*Lepus castroviejoi*, *L. europaeus*, *L. granatensis* and *L. capensis*) revealed by mitochondrial DNA phylogeny. Biochemical Genetics 32, 423–436.

Pierpaoli, M., Riga, F., Trocchi, V., Randi, E., 1999. Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. Molecular Ecology 8, 1805–1817.

Reitz, F., Leonard, Y., 1994. Characteristics of European hare *Lepus europaeus* use of space in a French agricultural region of intensive farming. Acta Theriologica 39, 143–157.

Rohlf, F.J., 1990. NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System. Exeter Software, New York.

Suchentrunk, F., Michailov, C., Markov, G., Haiden, A., 2000a. Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoogeographical crossroads. Acta Theriologica 45, 1–12.

Suchentrunk, F., Jaschke, C., Maiden, A. Little allozyme and MtDNA variability in brown hares (*Lepus europaeus*) from New Zealand and Britain — a legacy of bottlenecks? Zeitschrift für Säugetierkunde 2000b; 65, (in press).

Thulin, C.-G., Jaarola, M., Tegelstrom, H., 1997. The occurrence of mountain hare mitochondrial DNA in wild brown hares. Molecular Ecology 6, 463–467.

Waples, R.S., 1995. Evolutionary significant units and the conservation of biological diversity under the Endangered Species Act. American Fisheries Society Symposium 17, 8–27.

Wasilewski, M., 1991. Population dynamics of the European hare *Lepus europaeus* Pallas, 1778 in Central Poland. Acta Theriologica 36, 267–274.

Worthington-Wilmer, J., Moritz, C., Hall, L., Toop, J., 1994. Extreme population structuring in the threatened ghost bat *Macroderma gigas*: evidence from mitochondrial DNA. Proceedings of Royal Society of London B 257, 193–198.

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Assessment of Genetic Structure of Greek Brown Hare (*Lepus europaeus*) Populations Based on Variation in Random Amplified Polymorphic DNA (RAPD)

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*The RAPD method was used to assess the genetic differentiation of brown hare (*Lepus europaeus*) populations from Central Greece. Greek wild populations were compared with samples from Austria, Poland, Germany, France, and Bulgaria, as well as with reared/released hares to investigate the impact of the releases on the native populations' genetic structure. The absence of diagnostic bands distinguishing between* L. europaeus *populations confirmed the high level of gene flow between brown hare populations over long geographic distances reported by other authors. Phylogenetic trees, derived from genetic distances estimated by RAPD band frequencies, suggested one major partitioning event of nuclear DNA lineages found in the samples. The reared individuals clustered with the Austrian, Polish, German, and French populations, whereas the Greek populations clustered apart with the Bulgarian population. Within Greece the distribution of the six wild populations did not follow any geographical trend, since their genetic divergence did not seem to correlate to geographic distances. However, RAPD profiles of some reared and wild specimens were different from the common RAPD pattern observed in the vast majority of sampled hares, probably reflecting an admixture of genetically differentiated individuals. The RAPD analysis indicates that releases*

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might have begun to affect Greek population structure and reinforces the view that appropriate management is needed, adjusted to the local populations' biology and ecology.

KEY WORDS: *Lepus europaeus*; genetic diversity; genetic conservation; RAPDs.

INTRODUCTION

The brown hare (*Lepus europaeus* Pallas, 1778) is widely distributed throughout Europe, including the mainland and the islands of Greece, where it represents an important game species present in open woodland, farmland with pasture and grassland up to 1500 m (Mitchell-Jones *et al.*, 1999).

In a previous study, the genetic differentiation of brown hare populations from Central Greece was assessed using mtDNA RFLP-PCR analysis (Mamuris *et al.*, 2001). Extensive haplotype diversity within and among wild populations was observed, while significant genetic structuring was evident from heterogeneity of haplotypes frequencies among sampling sites. The average pairwise haplotype divergence of 2.0% for the sampled Greek brown hares was approximately five-fold higher than the highest value (0.38%) recorded in Scandinavian brown hares (Thulin *et al.*, 1997), almost twofold higher than the average (1.3%) of Italian hares (Pierpaoli *et al.*, 1999), and threefold lower than the average (6.2%) of Iberian brown hares (Perez-Suarez *et al.*, 1994). Therefore, mtDNA analysis indicates that brown hares, at least from continental Greece, are not separated into discernible phyletic groups. Furthermore, the Greek brown hares appeared highly polymorphic, exhibiting a high degree of population differentiation with limited maternal gene flow. This observation is in agreement with previous studies on Iberian (Perez-Suarez *et al.*, 1994), Scandinavian (Thulin *et al.*, 1997), and Italian (Pierpaoli *et al.*, 1999) brown hares, but contrasts with the study of Hartl *et al.* (1993), in which a survey with 131 brown hares from 18 different sampling sites in Austria revealed only six haplotypes.

Here, we extend the investigation of the genetic structure of brown hare populations to the nuclear level on the same samples from Central Greece using the RAPD method (Welsh and McClelland, 1990; Williams *et al.*, 1990). Brown hares from five other European countries (Poland, Austria, Germany, France, and Bulgaria) were also included in the analysis. Population genetic studies based on other nuclear markers, such as allozymes, did not reveal genetic differences among samples collected from Poland (Hartl *et al.*, 1992), Austria (Hartl *et al.*, 1993), and Bulgaria (Suchentrunk *et al.*, 2000), indicating a high level of gene flow among local and distant populations. However, allozymes are conservative genetic markers, evolving slowly, and perhaps they do not have the resolving power to reveal population differentiation. On the other hand, the RAPD markers are the amplified products of less functional part of the genome that do not strongly

respond to selection on the phenotypic level. Such DNA regions may accumulate more nucleotide mutations as compared with those encoded for allozymes, offering a wider potential in assessing interpopulation genetic differentiation.

The present study aims at assessing (a) the present level of nuclear genetic diversity within and among populations and (b) the genetic impact of releases to Greek wild populations. During the past decades, restocking programs with the introduction of allocthonous hares that have been carried out in several European countries might have seriously influenced the historical distribution and genetic integrity of indigenous hare species (Flux 1983; Pierpaoli *et al.*, 1999; Thulin *et al.*, 1997). In Greece, according to the records of the Ministry of Agriculture and the hunting associations, releases of reared hares imported from farms in Italy, Bulgaria, and Yugoslavia have been performed during the past decade. Imported hares have been used as breeding stocks in several Greek farms. In a previous study (Mamuris *et al.*, 2001) designed to assess the impact of the releases of reared individuals on the genetic structure of native populations, specific mtDNA profiles clearly differentiated reared from wild individuals and proved highly indicative for reared hares from past releases caught within wild populations. MtDNA analysis suggested the introgression of allochthonous gene pools into the native populations.

MATERIALS AND METHODS

Samples

A total of 327 brown hares, 26 reared and 301 wild were examined. Reared specimens came from two different farms (22 and four, respectively) and blood samples were collected before hares were released into the wild. Of the wild individuals, 172 belonged to six sampling areas (operationally called populations) from central Greece (Epirus and Thessaly) (Fig. 1 and Table I). The individuals under investigation were studied previously for mtDNA variation using RFLP-RCR analysis (Mamuris *et al.*, 2001). Specific mtDNA profiles that clearly differentiated reared from wild individuals permitted the identification of reared hares from past release within wild populations. These hares were separated from the wild samples and were assigned to a new group, named "likely-reared" population ($N = 19$). The remaining 129 wild hares came from Austria ($N = 10$), Poland ($N = 10$), Germany ($N = 46$), France ($N = 21$), and Bulgaria ($N = 42$). Samples from reared individuals were collected in 1999, whereas Greek wild samples were collected during two consecutive hunting seasons (1998 and 1999) and tissues were brought on ice or in ethanol to the laboratory immediately after hunting.

The main study area in Greece covers a surface of 23,100 km^2 and was chosen for the following reasons: (a) it comprises the whole range of brown hare habitats, (b) there is evidence that in the last 10 years reared brown hares, imported from abroad have been released by the hunting associations within the whole area,

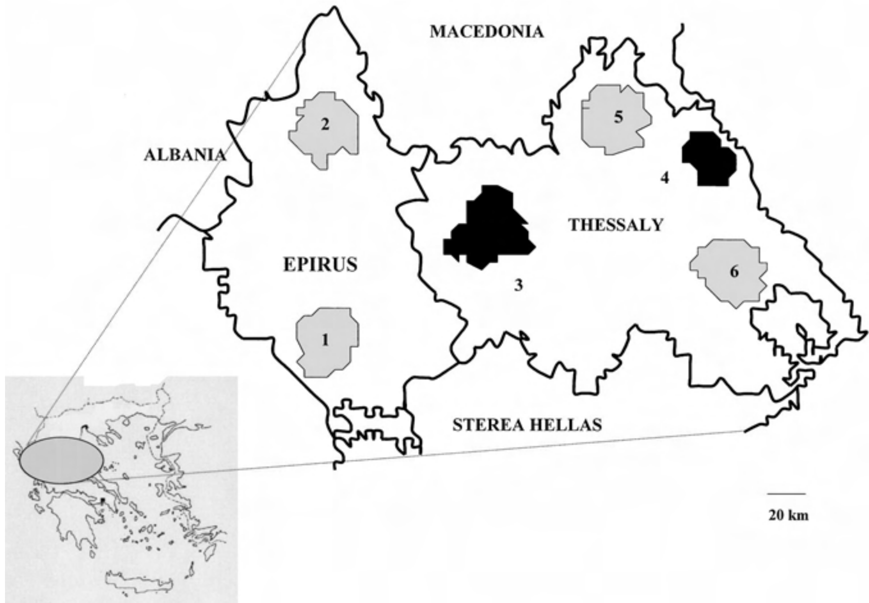

Fig. 1. Brown hare sampling sites within Greece: (1) Zallogo (ZA); (2) Vradeto (VR); (3) Pyrra (PY); (4) Spilia (SP); (5) Elassona (EL); (6) Velestino (VE). Black domains correspond to controlled-hunting areas.

Table I. Polymorphism in Populations of *L. europaeus*

		OPA-02		OPA-09		OPA-10		OPA-20		OPF-01	
Population	*N*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*
Zallogo	26	19	11	14	8	12	6	17	11	9	6
Vradeto	21	19	11	14	8	12	6	17	11	9	6
Pyrra	36	19	11	14	8	12	6	17	11	9	6
Spilia	23	19	11	14	8	12	6	17	11	9	6
Elassona	20 (4)	19	11	14	8	12	6	17	11	9	6
Velestino	23	19	11	14	8	12	6	17	11	9	6
Reared	24 (2)	19	9	14	5	12	5	17	9	9	4
Likely-reared	14 (5)	19	9	14	5	12	6	17	9	9	4
Austria–Poland	20	19	10	14	5	12	6	17	9	9	4
Germany	56	19	10	14	5	12	6	17	9	9	4
France	21	19	10	14	5	12	6	17	9	9	4
Bulgaria	42	19	11	14	8	12	6	17	10	9	6

Note. N: Number of specimens (numbers in parentheses indicate hares with “deviant” RAPD profiles); *n*: Number of RAPD bands analyzed per population and per primer; *P*: Number of polymorphic bands found per primer in each population of *L. europaeus*.

except into two controlled hunting areas (PY and SP) (Fig. 1), and (c) there is an ongoing parallel survey within the same area concerning hares ecology and biology.

RAPD Analysis

Total DNA extraction was performed using standard techniques (cell lysis using proteinase K, SDS, and EDTA; protein purification using chloroform followed by isopropanol precipitation). Forty decamer primers (OPA-01 to OPA-20 and OPF-01 to OPF-20) were used during this study, all purchased from Operon Technologies, Alameda, CA, U.S.A. Experiments were run with six individuals from each population to test the effect of DNA, dNTPs, Mg^{2+}, and Taq polymerase concentrations and to determine the optimal annealing temperature. Finally, consistent results were obtained with 31 primers and the same reaction conditions were applied to all individuals. PCR reactions were performed in 25 μL of reaction mixture containing 30 ng of template DNA, one unit of Taq polymerase, 0.25 mM dNTPs, 20 ng of each primer, 2.5 mM $MgCl_2$ and $1\times$ reaction buffer. Amplification conditions included a total of 35 cycles of 40 s at 94°C, 1 min at 38°C, 1 min at 72°C, using the fastest available transitions between each temperature.

To assess genetic markers capable of distinguishing populations six individuals from each population were screened with all 31 random primers. Amplification products were separated on 1.4% agarose gels containing 0.5 μL mL^{-1} of ethidium bromide in TAE buffer (40 mM Tris/acetate, 1 mM EDTA). In addition, to determine genetic relationships between populations, all individuals from each population were screened with five primers chosen arbitrarily (OPA-02, OPA-09, OPA-10, OPA-20, and OPF-01) and the amplification products were separated on 6% polyacrylamide gels. To test the reproducibility of each random primer, two RAPD-PCR replications were performed for every sample and every primer. Bands that were reproduced after the two replications were considered reliable. To avoid problems such as variations of number and intensity of the amplified products band mobility comparisons were made only within gels and based on the same PCR reaction.

Furthermore, pilot tests were performed to determine if fully reproducible comigrating bands could be considered homologous between populations and therefore scored as the same genetic marker. Twenty-five fully reproducible comigrating bands with different sizes, ranging from 400 to 800 bp, chosen arbitrarily after amplification with different primers, were excised from agarose gels and then digested with 4-base and 5-base cutter restriction endonucleases (*Alu*I, *Hae*III, *Msp*I, *Ava*II, and *Dde*I). This test was performed using five individuals from each population.

To calculate genetic parameters between and within populations, the equations 2a, 4a, 5, 7, 9a, 10a, 12, 13a, 14a, 15a proposed by Lynch and Milligan (1994) were applied. According to the authors, only polymorphic bands whose

frequency was less than $1 - (3/N)$, where N is the number of individuals analyzed for each population, were taken into consideration for estimating heterozygosities within populations. Gene identity within (J_i) and between (J_{ij}) populations were computed for all possible comparisons. Genetic distances (Nei, 1972) from all primers were used to estimate genetic relationships between populations, using the UPGMA and the Neighbour-joining phylogenetic trees. A parsimony analysis, based on Wagner method, was utilized, where all RAPD bands were considered as characters with one of two states, present (1) or absent (0). The binary-coded characters were used for the analysis. All dendrograms were constructed and confidence estimates were calculated using the PHYLIP 3.5 computer packages (Felsenstein, 1993). Given the absence of outgroup to root the phylogenetic trees, all data were treated using the "randomize input order of species" option.

RESULTS

Agarose Electrophoreses

All but nine primers yielded satisfactory amplification products with all specimens tested. Each primer produced a unique band pattern and most bands were between 100 and 2500 bp in size. No tissue specific RAPD marker was observed between blood (reared hares) and liver (wild hares). All primers failed to yield any diagnostic marker that could lead to unambiguous identification of the various populations. Banding pattern variation observed was analyzed on polyacrylamide gels. A problem linked to the application of the RAPD technique, is the homology between comigrating bands produced by the same random primer in different individuals. In the current study, 20 out of 25 fragments digested with the five restriction enzymes showed digestion patterns that indicated homology between comigrating bands. For five fragments the test was uninformative, because no restriction fragments were detected. These five RAPD bands were excluded from further analysis. Because similar tests were not conducted for all the comigrating bands scored, occasional misinterpretation of the origin of these bands might have occurred throughout this study. However, the analysis of the few comigrating bands suggests that misinterpretation would be unlikely or rare.

Although all primers produced similar DNA fragment patterns, 11 hares exhibited quite different but reproducible profiles with all primers (Fig. 2). Of these 11 hares, two came from the reared population, five from the "likely-reared" population and four from the EL population (Table I).

Polyacrylamide Electrophoreses

The number of individuals analyzed on polyacrylamide gels per population and per primer is shown in Table I. A preliminary χ^2 contingency analysis of RAPD marker frequencies showed no significant differences between the samples from Austria

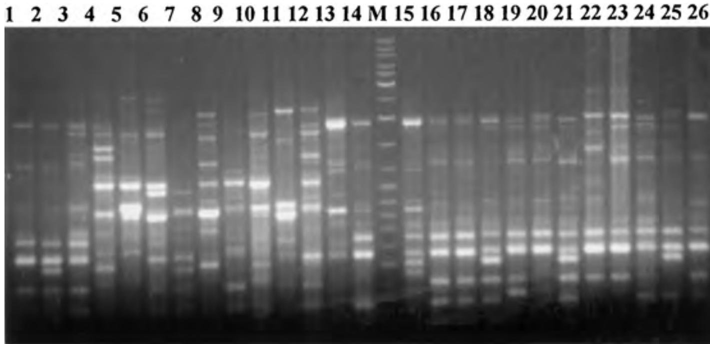

Fig. 2. RAPD profiles after amplification with the primer OPA-02. 1, 2, 3, and 14–26, common RAPD profile of some individuals from all populations. 4–13, individuals with "deviant" profiles from reared (4, 5), "likely-reared" (6, 7, 8, 9), and EL (10, 11, 12, 13) populations. $M = 100$ bp ladder.

and Poland and thus these two samples were pooled to increase the accuracy of comparisons. The number of reproducible and well-resolved bands analyzed per primer in all populations ranged from 9 to 19 (Table I). Initially, more bands were scored per primer and per population but due to the need for pruning polymorphic loci with low-frequency null alleles only 71 of them remained. Of the 71 bands analyzed for all the five random primers, 42 (59.15%) were polymorphic, and the rest were monomorphic, constantly present in all individuals (Table II).

The χ^2 contingency analysis of RAPD marker frequencies, after Bonferoni corrections, showed significant heterogeneity ($p < 0.001$) for 12 of the 42 polymorphic markers (28.6%) (Table II). This percentage was reduced to 19% (8/42) after the exclusion of the reared and the "likely-reared" samples, to 7% (3/42) among the reared, the "likely-reared" and the Central European samples and to 4.76% among the Greek samples. Overall analysis of marker frequencies, using χ^2-tests with a Monte-Carlo simulation, showed significant geographical heterogeneity ($p < 0.0001$) when all populations were included in the analysis. However the test showed no significant differences among the Greek samples ($p > 0.05$).

The estimated values of heterozygosity were slightly higher for all comparisons between populations (mean $H_{ij} = 0.269$) than for those observed within populations (mean $H_i = 0.246$) (Table II). Reared and "likely-reared" samples with $H_i = 0.202$ and $H_i = 0.200$, respectively had the lower estimated heterozygosity compared with the wild samples (mean $H_i = 0.255$) (Table II). Estimates of F_{ST} using the estimated heterozygosities for the 12 populations, suggested the presence of subdivision among populations ($F_{ST} = 0.086$). This subdivision was less pronounced when the reared and the "likely-reared" populations were excluded ($F_{ST} = 0.055$) and much lower within samples from Greek wild populations ($F_{ST} = 0.022$).

Table II. Frequencies of the Null Allele of Polymorphic RAPD Markers in *L. europaeus* Populations

RAPD marker	ZA ($n = 26$)	VR ($n = 21$)	PY ($n = 36$)	SP ($n = 23$)	EL ($n = 20$)	VE ($n = 23$)	Reared ($n = 24$)	Likely-reared ($n = 14$)	Austria–Poland ($n = 20$)	Germany ($n = 46$)	France ($n = 21$)	Bulgaria ($n = 42$)	Chi-squared value[a]
OPA-02_{1900}	0.518	0.872	0.420	0.722	0.670	0.417	0.500	0.462	0.387	0.361	0.378	0.556	40.387*
OPA-02_{1800}	0.679	0.487	0.514	0.551	0.547	0.510	0.577	0.654	0.591	0.571	0.577	0.598	8.965
OPA-02_{1400}	0.438	0.690	0.485	0.510	0.500	0.466	0.000	0.000	0.387	0.390	0.378	0.617	76.450*
OPA-02_{1200}	0.518	0.577	0.618	0.551	0.547	0.510	0.540	0.534	0.591	0.589	0.617	0.672	3.079
OPA-02_{1000}	0.679	0.816	0.685	0.722	0.774	0.659	0.500	0.534	0.547	0.532	0.534	0.772	17.929
OPA-02_{900}	0.554	0.534	0.420	0.589	0.591	0.510	0.456	0.462	0.000	0.000	0.000	0.636	47.735*
OPA-02_{850}	0.620	0.755	0.727	0.659	0.806	0.691	0.707	0.654	0.670	0.691	0.690	0.632	5.800
OPA-02_{500}	0.518	0.617	0.641	0.510	0.500	0.551	0.577	0.534	0.547	0.552	0.534	0.556	4.542
OPA-02_{400}	0.438	0.436	0.453	0.417	0.547	0.466	0.000	0.000	0.447	0.466	0.436	0.547	59.123*
OPA-02_{350}	0.733	0.872	0.874	0.884	0.836	0.884	0.707	0.707	0.670	0.659	0.654	0.774	18.344
OPA-02_{250}	0.759	0.755	0.618	0.780	0.806	0.691	0.816	0.845	0.741	0.766	0.756	0.756	10.684
OPA-09_{1250}	0.392	0.487	0.453	0.510	0.447	0.466	0.000	0.000	0.000	0.000	0.000	0.487	77.882*
OPA-09_{1100}	0.554	0.534	0.420	0.589	0.591	0.551	0.612	0.597	0.632	0.659	0.654	0.436	6.637*
OPA-09_{1000}	0.438	0.577	0.568	0.589	0.500	0.589	0.000	0.000	0.000	0.000	0.000	0.617	108.539*
OPA-09_{950}	0.518	0.487	0.485	0.466	0.632	0.510	0.500	0.462	0.591	0.625	0.617	0.547	4.690
OPA-09_{900}	0.392	0.617	0.594	0.722	0.707	0.691	0.000	0.000	0.000	0.000	0.000	0.723	145.629*
OPA-09_{850}	0.588	0.577	0.664	0.589	0.632	0.551	0.500	0.534	0.547	0.532	0.577	0.707	4.552
OPA-09_{680}	0.438	0.487	0.420	0.510	0.547	0.417	0.456	0.462	0.447	0.442	0.487	0.547	3.047
OPA-09_{350}	0.679	0.755	0.685	0.691	0.707	0.691	0.677	0.707	0.670	0.691	0.654	0.816	1.600
OPA-10_{1800}	0.518	0.436	0.420	0.466	0.500	0.417	0.577	0.534	0.547	0.552	0.534	0.654	7.738
OPA-10_{1600}	0.733	0.690	0.857	0.691	0.670	0.722	0.735	0.755	0.670	0.659	0.654	0.755	7.214
OPA-10_{850}	0.438	0.436	0.453	0.510	0.500	0.417	0.000	0.377	0.387	0.361	0.378	0.487	37.488
OPA-10_{750}	0.679	0.534	0.664	0.589	0.707	0.551	0.540	0.534	0.447	0.466	0.436	0.707	12.268
OPA-10_{650}	0.438	0.577	0.420	0.417	0.387	0.417	0.408	0.462	0.387	0.417	0.392	0.517	5.789
OPA-10_{350}	0.784	0.872	0.785	0.751	0.894	0.722	0.707	0.707	0.774	0.766	0.756	0.723	9.244
OPA-20_{2500}	0.480	0.487	0.485	0.510	0.500	0.417	0.577	0.597	0.591	0.608	0.617	0.577	7.193
OPA-20_{2000}	0.854	0.723	0.727	0.807	0.774	0.780	0.735	0.597	0.547	0.552	0.535	0.816	17.842
OPA-20_{1800}	0.554	0.487	0.568	0.691	0.741	0.510	0.500	0.654	0.447	0.490	0.487	0.547	14.630
OPA-20_{1200}	0.480	0.654	0.514	0.589	0.500	0.625	0.456	0.462	0.447	0.442	0.436	0.487	8.250
OPA-20_{1100}	0.679	0.577	0.641	0.625	0.632	0.659	0.645	0.597	0.632	0.625	0.617	0.690	1.335

OPA-20_{1000}	0.650	0.723	0.727	0.722	0.670	0.691	0.707	0.707	0.632	0.608	0.654	0.774	2.184
OPA-20_{950}	0.480	0.534	0.485	0.466	0.500	0.417	0.540	0.462	0.547	0.486	0.489	0.617	2.963
OPA-20_{700}	0.438	0.534	0.514	0.417	0.547	0.466	0.000	0.000	0.000	0.000	0.000	0.000	101.065*
OPA-20_{650}	0.518	0.436	0.420	0.466	0.632	0.466	0.577	0.654	0.547	0.571	0.617	0.632	10.975
OPA-20_{600}	0.438	0.577	0.568	0.417	0.387	0.625	0.000	0.000	0.000	0.000	0.000	0.632	104.972*
OPA-20_{450}	0.518	0.436	0.542	0.510	0.447	0.466	0.500	0.534	0.447	0.511	0.486	0.577	2.871
OPF-01_{2800}	0.877	0.872	0.685	0.884	0.707	0.884	0.889	0.845	0.866	0.860	0.816	0.755	21.065
OPF-01_{2000}	0.784	0.899	0.822	0.807	0.774	0.659	0.866	0.886	0.836	0.860	0.873	0.872	14.397
OPF-01_{1300}	0.438	0.436	0.594	0.417	0.500	0.510	0.456	0.462	0.500	0.466	0.436	0.534	5.404
OPF-01_{1150}	0.438	0.577	0.514	0.510	0.447	0.466	0.000	0.000	0.000	0.000	0.000	0.707	98.238*
OPF-01_{1000}	0.759	0.872	0.766	0.691	0.774	0.417	0.677	0.707	0.670	0.722	0.690	0.707	11.486
OPF-01_{950}	0.392	0.487	0.485	0.417	0.447	0.466	0.000	0.000	0.000	0.000	0.000	0.577	101.230*
H_i[b]	0.269	0.255	0.267	0.264	0.262	0.272	0.202	0.200	0.236	0.238	0.240	0.258	
J_i	0.731	0.745	0.733	0.736	0.738	0.728	0.798	0.800	0.764	0.762	0.760	0.742	

[a]Results of χ^2 contingency analysis for heterogeneity in RAPD marker frequencies between samples with 11 degrees of freedom (* indicates a significant result after Bonferroni correction).
[b]Estimated heterozygosity (H_i) and gene identities between individuals (J_i) within each population.

Table III. Nei's Genetic Distance Between 12 Populations of *L. europaeus* Based on RAPD Analysis

Populations	1	2	3	4	5	6	7	8	9	10	11	12
1. Zallogo	—											
2. Vradeto	0.013	—										
3. Pyrra	0.012	0.009	—									
4. Spilia	0.007	0.005	0.008	—								
5. Elassona	0.010	0.010	0.011	0.006	—							
6. Velestino	0.012	0.012	0.009	0.006	0.008	—						
7. Reared	0.074	0.085	0.076	0.075	0.082	0.077	—					
8. Likely-reared	0.069	0.086	0.076	0.077	0.084	0.081	0.005	—				
9. Austria–Poland	0.068	0.081	0.071	0.073	0.077	0.074	0.006	0.009	—			
10. Germany	0.067	0.077	0.068	0.071	0.075	0.072	0.006	0.007	0.002	—		
11. France	0.070	0.079	0.069	0.073	0.075	0.074	0.008	0.011	0.003	0.003	—	
12. Bulgaria	0.022	0.019	0.021	0.015	0.019	0.025	0.078	0.077	0.083	0.081	0.081	—

Values of intrapopulation gene identity (mean $J_i = 0.753$) (Table II) were very close to those estimated among populations (mean $J_{ij} = 0.726$). Reared and "likely-reared" sample with $J_i = 0.798$ and $J_i = 0.800$, respectively had higher intrapopulation gene identity compared with the wild samples (mean $J_i = 0.743$). Values of pairwise comparisons of Nei's genetic distance (D) between populations (Nei, 1972), computed from combined data for the five primers, ranged from $D = 0.002$ (Austria–Poland and Germany) to $D = 0.086$ ("likely-reared" and Vradeto) (Table III). Genetic distances among the six wild Greek samples ranged from $D = 0.005$ (Spilia and Vradeto) to $D = 0.013$ (Zallogo and Vradeto) with an average of $D = 0.009$, while the average of genetic distances among the three Central European (Austria–Poland, Germany, France) samples was 0.003. The reared and the "likely-reared" samples were genetically very close to the Central European samples ($D = 0.006$ and $D = 0.008$), but distinct from the Greek and the Bulgarian samples (mean $D = 0.078$ for all pairwise comparisons between the two groups of populations). All methods used to generate phylogenetic trees produced similar results (Fig. 3), except for some minor differences, and they suggested one major partitioning event of nuclear DNA lineages. The reared individuals clustered with the Austrian, Polish, German, and French populations, whereas the Greek populations clustered apart with the Bulgarian population. Within Greece the distribution of the six wild populations did not follow any geographical trend, because their genetic divergence did not seem to correlate to geographic distances.

DISCUSSION

The RAPD technique (Williams *et al.*, 1990), apart from single copy fractions, also amplifies highly repetitive regions that may accumulate more nucleotide mutations compared with those encoding allozymes. Thus, several authors reported

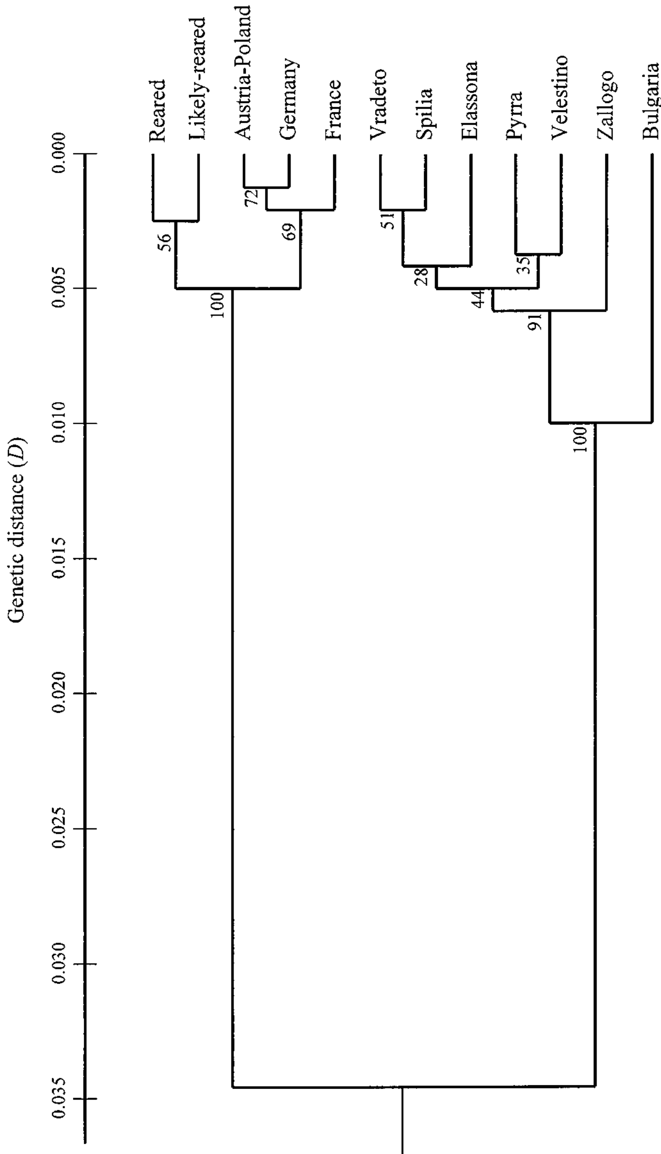

Fig. 3. UPGMA phenogram clustering the 12 brown hare populations according to Nei's (1972) genetic distance. Numbers above branches indicate bootstrap estimates as a percentage after 200 replications.

specific RAPD markers, useful for distinguishing intraspecies populations or between closely related species, in organisms where allozymes have been proven to have low resolution power to assess genetic differences (Black *et al.*, 1992; Cognato *et al.*, 1995). The absence of unique bands distinguishing populations at least between Greece and Central Europe may be indicative of a high level of nuclear gene flow over long geographic distances, pointed out by other authors after multilocus isozyme screening (Hartl *et al.*, 1993; Suchentrunk *et al.*, 2000). In this respect, RAPD data are not in concordance with the mtDNA study on Greek brown hares (Mamuris *et al.*, 2001). MtDNA analysis showed that the percentage of unique haplotypes within each region remained high, indicating that the populations are substantially isolated. However, the fact that females almost exclusively inherit mtDNA (Avise, 1994) combined with the fact that natal dispersal might be sex biased, i.e., males disperse while females tend to remain within their breeding group (Hulbert *et al.*, 1996; Reitz and Leonard, 1994), may result in an overall reduction in mitochondrial gene flow. Thus, because of sex-specific dispersal, it is possible that interpopulation diversity assessed by mtDNA markers is in disagreement with the RAPD markers. Our results are more in agreement with those of Fickel *et al.* (1999) who observed a distinct mtDNA differentiation across short geographic distances in German brown hare populations, but this differentiation was less evident when microsatellites were used to screen the same populations.

Despite the fact that all primers failed to produce specific markers that could discriminate between all populations, statistic analysis revealed genetic heterogeneity between them. Overall analysis of band frequencies suggested significant ($p < 0.0001$) genetic subdivision when all populations were considered. However, there were no significant differences between the six geographical populations within Greece. These results were further confirmed by F_{ST} analysis. F_{ST} values showed significant genetic structuring when all samples were considered ($F_{ST} = 0.086$) but very little between Greek samples ($F_{ST} = 0.022$). The latter is probably due to the absence of physical barriers that could prevent gene dispersal and to the presence of several subpopulations within each sampling area. Using radio-tracking it has been shown that brown hare home-range have a size of 200–340 ha (Homolka, 1985). In the present study, except for the SP region (3140 ha), each sampling area covered a surface ranging from 6400 (PY) to approximately 8000 ha (EL, VEL, VR, ZA).

Phylogenetic trees showed that the populations of *L. europaeus* clustered into two distinct clades, reflecting the partitioning of the RAPD band frequencies among samples (Fig. 3). Interestingly, the reared and the "likely-reared" populations clustered with the populations from Austria, Poland, France, and Germany, whereas the Greek populations clustered apart with the Bulgarian population. In a previous genetic study, after multilocus allozyme screening, Suchentrunk *et al.* (2000) did not observe any clear segregation between Bulgarian and Austrian brown hare populations. This difference between RAPDs and allozymes probably

arises from the fact that the two methods generate markers pertaining to different parts of the genome. Single copy regions of the genome, having a serious impact on important phenotypic characters and thus being more easily subject to selective pressure encode most of the allozymes. It is therefore probable that most of the RAPD markers are the amplified products of less functional part of the genome that do not strongly respond to selection on the phenotypic level. Such DNA regions may accumulate more nucleotide mutations as compared to those encoded for allozymes. Thus, RAPDs can detect a more pronounced genetic polymorphism between geographically distant brown hare samples than allozymes, provided that the previous conditions hold and also that the level of gene flow is unable to mask the consequences of new mutations.

Within Greece the distribution of the six wild populations did not follow any geographical trend, because their genetic divergence seems to be independent to geographic distances. The latter was also supported by the mtDNA analysis (Mamuris *et al.*, 2001).

Within the past decade, according to the records of hunting associations, more than 2000 reared brown hares have been released in the studied area and 826 of those have been released during the years 1998 and 1999. Although the released animals came from Greek farms, they had been originally imported from other European farms. Releases of imported brown hares might have begun to affect local gene pools, because both mitochondrial and nuclear markers showed that the "likely-reared" hares sampled in the wild were genetically very close to the reared sample. On the other hand, if "reared" hare genes were affecting local gene pools one would expect that the individuals identified as "likely-reared" by mtDNA would not clearly segregate with the reared individuals. The fact that the RAPD patterns segregate the reared and the "likely-reared" individuals from the other wild hare populations may indicate that the genetics of the "likely-reared" hares are staying intact. Therefore, it might be that the "likely-reared" animals were hunted just after they were released. Uncertain, however, remains the basis of the genetic similarity of the reared and the "likely-reared" hares only with those from Central Europe. Reared hares have also been imported from Bulgaria but less similarity was obsrved between reared hares and hares imported from the latter country. A possible explanation could be that the same breeding lines are recycled in many European farms and are established from animals with different genetic identity to that of the wild populations of each country.

The presence in the wild of nine individuals with RAPD patterns clearly different from the vast majority observed within all examined samples (Fig. 2) could be also indicative of the impact of the releases to the indigenous populations. Comparison with mtDNA data (Mamuris *et al.*, 2001) showed that these nine hares shared common or closely related *L. europaeus* haplotypes with other hares from the same populations, having common RAPD patterns. The presence

of two individuals in the reared population and of five individuals in the "likely-reared" population with different RAPD patterns indicates that this pattern detected in four individuals within a natural population came from the released hares. In the absence of genetic data on the past releases and of a complete record of the source of released animals, any hypothesis about the presence in the wild of these nine hares is difficult to check. It is possible that this "unique" pattern has been increasing in frequency by artificial "genetic drift" under captive breeding conditions. More intensive genetic studies, including brown hares from other countries and using codominant markers (i.e., microsatellites) could shed more light on this subject.

Ongoing studies in the sampling areas indicate that population densities do not exceed five individuals per 100 ha (VR area) with an average of two individuals per 100 ha (Sfougaris *et al.*, unpublished data). These values are very low compared with other European countries such as Poland (24–31 individuals per 100 ha; Wasilewski, 1991), France (23–71 individuals per 100 ha; Pepin, 1987), and North Italy (38–53 individuals per 100 ha; Meriggi and Verri, 1990). It is often assumed that, when a population goes through a severe bottleneck, random genetic drift will induce a massive loss of genetic variability. According to the records of the Ministry of Agriculture, during the years 1986–1990, a serious decline occurred in the brown hare populations due to the viral haemorrhagic disease. Certainly, RAPDs, because of their dominance property, are not the appropriate markers to assess the level of intrapopulation polymorphism with accuracy. Nevertheless, both reared and "likely-reared" populations, with estimated heterozygosity of $H_{\mathrm{i}} = 0.202$ and $H_{\mathrm{i}} = 0.200$, respectively and with intrapopulation similarity indices of $J_{\mathrm{i}} = 0.798$ and $J_{\mathrm{i}} = 0.800$, respectively showed relatively little genetic diversity compared with the wild populations (mean $H_{\mathrm{i}} = 0.257$ and mean $J_{\mathrm{i}} = 0.743$). A low level of genetic diversity within the reared and the "likely-reared" populations, in comparison with natural populations, has also been observed in mitochondial genome (Mamuris *et al.*, 2001). A crucial issue that remains to be checked, dealing also with the efficacy of restocking programs, is the survival of the released animals. High rates of virus infections due to different life conditions in captivity (mode of nutrition, administration of antibiotics) and/or unsuccessful adaptation in nature could seriously affect the survival of released animals. Whatever the fate of released individuals, our mtDNA data indicate that a percentage survives at least a year, between two hunting periods, enough to have one reproductive cycle and to transmit their genome. Therefore, uncontrolled introduction and releases of brown hares, at least at periods when natural populations go through bottleneck, could have a serious impact on their present genetic structure. On the long run, this may lead to a loss of the local and/or regional genetic diversity and conservation of genetic diversity is a major task in conservation and evolutionary biology, because genetic variation is the raw material for evolutionary changes within populations (Frankel and Soulé, 1981).

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REFERENCES

Avise, J. C. (1994). *Molecular Markers, Natural History and Evolution*, Chapman and Hall, New York.

Black, W. C., IV, DuTeau, N. M., Puterka, G. J., Nechols, J. R., and Pettorini, J. M. (1992). Use of the RAPD polymerase chain reaction to detect DNA polymorphisms in aphids (Homoptera: Aphididae). *Bull. Ent. Res.* **82:**151.

Cognato, A. I., Rogers, S. O., and Teale, S. A. (1995). Species diagnosis and phylogeny of the *Ips grandicollis* group (Coleoptera: Scotylidae) using random amplified polymorphic DNA. *Ann. Entomol. Soc. Am.* **88:**397.

Felsenstein, J. (1993). PHYLIP (*Phylogeny inference package*), *version 3.5c*, Department of Genetics, University of Washington, Seattle.

Fickel, J., Lieckfeld, D., and Pitra, C. (1999). Analyse der genetischen Diversität und Struktur in benachbarten Populationen des Feldhasen (*Lepus europaeus*, Pallas 1778). *Z. Jagdwiss.* **45:**230.

Flux, J. E. C. (1983). Introduction to taxonomic problems in hares. *Acta Zool. Fennica* **174:**7.

Frankel, O. H., and Soulé, M. E. (1981). *Conservation and Evolution*, Cambridge University Press, Cambridge.

Hartl, G. B., Markowski, J., Swiatecki, J., Janiszewski, T., and Willing, R. (1992). Genetic diversity in the Polish brown hare *Lepus europaeus* Pallas, 1778: implications for conservation and management. *Acta Theriol.* **37:**15–25.

Hartl, G. B., Suchentrunk, F., Nadlinger, K., and Willing, R. (1993). An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. *Acta Theriol.* **38:**33.

Homolka, M. (1985). Spatial activity of hares (*Lepus europaeus*). *Folia Zool.* **34:**217.

Hulbert, I. A. R., lason, G. R., Elston, D. A., and Racey, P. A. (1996). Home-range sizes in a stratified upland landscape of two lagomorphs with different feeding strategies. *J. Appl. Ecol.* **33:**1479.

Lynch, M., and Milligan, B. G. (1994). Analysis of population genetic structure with RAPD markers. *Mol. Ecol.* **3:**91.

Mamuris, Z., Sfougaris, A. I., and Stamatis, C. (2001). Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA RFLP-PCR analysis: Implications for conserving genetic diversity. *Biol. Conserv.* **101:**187.

Meriggi, A., and Verri, A. (1990). Population dynamics and habitat selection of the European hare on poplar monocultures in northern Italy. *Acta Theriol.* **35:**69.

Mitchell-Jones, A. J., Amori, G., Bogdanowicz, W., Krystufek, B., Reijnders, P. J. H., Spitzenberger, F., Stubbe, M., Thissen, J. B. M., Vohralik, V., and Zima, J. (1999). *The Atlas of European Mammals*, Poyser Natural History. Published by T & A. D. Poyser for the Societas Europaea Mammalogica.

Nei, M. (1972). Genetic distance between populations. *Am. Nat.* **106:**283–292.

Pepin, D. (1987). Dynamics of a heavily exploited population of brown hare in a large-scale farming area. *J. Appl. Ecol.* **24:**725.

Perez-Suarez, G., Palacios, F., and Boursot, P. (1994). Speciation and paraphyly in Western Mediterranean hares (*Lepus castroviejoi*, *L. europaeus*, *L. granatensis* and *L. capensis*) revealed by mitochondrial DNA phylogeny. *Biochem. Genet.* **32:**423.

Pierpaoli, M., Riga, F., Trocchi, V., and Randi, E. (1999). Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. *Mol. Ecol.* **8:**1805.

Reitz, F., and Leonard, Y. (1994). Characteristics of European hare *Lepus europaeus* use of space in a French agricultural region of intensive farming. *Acta Theriol.* **39:**143.

Suchentrunk, F., Michailov, C., Markov, G., and Haiden, A. (2000). Population genetics of Bulgarian brown hares *Lepus europaeus*: Allozymic diversity at zoogeographical crossroads. *Acta Theriol.* **45:**1.

Thulin, C.-G., Jaarola, M., and Tegelstrom, H. (1997). The occurrence of mountain hare mitochondrial DNA in wild brown hares. *Mol. Ecol.* **6:**463.

Wasilewski, M. (1991). Population dynamics of the European hare *Lepus europaeus* Pallas, 1778 in Central Poland. *Acta Theriol.* **36:**267.

Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18:**7213.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18:**6531.

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Biochemical Genetic Variability in Brown Hares (*Lepus europaeus*) From Greece

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*Allozyme variability of 91 brown hares (*Lepus europaeus*) from seven regions in Greece was compared to existing data of Bulgarian populations to test the hypothesis of the occurrence of specific alleles in Greece, likely stemming from an isolated Late Pleistocene refugial population in the southern Balkans. This hypothesis is particularly suggested by some subfossil Late Pleistocene hare remains in Greece and the reported high mtDNA diversity in Greek hares. Allozymic diversity could be higher in Greek hares than in hares from neighboring regions as a result of the accumulation of variants in a long-lasting Pleistocene refugium. Conversely, Greek hares could exhibit reduced genetic diversity because of long-lasting low effective population sizes during the Late Glacial Maximum and a lower chance of postglacial gene flow from other populations into this rather marginal part in the southern Balkans. Horizontal starch gel electrophoresis of proteins from 35 loci revealed three alleles ($Es\text{-}1^{-162}$, $Pep\text{-}2^{114}$, Mpi^{88}) at low frequencies, which were not found in Bulgarian or any other brown hare population. In contrast, some alleles from the populations from Bulgaria and other regions of Europe were absent in the Greek samples. Population genetic statistics indicated only a slight tendency of increased gene pool diversity in Greek hares, little substructuring in Greek and Bulgarian populations, respectively, as well as an only slightly lower level of gene flow between the two neighboring regions, as compared to the gene flow within each region. The results conform to the hypothesis of a Late Pleistocene refugial population in the southern Balkans, with some few specific*

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nuclear gene pool characteristics, but little effect on the overall genetic differentiation between Greek and Bulgarian hares.

KEY WORDS: brown hare; *Lepus europaeus*; allozymes; population genetics; Greece; Mediterranean.

INTRODUCTION

Hares from Greece have been described as various subspecies (*Lepus europaeus carpathous, L. e. creticus, L. e. cyrensis, L. e. ghigii, L. e. meridiei, L. e. niethammeri, L. e. parnassius, L. e. rhodius, L. e. transsylvanicus*), based on coat coloration, body size, external body measurements, as well as skull and tooth characteristics (De Beaux, 1929; Hilzheimer, 1906, 1908; Miller, 1912; von Wettstein, 1943; see also Chaworth-Musters, 1932; Kattinger, 1972; Ondrias, 1965; Zimmermann *et al*., 1953; overview in De Beaufort, 1991). But apart from the old rather vague or anecdotal descriptions used for this classification, no comprehensive data exist to allow assessing the evolutionary position of these hares, their systematic status, and their possible subspecific ranges.

Greek hares exhibit a bewildering mtDNA variability as assessed by PCR–RFLPs and all detected haplotypes, apart from those considered originating from imported and released hares, were absent in brown hares from central Europe (Mamuris *et al*., 2001). In southern and central Greece, a mosaic of steppe and pockets of forest or scattered tree stands (e.g., Bennett *et al*., 1991; Lang, 1994; see also, e.g., Geraga *et al*., 2000; Roberts *et al*., 1999; Roberts and Wright, 1993) could have provided a refuge for brown hares during the Late Pleistocene, when large parts of Europe were either covered with ice or were otherwise unsuitable for this species (cf., e.g., Frenzel *et al*., 1992). Similar refugia of brown hares also might have existed over millennia in the south of the Italian and Iberian peninsulas (Corbet, 1986). In the absence of significant gene flow, refugial Mediterranean populations could have acquired sufficiently differentiated gene pools, warranting subspecific status of the respective hares.

In this study, we assess multilocus allozyme variability in Greek hares. We particularly test the hypothesis of the occurrence of indigenous alleles possibly resulting from multiple mutations during phases of isolation in a Late Pleistocene refuge in the south of the Balkan peninsula by comparing the present allozyme data to already published data of brown hares (Hartl *et al*., 1989, 1990, 1992, 1993, 1994, 1995). However, a Late Pleistocene refugial population in the southern Balkans could have lost variant alleles because of repeated range restrictions and concomitant reductions of effective population size under unfavorable habitat conditions and restricted gene flow. Also, such southern refugial hares would have had less chance of receiving genes from populations that possibly have spread after the Late Glacial Maximum from eastern Europe, because of their rather marginal

range in the south of the Balkan peninsula. Hence, we specifically analyze allozyme data of hares from sample regions in Greece together with data of brown hares from eight populations in Bulgaria produced earlier in the same laboratory (Suchentrunk *et al.*, 2000a). The latter hares can be considered as populations at a zoogeographical crossroad and might therefore have a richer genetic diversity than Greek hares have.

MATERIAL AND METHODS

Samples and Isozyme Electrophoresis

Liver tissue samples of 91 brown hares were collected during the hunting seasons (mid-September to early January 1998–2000) in seven regions (operationally called populations) of mainland Greece and in southeastern Crete. In mainland Greece, hares were shot mainly in shrubland at altitudes of ca. 300–1000 m asl in typical Eastern Mediterranean-type ecosystems, dominated by Kermes oak (*Quercus coccifera*) in four regions (Loutraki, $n = 10$; Velestino, $n = 5$; Epirus, $n = 5$; Serres, $n = 12$) and in landscapes with mixed fir forest–subalpine pastures, dominated by *Abies borisii-regis*, between 1500–1700 m asl (Spilia, $n = 29$; Pyrra, $n = 26$). Four hares were shot in dry scrubland around Ierapetra, southeastern Crete, between 100–400 m asl. The sample regions are shown in Fig. 1.

The samples were frozen within a few hours after shooting and stored at $-20°$C until further treatment in the laboratory. Horizontal starch gel electrophoresis was used to reveal allelic variation at isozyme loci. Poor quality of some samples and lack of spleen and blood samples, however, allowed scoring of only a limited number out of the loci studied earlier in hares from Europe (e.g., Alves *et al.*, 2000; 2001; Alves and Ferrand, 1999; Bonhomme *et al.*, 1986; Hartl *et al.*, 1989, 1990, 1992, 1993, 1994, 1995; Suchentrunk, 1993; and Suchentrunk *et al.*, 1998; 2000a; 2001).

The following 26 enzymes/enzyme systems corresponding to 35 putative structural gene loci (isozyme-system abbreviation, E.C. number, and respective structural gene loci in parentheses) were studied: sorbitol dehydrogenase (SDH, 1.1.1.14, *Sdh*), lactate dehydrogenase (LDH, 1.1.1.27, *Ldh-2*), malate dehydrogenase (MOR, 1.1.1.37, *Mor-1,-2*), malic enzyme (MOD, 1.1.1.40, *Mod-1,-2*), isocitrate dehydrogenase (IDH, 1.1.1.42, *Idh-1,-2*), glucose dehydrogenase (GDH, 1.1.1.47, *Gdh-2*), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.2.1.12, *Gapdh*), xanthine dehydrogenase (XDH, 1.2.3.2, *Xdh*), glutamate dehydrogenase (GLUD, 1.4.1.3, *Glud*), catalase (CAT, 1.11.1.6, *Cat*), superoxide dismutase (SOD, 1.15.1.1, *Sod-1,-2*), purine nucleoside phosphorylase (NP, 2.4.2.1, *Np*), aspartate aminotransferase (AAT, 2.6.1.1, *Aat-1,-2*), hexokinase (HK, 2.7.1.1, *Hk-1,-3*), pyruvate kinase (PK, 2.7.1.40, *Pk-1*), adenylate kinase (AK, 2.7.4.3, *Ak-1,-2*),

Fig. 1. Locations of study populations of brown hares in Greece: *EPI* = Epirus, *IER* = Ierapetra, *LOU* = Loutraki, *PYR* = Pyrra, *SER* = Serres, *SPI* = Spilia, *VEL* = Velestino.

phosphoglucomutase (PGM, 2.7.5.1, *Pgm-1*), esterases (ES, 3.1.1.1, *Es-1*; 4.2.1.1, *Es-D*), fructose-1,6-diphosphatase (FDP, 3.1.3.11, *Fdp-1*), peptidases (PEP, 3.4.11, *Pep-1,-2*), guanine deaminase (GDA, 3.5.4.3, *Gda*), aldolase (ALDO, 4.1.2.13, *Aldo*), fumarate hydratase (FH, 4.2.1.2, *Fh*), aconitase (ACO, 4.2.1.3, *Aco-1*), mannose phosphate isomerase (MPI, 5.3.1.8, *Mpi*), glucose phosphate isomerase (GPI, 5.3.1.9, *Gpi-1*).

Tissue preparation, electrophoresis, and protein-specific staining were carried out following Hartl and Höger (1986) and Grillitsch *et al.* (1992). Direct side-by-side comparisons of migrating allozymes were made including samples of Austrian and Bulgarian brown hares on the same gels. This enabled allele designations consistent with Hartl *et al.* (1993), Suchentrunk *et al.* (1999, 2000a, 2001), and Alves *et al.* (2001). Genotypic interpretations of band patterns were carried out in accordance with the respective quaternary enzyme structure (e.g., Harris and Hopkinson, 1976; Rothe, 1994). Because of poor tissue quality, some hares could not be genotyped for the whole array of loci because of dubious band patterns. For the combined data analysis, the allozyme data of Bulgarian hares (Suchentrunk *et al.*, 2000a) were adjusted to the earlier listed 35 loci, by using only those loci screened presently in the Greek hares.

Data Analysis

The BIOSYS-1 pc package, release 1.7 (Swofford and Selander, 1989) was used to calculate allele frequencies, average heterozygosity (H_o: observed, H_e: expected), proportion of polymorphic loci ($P_{95\%}$), mean number of alleles per locus based on all 35 loci (A), pairwise Nei's F_{st} values (Nei, 1977), hierarchical F-statistics for estimation of partitioning of relative genetic variability among Greek and Bulgarian populations, Nei's genetic distances (D) corrected for small sample sizes (Nei, 1978), modified Rogers' distances (Wright 1978), and to construct a Wagner dendrogram from pairwise Rogers' distances (Fig. 2). Significance of deviation of F_{st} values from zero was tested following Chesser (1993). A G-test was used to test significance of different total rates of polymorphism (no criterion) for Greek and Bulgarian hares.

The FSTAT program, version 2.9.3 (Goudet, 2001; see also Goudet, 1995) was used to test for significant deviations of genotype frequencies from Hardy–Weinberg (HW) expectations and linkage disequilibrium (LD) between pairs of polymorphic loci, separately for each population. The same program was used to calculate overall and population-specific Weir and Cockerham (1984) estimators of F_{ST} θ and F_{is} (f) and respective significance levels for difference from zero by randomization tests. It was further used to test for significant differences in allelic richness (Rs) with a rarefaction approach that corrects for unbalanced sample sizes, as well as observed heterozygosity (H_o), gene diversity (H_s), f, and Θ values for Greek and Bulgarian hares, respectively. These tests were based on randomly (10,000 permutations) allocating single populations to one of these two groups (Greek vs. Bulgarian hares) and comparing the permutation results with the original results. To account for multiple tests, significance levels were determined for all test series according to sequential or strict Bonferroni procedures with a nominal α of 0.05 (Rice, 1989).

RESULTS

Of all 35 loci assayed, five (14.3%) were polymorphic with two to five alleles in at least one Greek population and seven (20%) were polymorphic with two to four

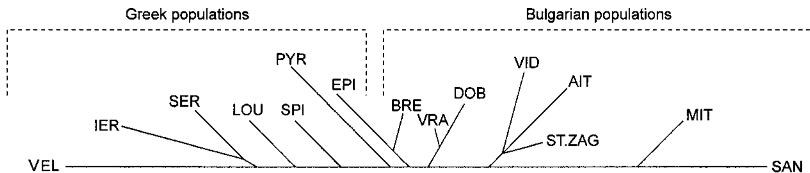

Fig. 2. Unrooted Wagner tree (Farris, 1972) based on pairwise modified Rogers' distances and 35 loci. For acronyms of Greek populations see Fig. 1 and for Bulgarian populations see Suchentrunk *et al.* (2000a). Cophenetic correlation coefficient = 0.946; total tree length = 0.454.

Table I. Allele Frequencies at Polymorphic Loci and Indices of Genetic Variability in Brown Hare Populations From Greece

	Greek populations [Locus]							8 Bulgarian populations
Alleles	*SER* (11.2)	*PYR* (25.6)	*IER* (3.8)	*VEL* (4.8)	*SPI* (28.6)	*LOU* (9.9)	*EPI* (4.9)	(15.0–24.8)
Sdh								
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.975–1.000
300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000–0.025
Ldh-2								
83	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000–0.031
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.969–1.000
Idh-2								
100	0.792	0.917	1.000	1.000	0.946	1.000	0.875	0.500–1.000
130	0.208	0.083	0.000	0.000	0.054	0.000	0.125	0.000–0.500
Es-1								
−42	0.100	0.184	0.000	0.500	0.050	0.000	0.125	0.000–0.139
−75	0.200	0.342	0.333	0.000	0.225	0.250	0.375	0.450–0.794
−100	0.700	0.395	0.667	0.500	0.425	0.583	0.500	0.083–0.450
−108	0.000	0.026	0.000	0.000	0.275	0.167	0.000	0.000–0.079
−162	0.000	0.053	0.000	0.000	0.025	0.000	0.000	0.000
Es-D								
100	0.958	0.900	1.000	0.875	0.911	1.000	1.000	0.781–1.000
141	0.042	0.100	0.000	0.125	0.089	0.000	0.000	0.000–0.219
Pep-2								
94	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000–0.056
100	0.864	0.604	0.833	1.000	0.932	0.900	0.750	0.725–0.938
104	0.045	0.083	0.000	0.000	0.034	0.050	0.250	0.063–0.275
114	0.091	0.313	0.167	0.000	0.034	0.050	0.000	0.000
Mpi								
77	0.000	0.000	0.000	0.000	0.020	0.100	0.000	0.000–0.025
88	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000
100	0.792	0.840	0.625	0.750	0.920	0.700	1.000	0.806–0.977
126	0.208	0.160	0.375	0.250	0.040	0.200	0.000	0.023–0.194
H_o	4.6	4.6	5.0	4.8	4.0	4.1	5.0	1.5–4.3
H_e	4.4	5.3	4.0	5.0	3.6	3.7	3.9	2.6–5.2
$P_{95\%}$	11.43	14.29	8.57	8.57	14.29	8.57	8.57	5.71–11.43
A	1.20	1.26	1.09	1.09	1.31	1.17	1.11	1.11–1.20
Rs	1.192	1.233	1.175	1.210	1.158	1.163	1.170	1.111–1.228
f	−0.063	0.14	−0.313	−0.125	−0.121	−0.117	−0.355	−0.081 – +0.426

Note. For abbreviations and locations see Fig. 1. For comparison, ranges of respective values for eight Bulgarian populations (Suchentrunk *et al.*, 2000a) are also given. H_o: observed population-specific heterozygosity, H_e: expected population-specific heterozygosity, $P_{95\%}$: rate of polymorphism (95% criterion), A: mean number of alleles per locus, Rs: allelic richness, f: Weir and Cockerham's estimators of F_{is} (no value differed significantly from zero; Weir and Cockerham, 1984); mean number of hares (averaged over all loci scored) is given in parentheses for each population.

alleles in at least one Bulgarian population (Table I). The alleles $Es\text{-}1^{-162}$, $Pep\text{-}2^{114}$, and Mpi^{88} that occurred at low frequencies respectively in one or more Greek populations, were not found previously in any other study on brown hares. Overall

rates of polymorphism (no criterion) did not differ significantly ($p > 0.05$, two-sided exact Fisher's test) between Greek (14.3%) and Bulgarian (20.0%) hares. For the Greek populations, the mean number of alleles per locus ranged between 1.09 and 1.26, and the rate of polymorphism (95% criterion) ranged between 8.57 and 14.29%. Average observed (H_o) and expected (H_e) heterozygosity ranged between 4.0–5.0 and 4.1–5.0, respectively. No f value (F_{is} according to Weir and Cockerham, 1984) differed significantly from zero in any population. There was neither a significant deviation of HW proportions of genotypes at any locus and in any population, nor a significant LD for any pair of loci, when tested separately for each population. Population indices of genetic variability and Nei's F_{is} values are detailed in Table I for Greek populations (Nei, 1977), along with the respective ranges for the Bulgarian populations.

Mean Nei's F_{st} and Θ values of Greek and Bulgarian hare populations (Nei, 1977; Greece: $F_{st} = 0.095$, $\Theta = 0.038$; Bulgaria: $F_{st} = 0.100$; $\Theta = 0.086$) differed significantly from zero, respectively. Among Greek hare populations, however, only 1 (4.76%) out of 21 pairwise Θ values differed significantly from zero, whereas 8 (28.57%) out of 28 values differed significantly from zero in pairwise comparisons between the Bulgarian populations, and 19 (33.93%) out of 56 values differed significantly from zero in pairwise comparisons between the Greek and Bulgarian populations (adjusted nominal level of 0.000476 after sequential Bonferroni corrections based on 105 pairwise tests). Significance for Nei's F_{st} values of Greek populations was proved for 23.8% of all pairwise comparisons (Nei, 1977; Table II). Pairwise Nei's D (Nei, 1978) and modified Rogers' distance values were generally very low for Greek populations and of the same magnitude or somewhat greater for Greek/Bulgarian population pairs (Table II).

Under sequential Bonferroni correction, population-specific Rs values, rate of polymorphism (95% criterion), H_s, f, and Θ values did not differ significantly between Greek and Bulgarian hares, respectively. Only population-specific H_o values were significantly different in Greek and Bulgarian hares, when based on two-sided p values after 10,000 permutations (Table III). Rs values did not vary significantly among Greek populations ($p = 0.949$, df $= 6$, Kruskal Wallis test based on locus-specific Rs values). Wright's hierarchical analysis (Wright, 1978) of population differentiation revealed that only 3.3% of the relative genetic variation was due to partitioning among Greek and Bulgarian hares, whereas 6% was due to partitioning among all populations studied (Table IV).

DISCUSSION

Both the level of allozymic variability and the population genetic structure of hares from Greece largely conform to those found in brown hares from Bulgaria and central Europe (Suchentrunk *et al.*, 2000a; see also Hartl *et al.*, 1989, 1990, 1992, 1993, 1994, 1995; Suchentrunk *et al.*, 2001). Rs and other indices of genetic

Table II. Genetic Differentiation Among Greek Hare Populations

	Between populations from Greece							Between Greek and Bulgarian populations
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	
SER (1)		0.002	0.000	0.003	0.002	0.000	0.000	0.003–0.011
	—	0.064	0.051	0.073	0.061	0.049	0.058	0.068–0.111
								0.054–0.252
PYR (2)			0.001	0.006	0.003	0.003	0.000	0.000–0.011
	0.042	—	0.067	0.087	0.066	0.068	0.059	0.059–0.106
	0.033							0.035–0.167
IER (3)				0.003	0.002	0.000	0.001	0.000–0.014
	0.034	0.050	—	0.085	0.075	0.039	0.081	0.072–0.129
	−0.032	−0.012						0.015–0.274
VEL (4)					0.005	0.004	0.005	0.004–0.023
	0.068	0.082*	0.100	—	0.078	0.076	0.092	0.081–0.152
	−0.005	0.050	−0.014					−0.021–0.179
SPI (5)						0.000	0.000	0.000–0.013
	0.046*	0.046	0.076*	0.083*	—	0.045	0.057	0.054–0.117
	0.054	0.071*	0.085	0.009				0.049–0.248
LOU (6)							0.001	0.001–0.014
	0.050	0.051*	0.022	0.081	0.029	—	0.066	0.060–0.123
	0.003	0.047	0.046	0.047	0.018			0.041–0.238
EPI (7)								0.000–0.007
	0.043	0.038	0.087	0.115	0.044	0.059	—	0.031–0.097
	0.007	0.010	0.073	0.146	0.030	0.046		−0.064–0.123

Note. Nei's pairwise genetic D distances (Nei, 1978) (first row) and Rogers' modified genetic distances (second row) are given above the diagonal. Nei's F_{st} values (Nei, 1977) and Weir and Cockerham's estimators of F_{st} (Weir and Cockerham, 1984; Θ values) for relative genetic differentiation are given below the diagonal in first and second rows, respectively. Asterisks denote significance at the nominal 5% level under Bonferroni corrections for multiple tests. Respective ranges for pairwise values of D and Rogers' distances as well as Θ values between the Greek and eight Bulgarian populations (see Suchentrunk *et al.*, 2000a) are given in the rightmost part of the table for comparison.

Table III. Comparison of Overall Population Genetic Parameters for Greek and Bulgarian Hares

Parameter	Greek hares	Bulgarian hares	p
Rs	1.042	1.038	0.2518
H_o	0.044	0.033	0.0064
H_s	0.043	0.038	0.3542
f	−0.035	0.128	0.0348
Θ	0.038	0.086	0.3387

Note. Rs: allelic richness, H_o: observed heterozygosity, H_s: gene diversity, f: Weir and Cockerham's estimator of F_{is}, (Weir and Cockerham, 1984), Θ: Weir and Cockerham's estimator of F_{st} (Weir and Cockerham, 1984), p: two-sided significance value after 10,000 permutations.

Table IV. Wright's Hierarchical F-statistics for Greek and Bulgarian Hares (Wright, 1978), Based on 35 Loci

Comparison	Variance component	F-statistics
Population × region[a]	0.08714	0.060
Population × total variance	0.13652	0.091
Region × total variance	0.04938	0.033

Note. Variance components and F-statistics combined across loci.
[a]Region: Greece vs. Bulgaria.

diversity did not differ significantly between Greek and Bulgarian hares, except for H_o, which was marginally elevated in Greek hares. However, three new alleles, namely *Es-1*$^{-162}$, *Pep-2*^{114}, and *Mpi*^{88}, were detected in the Greek hares with low frequencies. Since they were absent in all brown hares from regions in central and southern Europe (Hartl *et al.*, 1994; Suchentrunk *et al.*, 2000a, 2001; unpublished data) they might indeed be indicative of a Late Pleistocene refuge population in the southern Balkan. Subfossil remains from several sites in Greece (e.g., Reisch, 1976) prove at least the occasional occurrence of brown hares during the Late Pleistocene in this part of the Mediterranean.

Among these three perhaps endemic alleles, the *Pep-2*^{114}allele apparently is widespread at low frequency among hares from mainland Greece, whereas the other two were found only in two populations. They might also occur at low frequencies in a wider range in Greece but could have been missed in the small population samples. On the other hand, while the alleles *Sdh*^{300}, *Ldh-2*^{83}, and *Pep-2*^{94} are present in brown hares from Bulgaria and central Europe (Suchentrunk *et al.*, 2000a) at low frequencies, respectively, they are absent in the Greek hares. While the *Idh-2*^{83} allele of some central European populations (Hartl *et al.*, 1993; Suchentrunk *et al.*, 2000a) is absent both in the Greek and Bulgarian samples, the quite common *Pep-2*^{114} allele of Greek hares likely is absent in Bulgarian hares. The *Pep-2*^{94} allele of one southwestern Bulgarian population was not detected in any Greek population. Hence, the earlier hypothesis of its origin through gene flow from a northern Greek population into the Struma valley in southwestern Bulgaria (Suchentrunk *et al.*, 2000a) is not supported by this evidence.

In summary, this distributional pattern of alleles with low frequency of occurrence indicates some gene pool differentiation both between Greek and Bulgarian hares and between Greek and central European populations. It parallels the phylogenetic divergence of mtDNA haplotypes found between indigenous Greek hares and those that had been imported to Greece from central Europe, reared in breeding stations and released to the wild (see Mamuris *et al.*, 2001). Since the presently studied Greek hares were collected in areas well away from such restocking regions, and released hares presumably survive for relatively short periods of time

in Greece (Mamuris *et al.*, 2001), we consider our allozyme data as typical for indigenous Greek hares. However, all common alleles of central European brown hares were also common in Greek hares. Thus, among the studied loci, apparently no allele is useful to trace possible specimens imported from central European populations and released in the course of restocking programs in Greece. We also cannot allocate particular allozymic characteristics to certain nominal subspecies of Greek hares, because we did not have the opportunity of morphological analyzes of the studied hares to determine subspecies and because of the incomplete knowledge on the respective ranges of these subspecies. Moreover, some of these nominal subspecies may not hold or be combined in the long run. As regards hares from Crete which are considered a separate subspecies (*L. e. creticus*), the few specimens we examined do not particularly differ in their allozyme pattern from the hares from mainland Greece. Apparently they do not exhibit reduced genetic diversity, as might be expected from an island population, that originates from imported hares and likely has experienced a founder effect.

According to the similar values of allelic richness, a parameter of genetic diversity which accounts for unbalanced sample sizes, and the other indices of genetic diversity, none of the presently studied Greek populations can be identified as one with particularly rich genetic resources. Levels of gene pool variability for single Greek populations are moderate to somewhat elevated as compared to mammalian standards (e.g. Tiedemann *et al.*, 1996) and compatible with allozymic diversity in Bulgarian and central European brown hares (Hartl *et al.*, 1993, 1994; Suchentrunk *et al.*, 1999, 2000a, 2001; contrary to the misinterpretation of low genetic diversity of European populations by Thulin and Tegelström, 2001). These results suggest that the long–term low population densities reported for the Greek hares (Mamuris *et al.*, 2001; Sfougaris *et al.*, 1999) so far did not have any negative impact on genetic diversity. However, to substantiate this supposition, we would need allozymic data of Greek populations with distinctly higher population densities.

Both the mostly insignificant F-statistics and the low pairwise genetic distance values indicated a low level of partitioning of the encountered allozymic variability among the Greek populations. The high number of insignificant F_{st} and Θ values for pairwise comparisons of populations, however, might in part be due to the low sample sizes for several populations. The only slightly greater genetic distance values for pairwise comparisons between Greek and Bulgarian populations, and the hierarchical F-statistics indicated an only marginally increased gene pool differentiation across these two regions. Low levels of allozymic divergence appear to be common among populations of brown hares or other hare species, even across large geographic distances in Europe (Alves and Ferrand, 1999; Hartl *et al.*, 1993, 1994; Suchentrunk *et al.*, 1999, 2000a, 2001; contrary to the misinterpretation of a moderate geographic substructuring by Thulin and Tegelström, 2001). Low nuclear gene pool divergence was also indicated by minor

differences in RAPD band patterns between brown hares from Greece and central Europe (Mamuris *et al.*, 2002), and even highly resolving nuclear markers such as microsatellites revealed substantial gene flow between neighboring brown hare populations in northern continental Europe, contrary to clearly restricted mtDNA gene flow (Fickel *et al.*, 1999). This discrepancey in geographical structuring of nuclear and mtDNA might be due to a more philopatric behaviour of females, which should result in less spreading of the maternally inherited mtDNA.

The F-statistics indicate a high level of gene flow among the Greek populations. Based on the relationship between the fixation index and the number of migrating alleles among populations under the island model of populations ($Nm \sim [1 - Fst]/4Fst$; Wright, 1943), an average of 2.38 (based on F_{st} values) or 6.33 (based on Θ values) hares per generation exchange genes among populations. This is well above the value (1.0) considered to balance drift effects in populations. Drift might be expected particularly in the Greek populations, because of their very low densities with less than five hares per 100 hectares (Mamuris *et al.*, 2001; Sfougaris *et al.*, 1999). Absence of pronounced drift together with the low genetic distances suggest little genetic differentiation among nominal subspecies. However, particularly in the face of the wide morphological and morphometrical variability within and among hares conventionally considered to belong to *L. europaeus* or *L. capensis* (e.g. Angermann 1983; see also Suchentrunk *et al.*, 2000b) and unpublished data on mtDNA characteristics (Kasapidis *et al.*, submitted; Mamuris *et al.*, unpublished manuscript), the existing old descriptions of Greek subspecies should be considered insufficient. A validation or revision of the various currently listed Greek subspecies and an evaluation of their evolutionary relationships would necessitate combined morphological and molecular examinations based on large numbers of hares from many locations, with a particular emphasis on both nuclear and mitochondrial marker systems.

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REFERENCES

Alves, P. C., Branco, M., Matias, O., and Ferrand, N. (2000). New genetic variation in the European hares, *Lepus granatensis* and *L. europaeus*. *Biochem. Genet.* **38**:87–96.

Alves, P. C., and Ferrand, N. (1999). Genetic variability in Portugese populations of the Iberian hare, *Lepus granatensis*. *Folia Zool.* **48**(Suppl. 1):3–10.

Alves, P. C., Ferrand, N., and Suchentrunk, F. (2001). Developmental stability and protein heterozygosity in a local population of Iberian hares (*Lepus granatensis*). *Mamm. Biol.* **66**:238–250.
Angermann, R. (1983). The taxonomy of Old World Lepus. *Acta Zool. Fenn.* **174**:17–21.
Bennett, K. D., Tzedakis, P. C., and Willis, K. J. (1991). Quaternary refugia of north European trees. *J. Biogeogr.* **18**:103–115.
Bonhomme, F., Fernandez, J., Palacios, F., Catalan, J., and Machordon, A. (1986). Charactérisation du complex d'espèces du genre *Lepus* en Espagne. *Mammalia* **50**:495–506.
Chaworth-Musters, J. L. (1932). A contribution to our knowledge of the mammals of Macedonia and Thessaly. *Ann. Mag. Nat. Hist. Ser.* **10**(9):169.
Chesser, R. K. (1993). Genetic variability within and among populations of the black-tailed prairie dog. *Evolution* **37**:320–331.
Corbet, G. B. (1986). Relationships and origins of the European lagomorphs. *Mamm. Rev.* **16**:105–110.
De Beaufort, F. (1991). La faune des mammifères de Grèce: Caractéristiques, endèmisme, particularismes. *Biologia Gallo-hellenica* **18**:99–106.
De Beaux, O. (1929). Ricerche faunistiche nelle isole Italiane dell' Egeo. Mammiferi. *Arch. Zool. Ital.* **13**:1–25.
Farris, J. S. (1972). Estimating phylogenetic trees from distance matrices. *Am. Nat.* **106**:645–668.
Fickel, J., Lieckfeld, D., and Pitra, C. (1999). Analyze der genetischen Diversität und Struktur in benachbarten Populationen des Feldhasen (*Lepus europaeus*, Pallas 1778). *Z. Jagdwiss.* **45**:230–237.
Frenzel, B., Pécsi, M., and Velichko, A. A. (1992). *Atlas of Paleoclimates and Paleo-environments of the Northern Hemisphere, Late Pleistocene—Holocene*, Geographical Research Institute, Hungarian Academy of Sciences, Budapest, G. Fischer Verlag, Budapest, pp. 1–153.
Geraga, M., Tsaila-Monopolis, S., Ioakim, C., Papatheodorou, G., and Ferentinos, G. (2000). Evaluation of palaeoenvironmental changes during the last 18,000 years in the Myrtoon basin, SW Aegean Sea. *Palaeogeogr. Palaeoclim. Palaeoecol.* **156**:1–17.
Goudet, J. (1995). Fstat Version 1.2. A computer program to calculate F-statistics. *J. Hered.* **86**:485–486.
Goudet, J. (2001). Fstat, a program to estimate and test gene diversities and fixation indices, version 2.9.3. Available from http://www.unil.ch/izea/softwares/fstat.html. Updated from Goudet (1995).
Grillitsch, M., Hartl, G. B., Suchentrunk, F., and Willing, R. (1992). Allozyme evolution and the molecular clock in the Lagomorpha. *Acta Theriol.* **37**:1–13.
Harris, H., and Hopkinson, D. A. (1976). *Handbook of Enzyme Electrophoresis in Human Genetics*, North-Holland Publishing, Amsterdam.
Hartl, G. B., and Höger, H. (1986). Biochemical variation in purebred and crossbred strains of domestic rabbit *Oryctolagus cuniculus* L. *Genet. Res. (Camb.)* **48**:27–34.
Hartl, G. B., Markowski, J., Kovacs, G., Grillitsch, M., and Willing, R. (1990). Biochemical variation and differentiation in the brown hare (*Lepus europaeus*) of Central Europe. *Z. Säugetierk.* **55**:186–193.
Hartl, G. B., Markowski, J., Swiatecki, A., Janiszewski, T., and Willing, R. (1992). Genetic diversity in the Polish brown hare *Lepus europaeus* Pallas, 1778: Implications for conservation and management. *Acta Theriol.* **37**:15–25.
Hartl, G. B., Suchentrunk, F., Nadlinger, K., and Willing, R. (1993). An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. *Acta Theriol.* **38**(Suppl. 2):33–57.
Hartl, G. B., Suchentrunk, F., Willing, R., and Grillitsch, M. (1989). Biochemisch-genetische Variabilität und Differenzierung beim Feldhasen (*Lepus europaeus*) in Niederösterreich. *Wien. Tierärztl. Monatsschr.* **76**:279–284.
Hartl, G. B., Suchentrunk, F., Willing, R., and Petznek, R. (1995). Allozyme heterozygosity and fluctuating asymmetry in the brown hare (*Lepus europaeus*): A test of the developmental homeostasis hypothesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **350**:313–323.
Hartl, G. B., Willing, R., and Nadlinger, K. (1994). Allozymes in mammalian population genetics and systematics: Indicative function of a marker system reconsidered. In Schierwater, B., Streit, B., Wagner, G. P., and DeSalle, R. (eds.), *Molecular Ecology and Evolution: Approaches and Applications*, Birkhäuser Verlag, Basel, Switzerland, pp. 299–310.

Hilzheimer, M. (1906). Die europäischen Hasen. *Zool. Anz.* **30**:510–513.
Hilzheimer, M. (1908). Die Hasenarten Europas. *Mitt. Kgl. Naturalienkab. zu Stuttgart* **59**:1–39.
Kasapidis, P., Suchentrunk, F., Magoulas, A., and Kotoulas, G. (submitted). The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations.
Kattinger, E. (1972). Beiträge zur Säugetierkunde der südlichen Balkaninsel und des Vorderen Orients (Syrien und Unterägypten). *Ber. d. Naturf. Ges. Bamberg* **46**:11–32.
Lang, G. (1994). Quartäre vegetations Geschichte europas. Methoden und ergebnisse. G. Fischer, Jena, 62 pp.
Mamuris, Z., Sfougaris, A. I., and Stamatis, C. (2001). Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDBNA–PCR analysis: Implications for conserving genetic diversity. *Biol. Conserv*. **101**:187–196.
Mamuris, Z., Sfougaris, A. I., Stamatis, C., and Suchentrunk, F. (2002). Genetic structure of Greek brown hare (*Lepus europaeus*) populations based on the Random Amplified Polymorphic DNA (RAPD) method. *Biochem. Genet*. **40**:323–338.
Miller, G. S. (1912). *Catalogue of the Mammals of Western Europe (Europe exclusive of Russia) in the collection of the British Museum*. British Museum, London, 1019 pp.
Nei, M. (1977). *F*-statisitcs and analysis of gene diversity in subdivided populations. *Ann. Human Genet*. **41**:225–233.
Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**:583–590.
Ondrias, J. C. (1965). Die Säugetiere Griechenlands. *Säugetierk. Mitt.* **13**:109–127.
Reisch, L. (1976). Beobachtungen an Vogelknochen aus dem Spätpleistozän an der Höhle Kephalari (Argolis, Griechenland). *Archäolog. Korrespondenzblatt* **6**:261–265.
Rice, W. S. (1989). Analyzing tables of statistical tests. *Evolution* **43**:223–225.
Roberts, N., Kuzucuoglu, C., and Karabiyikoglu, M. 1999. The Late Quaternary in the Eastern Mediterranean region: An introduction. *Q. Sci. Rev*. **18**:497–499.
Roberts, N., and Wright, H. E., Jr. (1993). Vegetational, lake-level, and climatic history of the Near East and Southwest Asia. In Wright, H. E., Jr., Kutzbach, J. E., Webb, T., III, Ruddiman, W. F., Street-Perrot, F. H., and Bartlein, P. J. (eds.), *Global Climates Since the Last Glacial Maximum*, University of Minnesota Press, Minneapolis, pp. 194–220.
Rothe, G. M. (1994). Electrophoresis of Enzymes: Laboratory Methods. *Springer LAB Manual*, Springer, Berlin, Germany, 307 pp.
Sfougaris, A., Papageorgiou, N., Giannakopoulos, A., Goumas, H., Papaevangelou, E., and Anni A. (1999). Distribution, populations and habitat of the European hare (*Lepus europaeus* Pallas, 1778) in central and western Greece. In Thomaides, C., and Kypridimos, N. (eds.), *Proceedings of 24th Congress of International Union of Game Biology: Agriculture–Forestry–Game: Integrating Wildlife in Land Management*, Sept. 20–24, 1999, Thessaloniki, Greece, pp. 423–430.
Swofford, D. L., and Selander, R. B. (1989). *BIOSYS-1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics, Release 1.7. Users manual.* Champaign: Illinois Natural History Survey.
Suchentrunk, F. (1993). Variability of minor tooth traits and allozymic diversity in brown hare *Lepus europaeus* populations. *Acta Theriol*. **38**(Suppl. 2):59–69.
Suchentrunk, F., Alkon, P. U., Willing, R., and Yom-Tov, Y. (2000b). Epigenetic dental variability of Israeli hares (*Lepus* sp.): Ecogenetic or phylogenetic causation? J. Zool. **252**:503–515.
Suchentrunk, F., Hartl, G. B., Flux, J. E. C., Parkes, J., Haiden, A., and Tapper, S. (1998). Allozyme heterozygosity and fluctuating asymmetry in brown hares *Lepus europaeus* introduced to New Zealand: Developmental homeostasis in populations with a bottleneck history. *Acta Theriol.* **5**(Suppl.):35–52.
Suchentrunk, F., Jaschke, C., and Haiden, A. (2001). Little allozyme and mtDNA variability in brown hares (*Lepus europaeus*) from New Zealand and Britain—A legacy of bottlenecks? *Mamm. Biol.* **66**:48–59.
Suchentrunk, F., Michailov, C., Markov, G., and Haiden, A. (2000a). Population genetics of Bulgarian brown hares *Lepus europaeus*: Allozymic diversity at zoogeographical crossroads. *Acta Theriol.* **45**:1–12.

Suchentrunk, F., Polster, K., Giacometti, M., Ratti, P. C.-G., Thulin, C., Ruhlé, C., Vasil'ev, A. G., and Slotta-Bachmayr, L. (1999). Spatial partitioning of allozyme variability in European mountain hares (*Lepus timidus*): Gene pool divergence across a disjunct distributional range? *Z. Säugetierkunde* **64**:1–11.

Thulin, C. G., and Tegelström, H. (2001). High mtDNA haplotype diversity among introduced Swedish brown hares *Lepus europaeus*. *Acta Theriol*. **46**:375–384.

Tiedemann, R., Hammer, S., Suchentrunk, F., and Hartl, G. B. (1996). Allozyme variability in medium-sized and large mammals: determinants, estimators, and significance for conservation. *Biodiv. Lett.* **3**:81–91.

Von Wettstein, O. (1943). Eine neue Hasenrasse vom Peloponnes. *Zool. Anz*. **143**:282–284.

Weir, B. S., and Cockerham, C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**:1358–1370.

Wright, S. (1943). Isolation by distance. *Genetics* **28**:114–138.

Wright S. (1978). *Evolution and Genetics of Populations, Vol. IV: Variability Within and Among Natural Populations*, University Chicago Press, Chicago, 580 pp.

Zimmermann, K., von Wettstein, O., and Pohle, O. (1953). Die Wildsäuger von Kreta. *Z. Säugetierk*. **17**:1–72.

3. ΣΥΖΗΤΗΣΗ

3.1 Γενετική δομή και φυλογενετικές σχέσεις των πληθυσμών του ευρωπαϊκού λαγού με ανάλυση του μιτοχονδριακού DNA

Στη μελέτη των Stamatis *et al*., (2009) πραγματοποιήθηκε ανάλυση του mtDNA με τη μέθοδο RFLP σε τρία τμήματα του γονιδιώματος (*cytb*/*CR*, *COI* και *12S/16S rRNA*). Η ανάλυση πραγματοποιήθηκε σε 926 δείγματα ευρωπαϊκού λαγού, από 33 περιοχές δειγματοληψίας που προέρχονταν από 15 χώρες της Ευρώπης και της Ασίας. Επιπλέον αναλύθηκαν αλληλουχίες της περιοχής ελέγχου (*CR*) του μιτοχονδριακού DNA από 69 δείγματα λαγού, αντιπροσωπευτικά από τις περιοχές δειγματοληψίας, οι οποίες συγκρίθηκαν με 137 αλληλουχίες που προήλθαν από την τράπεζα δεδομένων GenBank. Από την ανάλυση του mtDNA με τη μέθοδο RFLP προέκυψαν 112 απλότυποι οι οποίοι ομαδοποιούνται σε πέντε, εμφανώς διαχωρισμένες, φυλογεωγραφικές απλοομάδες, οι οποίες είναι οι εξής:

1. Νοτιοανατολικού Ευρωπαϊκού τύπου Απλοομάδα (SEEh)
2. Ανατολικού – Μεσανατολικού τύπου Απλοομάδα (AMh)
3. Ευρωπαϊκού τύπου Απλοομάδα – Υποομάδα Α (EUh-A)
4. Ευρωπαϊκού τύπου Απλοομάδα – Υποομάδα Β (EUh-B)
5. Ενδιάμεση Απλοομάδα (INTERh)

Το 59,8% της συνολικής ποικιλότητας του μιτοχονδριακού DNA, είναι αποτέλεσμα της ομαδοποίησης των απλοτύπων στις πέντε απλοομάδες. Επίσης, τα δεδομένα που προέκυψαν από την ανάλυση των αλληλουχιών συμφωνούν με τα αποτελέσματα της ανάλυσης του mtDNA με τη μέθοδο RFLP, καθώς όλες οι αλληλουχίες που αναλύθηκαν, προσαρμόστηκαν φυλογεωγραφικά στις απλοομάδες, ενισχύοντας την παραπάνω ομαδοποίηση.

Από την ανάλυση αποδεικνύεται η ύπαρξη μίας μεγάλης ζώνης επικάλυψης στη Βορειανατολική Ελλάδα και τη Βουλγαρία, λόγω της παρουσίας απλοτύπων και από τις πέντε απλοομάδες, το οποίο έχει αναφερθεί και σε προηγούμενη μελέτη στον ευρωπαϊκό λαγό από τους Kasapidis *et al*., (2005). Η περιοχή αυτή παρουσιάζει την υψηλότερη νουκλεοτιδική διαφοροποίηση από όλες τις περιοχές, καθώς, εντοπίστηκε το μεγαλύτερο

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ποσοστό απλοτύπων (31,3%) από οποιαδήποτε άλλη περιοχή. Τα δεδομένα δεν αποδεικνύουν την επέκταση της ζώνης επικάλυψης στην Ανατολή, όπως υποστηρίζεται από την εργασία των Kasapidis *et al*., (2005). Ωστόσο, για να επιβεβαιωθούν αυτά τα ευρήματα, θα πρέπει να αναλυθούν περισσότερα δείγματα λαγών από τη Βορειοδυτική Τουρκία.

Από τους απλότυπους της ζώνης επικάλυψης, τρεις απλότυποι (Ενδιάμεση Απλοομάδα INTERh), εμφανίζουν μία ενδιάμεση θέση σε σχέση με την Απλοομάδα (SEEh) και την Απλοομάδα (AMh). Η σύνδεση των απλοτύπων αυτών με τις Απλοομάδες SEEh και AMh και η παρουσία τους μόνο στην περιοχή επικάλυψης, υποδεικνύει ότι πιθανόν να έχουν διασωθεί από μία αρχαία γονιδιακή δεξαμενή από την οποία εξελίχθηκαν οι Απλοομάδες SEEh και AMh. Ωστόσο, για την ενίσχυση αυτής της θεωρίας είναι απαραίτητη η ανάλυση πληθυσμών λαγού από περιοχές γειτονικές της ζώνης επικάλυψης, όπως της Ρουμανίας, της Κεντρικής Βαλκανικής Χερσονήσου, περιοχές βόρεια της Μαύρης Θάλασσας, καθώς και περιοχές της Τουρκίας που γειτνιάζουν με την Ελλάδα.

Από την ανάλυση των δικτύων παρατηρείται ότι μόνο τρεις απλότυποι από την Απλοομάδα SEEh είναι πρόγονοι των απλοτύπων της Απλοομάδας EUh-B. Επίσης, τρεις απλότυποι της Απλοομάδας EUh-B, οι οποίοι βρέθηκαν στους πληθυσμούς της Βουλγαρίας, της Βόρειας και Βορειοανατολικής Ελλάδας, συσχετίζονται στενά με τρεις απλότυπους της απλοομάδας EUh-A, οι οποίοι βρέθηκαν σε άτομα των πληθυσμών της Βουλγαρίας, της Σερβίας και στην πλειονότητα των ατόμων της Κεντρικής Ευρώπης. Όλοι οι απλότυποι που βρέθηκαν στην Κεντρική, Βορειοδυτική και Νοτιοδυτική Ευρώπη προέρχονται από δύο απλότυπους της απλοομάδας EUh-A, οι οποίοι βρέθηκαν, επίσης, στη Βουλγαρία. Οι πολύ στενές φυλογενετικές σχέσεις όλων αυτών των απλοτύπων, που απαντώνται σε μεγάλα τμήματα της Βόρειας, της Βορειοδυτικής και της Νοτιοδυτικής Ευρώπης, ενισχύουν τη θεωρία του ταχύτατου εποικισμού των περιοχών αυτών από ευρωπαϊκούς λαγούς με την εξασθένηση των κλιματικών φαινομένων της τελευταίας Περιόδου των Παγετώνων (Corbet, 1986).

Η μειωμένη γενετική διαφοροποίηση των απλοτύπων της απλοομάδας EUh-A στους πληθυσμούς της Βόρειας, Βορειοδυτικής και Νοτιοδυτικής Ευρώπης, έρχεται σε αντίθεση με τη θεωρία ύπαρξης καταφυγίου *L. europaeus* στην Ιβηρική Χερσόνησο κατά

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τη διάρκεια της τελευταίας Περιόδου των Παγετώνων, καθώς και με την ύπαρξη γονιδιακής ροής από την Ανατολική Ευρώπη και τις περιοχές της Σιβηρίας προς την Κεντρική Ευρώπη, μετά την Περίοδο των Παγετώνων (Corbet, 1986, Suchentrunk *et al*., 2000). Ως εκ τούτου, τα δεδομένα δεν συμφωνούν με την υπόθεση των Bilton *et al*., (1998), ότι πολλά είδη θηλαστικών μετανάστευσαν από την Ανατολική Ευρώπη ή από περιοχές της Σιβηρίας προς την Κεντρική Ευρώπη, μετά την Περίοδο των Παγετώνων. Αντιθέτως, ενισχύεται η θεωρία του εποικισμού μεγάλων περιοχών της Ευρώπης από πληθυσμούς λαγού από τις κεντρικές και νότιες περιοχές των Βαλκανίων μετά την Περίοδο των Παγετώνων. Το σενάριο αυτό ταιριάζει με το πρότυπο εξάπλωσης των πληθυσμών από Μεσογειακά καταφύγια (Taberlet *et al*., 1998, Hewitt, 2000).

Οι ευρωπαϊκοί λαγοί των Βρετανικών νησιών, οι οποίοι θεωρούνται ξεχωριστό υποείδος (*L. e. occidentalis*), βρέθηκαν να έχουν στενή φυλογενετική σχέση με τους λαγούς της Βόρειας Ηπειρωτικής Ευρώπης. Η γενετική ποικιλότητα των πληθυσμών της Μ. Βρετανίας ήταν πολύ χαμηλή. Ο πληθυσμός που προερχόταν από τη Βόρεια Μ. Βρετανία εμφάνισε μόνο έναν απλότυπο, ο οποίος βρέθηκε μόνο στον πληθυσμό της Βόρειας Γερμανίας, κοντά στα σύνορα με τη Δανία ενώ ο πληθυσμός της Νότιας Μ. Βρετανίας εμφάνισε έναν, επίσης, μοναδικό απλότυπο, τον πιο κοινό και ευρέως εξαπλωμένο απλότυπο της απλοομάδας EUh-A, ο οποίος απαντάται, επίσης, στον πληθυσμό της Βόρειας Γερμανίας. Επομένως, είναι πολύ πιθανό οι πληθυσμοί των λαγών της Μ. Βρετανίας να προέρχονται είτε από πληθυσμούς της Βόρειας Γερμανίας, οι οποίοι μεταφέρθηκαν στα νησιά αυτά είτε από τους αρχαίους Ρωμαίους ή από νεώτερες αποικίσεις, είτε εξαιτίας φυσικής μετανάστευσης του είδους μέσω μίας ζώνης-γέφυρας ξηράς, που υπήρχε κατά τη διάρκεια του Νεώτερου Ολόκαινου και συνέδεε τα Βρετανικά Νησιά και την Ηπειρωτική Ευρώπη (Corbet, 1986, Suchentrunk *et al*., 2006).

Σε πέντε λαγούς από τις νότιες Ελβετικές Άλπεις βρέθηκαν τρεις απλότυποι οι οποίοι, φυλογενετικά, ήταν αρκετά συνδεδεμένοι με τους απλότυπους της Απλοομάδας SEEh. Επίσης, ένας απλότυπος από τους παραπάνω, αντιπροσώπευε και τα τέσσερα άτομα που προέρχονταν από την Ιταλία, αλλά εντοπίστηκε και στον πληθυσμό του Βραδέτου της Δυτικής Ελλάδας. Αυτό το πρότυπο διασποράς του μιτοχονδριακού DNA υποδηλώνει τη φυσική γονιδιακή ροή μεταξύ των πληθυσμών λαγού από τη Βορειοδυτική Ελλάδα προς την Κεντρική Ιταλία μέσω μίας ζώνης-γέφυρας ξηράς στη

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Βόρεια Αδριατική, ανάμεσα στην Ιταλική και Βαλκανική Χερσόνησο, κατά τη διάρκεια του Νεώτερου Ολόκαινου και του Τελευταίου Πλειστόκαινου. Αυτή η θεωρία συμφωνεί με τα αποτελέσματα που προέκυψαν από την ανάλυση των αλληλουχιών, τα οποία δείχνουν ότι δύο απλότυποι από την Ιταλία είναι φυλογενετικά συνδεδεμένοι με τους απλότυπους της Απλοομάδας SEEh και δεν συμφωνούν με την ύπαρξη του υποείδους *L. e. meridiei* στην Ιταλία (Pierpaoli *et al*., 1999). Όσον αφορά στους λαγούς της Ελβετίας που ανήκουν στην Απλοομάδα SEEh, πιθανόν να προήλθαν από έναν αρχέγονο πληθυσμό της Ιταλίας, ο οποίος μετακινήθηκε προς τις Νότιες Άλπεις μετά την υποχώρηση των Παγετώνων. Η παρουσία λαγών που ανήκουν στην Απλοομάδα SEEh στην Ιταλία, την Ελβετία και την Αυστρία μπορεί, επίσης, να είναι αποτέλεσμα εισαγωγών και απελευθερώσεων στις περιοχές αυτές. Οι επικρατέστεροι απλότυποι στην Ιταλία είναι οι απλότυποι της Απλοομάδας EUh-A, σύμφωνα με τη συνδυασμένη ανάλυση των αλληλουχιών της μελέτης μας και αυτών της μελέτης των Pierpaoli *et al*., (1999). Ένα πιθανό φυλογενετικό σενάριο θα ήταν ότι οι απλότυποι αυτοί έφτασαν εκεί μετά την εξάπλωση των λαγών προς την Ιταλική Χερσόνησο μετά το Τελευταίο Μέγιστο των Παγετώνων (Pierpaoli *et al*., 1999). Εναλλακτικά ή συμπληρωματικά, η αυξημένη παρουσία απλοτύπων της Απλοομάδας EUh-A στις περιοχές αυτές, θα μπορούσε να εξηγηθεί και από τη αθρόα εισαγωγή λαγών από γειτονικές χώρες, η οποία διήρκησε για περίπου έναν αιώνα, μέχρι τη δεκαετία του ’90. Παρόμοιες δράσεις πιθανόν να αντικατέστησαν τους γηγενείς απλότυπους της Απλοομάδας SEEh, στο μεγαλύτερο τμήμα της Ιταλίας (Pierpaoli *et al*., 1999).

Η παρουσία απλοτύπων της Απλοομάδας (AMh) στις νοτιοανατολικές περιοχές της Βαλκανικής Χερσονήσου υποδηλώνει τη γονιδιακή ροή από περιοχές της Ανατολής προς την Ευρώπη, μέσω γέφυρας ξηράς στην περιοχή του Βοσπόρου την Τελευταία Περίοδο του Πλειστόκαινου, η οποία εξαφανίστηκε λόγω της αύξησης της στάθμης της θάλασσας 8000 χρόνια π.Χ. (Gökaşan *et al*., 1997). Η θεωρία αυτή συμφωνεί με την υπόθεση ότι οι ευρωπαϊκοί λαγοί μετανάστευσαν από την Ανατολή προς περιοχές της Νοτιοανατολικής Βαλκανικής Χερσονήσου αλλά και από τις ακτές της Μικράς Ασίας προς τα νησιά του Ανατολικού Αιγαίου, όταν οι περιοχές αυτές ήταν ενωμένες με τμήματα ξηράς, την Τελευταία Περίοδο των Παγετώνων ή την Περίοδο του Νεώτερου Ολόκαινου (Kasapidis *et al.,* 2005). Η νουκλεοτιδική ποικιλότητα των λαγών στις

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περιοχές αυτές παρουσιάστηκε στα ίδια επίπεδα με αυτή των λαγών της Τουρκίας και του Ισραήλ, αποδεικνύοντας ότι δεν επήλθε καμία μείωση της ποικιλομορφίας του mtDNA εξαιτίας της μεταναστευτικής πορείας των πληθυσμών του λαγού από την Ανατολή προς την Νοτιοανατολική Ευρώπη. Σε αντίθεση με την Κεντρική Ευρώπη και την Απλοομάδα EUh-A, η γενετική ανάλυση του mtDNA χρησιμοποιώντας τα δεδομένα των RFLP αλλά και των αλληλουχιών, δείχνει ότι η περιοχή της Ανατολής παρουσιάζει την υψηλότερη γενετική διαφοροποίηση, εκτός της ζώνης επικάλυψης, στη Νοτιοανατολική Βαλκανική Χερσόνησο, αποδεικνύοντας τη συνεχή παρουσία του είδους στις περιοχές αυτές. Το πιθανότερο σενάριο είναι ότι οι περιοχές της Ανατολής υπήρξαν διαρκώς ένα βιογεωγραφικό σταυροδρόμι για πολλά θηλαστικά και δέχθηκαν μεγάλη γονιδιακή ροή από παρακείμενες περιοχές της Ευρασίας και της Αφρικής κατά τη διάρκεια του Πλειστόκαινου και του Ολόκαινου (Cheylan, 1991, Sert *et al*., 2005).

Λαμβάνοντας υπόψη τις υψηλές τιμές F_{ST} που παρατηρήθηκαν στους αυτόχθονες πληθυσμούς της Ελλάδας, που υποδηλώνει τη μικρή γονιδιακή ροή του mtDNA και αυξημένη φιλοπατρία των θηλυκών ατόμων (Mamuris *et al*., 2001), η ζώνη επικάλυψης φαίνεται να είναι αρκετά εκτεταμένη. Ακούσιες, ανθρωπογενείς μετακινήσεις λαγών από τις νοτιοανατολικές προς τις βόρειες και δυτικές περιοχές της Βουλγαρίας ίσως είχαν ως αποτέλεσμα την τεχνητή επέκταση της ζώνης επικάλυψης και προς ολόκληρη τη Βουλγαρία. Επιπλέον, η ύπαρξη μιας εκτεταμένης περιοχής επικάλυψης και η παρουσία των απλοτύπων της Ενδιάμεσης Απλοομάδας INTERh μόνο στην περιοχή της ζώνης επικάλυψης υποδεικνύει ότι η ανάμειξη πολύ διαφορετικών τύπων γονιδιώματος λαγού στην περιοχή αυτή δεν είναι, απλά, ένα περιστασιακό φαινόμενο, με μικρή εξελικτική επίπτωση. Τα διαφορετικά αυτά γονιδιώματα, πιθανόν, να μεταβιβάζονται επιτυχώς από γενιά σε γενιά για μεγάλο χρονικό διάστημα και μπορούν να επηρεάσουν και να διαστρεβλώσουν σημαντικά τα φυλογενετικά και ταξινομικά συμπεράσματα, εάν μελετάται μικρό μόνο δείγμα ατόμων από μια περιορισμένη περιοχή. Ένα παράδειγμα έντονης εισδοχής γονιδιώματος, στο γένος *Lepus*, μετά από υβριδισμό, είναι η περίπτωση του *L. granatensis* που δέχτηκε mtDNA γονιδίωμα από το *L. timidus* (Melo-Ferreira *et al*., 2005; Alves *et al*., 2006) και από διάφορα άλλα είδη *Lepus* της κεντρικής και άπω Ανατολικής Ασίας (Ben Slimen *et al*., 2007).

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Στην εργασία των Stamatis *et al*., (2008) αναλύθηκαν, μετά από αλληλούχιση tRNA γονίδια του mtDNA, σε δείγματα ευρωπαϊκού λαγού από διαφορετικές περιοχές δειγματοληψίας. Μελετήθηκαν οι ένδο- και δια-ειδικές μεταλλάξεις με σκοπό να εξηγηθούν οι φυλογενετικές διαφοροποιήσεις και η διαφορετική γεωγραφική κατανομή. Η ανάλυση επικεντρώθηκε στα γονίδια που κωδικοποιούν για το $tRNA^{Thr}$ και το $tRNA^{Pro}$, δύο γονίδια που εντοπίζονται κοντά στην περιοχή ελέγχου (D-loop), η οποία χρησιμοποιείται ευρέως σε μελέτες που εξετάζουν τις εξελικτικές, φυλογενετικές και φυλογεωγραφικές σχέσεις μεταξύ διαφορετικών ειδών αλλά και μέσα στο ίδιο είδος. Επιπλέον, τα δύο γονίδια κωδικοποιούν αντιπροσωπευτικά μόρια tRNA, εφόσον το ένα μεταγράφεται από την «ελαφριά» αλυσίδα του mtDNA (αλυσίδα πλούσια σε C που κωδικοποιεί για 14 tRNA) και έτσι καταλήγει σε ένα «βαρύ» $tRNA^{Pro}$, ενώ το άλλο μεταγράφεται από την «βαριά» αλυσίδα mtDNA (αλυσίδα πλούσια σε G που κωδικοποιεί για οκτώ tRNA) και έτσι καταλήγει σε ένα «ελαφρύ» $tRNA^{Thr}$ (Anderson *et al*., 1981). Στόχος επίσης ήταν και η διερεύνηση του κατά πόσο αυτά τα γονίδια θα μπορούσαν να χρησιμοποιηθούν ως μοριακοί δείκτες για την εξαγωγή χρήσιμων φυλογενετικών και φυλογεωγραφικών πληροφοριών με ταυτόχρονη παραγωγή αποτελεσμάτων σε βιοχημικό επίπεδο. Βάσει αποκλειστικά των δεδομένων αλληλούχισης που προέκυψαν από τα μιτοχονδριακά γονίδια tRNA, η ανάλυση εστιάστηκε σε θέσεις που δεν θα μπορούσαν να επηρεάσουν τη συνολική δομή των μορίων, αλλά θα μπορούσαν πιθανά να συνεισφέρουν σε διαφορετική φυλογένεια μέσα στο είδος *L. europaeus* που παρουσιάζει διαφορετική γεωγραφική κατανομή. Τέλος αυτά τα αποτελέσματα συγκρίθηκαν με προηγούμενες μελέτες (Kasapidis *et al*., 2005).

Από την ανάλυση των αλληλουχιών ανιχνεύτηκε μία μετάλλαξη για το γονίδιο $tRNA^{Pr}$ στη θέση 13 (U13C) και μία μετάλλαξη στη θέση 39 (A39G) για το γονίδιο $tRNA^{Thr}$. Τα αποτελέσματα της πληθυσμιακής ανάλυσης, στηριζόμενα στα προηγούμενα ευρήματα, ομαδοποίησαν τους πληθυσμούς του λαγού σε δύο κύριες φυλογεωγραφικές ομάδες: η πρώτη ομάδα περιλαμβάνει τους λαγούς από τη Βόρεια, την Κεντρική και τη Νοτιοανατολική Ευρώπη, η δεύτερη ομάδα περιλαμβάνει τους λαγούς της Τουρκίας και του Ισραήλ ενώ στη Βουλγαρία και τη Βορειοανατολική Ελλάδα απαντώνται και οι δύο ομάδες (ζώνη επικάλυψης). Τα αποτελέσματα αυτά συμφωνούν με τη φυλογεωγραφική κατανομή των πληθυσμών του λαγού, που προέκυψε από την ανάλυση των αλληλουχιών

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της περιοχής D-loop (Stamatis *et al*., 2009). Δεδομένου ότι, εξελικτικά, τα γονίδια tRNA είναι πολύ συντηρημένα, οι μεταλλάξεις οι οποίες επιφέρουν αλλαγές στα μόρια των tRNA, μπορεί έχουν σημαντική επίδραση στην εξελικτική προσαρμογή συγγενικών ειδών αλλά και μέσα στο ίδιο είδος. Από την ανάλυση προκύπτει ότι οι μεταλλάξεις στα δύο αυτά γονίδια σχετίζονται με μία ευδιάκριτη γεωγραφική κατανομή των πληθυσμών του λαγού. Το γεγονός ότι οι νουκλεοτιδικές αλλαγές των γονιδίων tRNA που μελετήθηκαν, εμφανίζονται σε ένα μεγάλο αριθμό ατόμων *L. europaeus*, υποδεικνύει ότι οι αλλαγές αυτές δεν επηρεάζονται, προς το παρόν, από την πίεση της φυσικής επιλογής.

Στην εργασία των Mamuris *et al*., (2010) πραγματοποιήθηκε ανάλυση των αλληλουχιών του κυτοχρώματος *b*, σε δείγματα 75 λαγών, αντιπροσωπευτικών των χωρών και των απλοομάδων, όπως περιγράφηκαν στην εργασία των Stamatis *et. al*., (2009). Από την ανάλυση προέκυψαν 57 διαφορετικές αλληλουχίες, οι οποίες ομαδοποιήθηκαν σε τέσσερις απλοομάδες: την απλοομάδα CBEU-A, που βρέθηκε στην Ευρώπη, εκτός της Ελλάδας, τις απλοομάδες CBSEE και CBEU-B, οι οποίες εντοπίστηκαν στην Ελλάδα και τη Βουλγαρία και την απλοομάδα CBAM, η οποία εντοπίστηκε στη Βουλγαρία, τη Βορειοανατολική Ελλάδα, τη Τουρκία και το Ισραήλ.

Η φυλογενετική ανάλυση του κυτοχρώματος *b* επιβεβαιώνει την ύπαρξη τεσσάρων διαφορετικών απλοομάδων, γεωγραφικά καθορισμένων στην Ευρώπη και την Ανατολή, αποτελέσματα τα οποία συμφωνούν με τις προηγούμενες μελέτες σε διαφορετικές περιοχές του μιτοχονδριακού DNA (Stamatis *et al*., 2008, 2009). Επίσης, από την ανάλυση των γενετικών αποστάσεων επιβεβαιώνεται ο σαφής διαχωρισμός των ευρωπαϊκών λαγών της Ανατολής από τους λαγούς της Ευρώπης στο επίπεδο του mtDNA (Stamatis *et al*., 2009). Η υψηλότερη τιμή νουκλεοτιδικής διαφοροποίησης, ανάμεσα στις τέσσερις απλοομάδες, παρατηρήθηκε στην απλοομάδα CBAM, αποτέλεσμα το οποίο ενισχύει την άποψη ότι οι περιοχές της Ανατολής δέχθηκαν μεγάλη γονιδιακή ροή από παρακείμενες περιοχές της Ευρασίας και της Αφρικής κατά τη διάρκεια του Πλειστόκαινου και του Ολόκαινου (Cheylan, 1991, Sert *et al*., 2005). Αντιθέτως, η χαμηλότερη τιμή νουκλεοτιδικής διαφοροποίησης, που παρατηρήθηκε στην απλοομάδα CBEU-A, υποδεικνύει τον γρήγορο, σχετικά, εποικισμό ευρύτερων περιοχών της Ευρώπης και την άμεση εξάπλωση των ειδών μετά την Περίοδο των Παγετώνων (Kasapidis *et al.,* 2005, Fickel *et al.,* 2008, Stamatis *et al*., 2009).

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3.2 Πληθυσμιακή μελέτη του *Lepus europaeus* στο χρωμόσωμα *Y*

Στην εργασία των Mamuris *et al*., (2010), παράλληλα με την ανάλυση του κυτοχρώματος *b*, πραγματοποιήθηκε ανάλυση μοριακών δεικτών του χρωμοσώματος *Y* του ευρωπαϊκού λαγού, οι οποίοι κληρονομούνται μονογονεϊκά, με σκοπό να συγκριθεί το πρότυπο κατανομής της γονιδιακής τους ροής με αυτό του μιτοχονδριακού DNA αλλά και με δείκτες οι οποίοι κληρονομούνται διγονεϊκά (αλλοένζυμα, μικροδορυφόροι, RAPDs). Το mtDNA εξασφαλίζει πληροφορίες μόνο για την μητρική σειρά και η γρήγορη εξέλιξή του το κάνει ευάλωτο σε φαινόμενα μεταλλακτικού κορεσμού (ομοπλασία) σε βάθος πολύ μεγάλων εξελικτικών χρονικών κλιμάκων. Επιπροσθέτως, είναι καλά τεκμηριωμένο ότι τα εξελικτικά πρότυπα ενός μόνο γονιδίου ή αλληλουχίας δεν μπορούν να συγκριθούν απαραιτήτως και δεν αποδίδουν με ακρίβεια την εξέλιξη του οργανισμού (Avise, 2004). Σε αντιδιαστολή, το *Y* χρωμόσωμα των θηλαστικών έχει αυστηρά πατρική κληρονόμηση και μικρότερο ρυθμό μεταλλάξεων σε σχέση με το mtDNA (Schaffner, 2004). Αν και οι γενετικοί τόποι του mtDNA και του χρωμοσώματος *Y* είναι απλοειδή συστήματα που κληρονομούνται μονογονεϊκά , το πρότυπα διασποράς του ευρωπαϊκού λαγού θα μπορούσαν να οδηγήσουν σε σημαντική γεωγραφική διαφοροποίηση των απλοτύπων του mtDNA, αλλά σε μια ενιαία ευρέως ομογενοποιημένη γονιδιακή δεξαμενή, όσον αφορά στους απλότυπους του χρωμοσώματος *Y* (Melnick & Hoelzer, 1992). Επομένως, μελετώντας ταυτόχρονα και τα δύο συστήματα θα μπορούσε να διευκολύνει τη συγκριτική ανάλυση, ώστε (α) να διερευνηθούν οι διαδρομές της γονιδιακής ροής σε όλη την κατανομή του είδους σε Ευρώπη και Ανατολία και (β) να κατανοηθεί το κατά πόσο οι γενετικοί δείκτες του χρωμοσώματος *Y*, που έχουν ένα πρότυπο διασποράς συνδεδεμένο με πυρηνική γονιδιακή ροή των αρσενικών, παράγει φυλογένειες που προσομοιάζουν με τα αλλοένζυμα, άλλους δείκτες πυρηνικού DNA και τη μορφολογία, ή ταιριάζουν με τις φυλογένειες του mtDNA, με το οποίο μοιράζονται την μονογονεϊκή κληρονόμηση.

Οι δείκτες που χρησιμοποιήθηκαν ήταν ένα τμήμα του εξωνίου του γονιδίου *SRY* (sex determination region- φυλο-καθοριστικός παράγοντας) και μία περιοχή ιντρονίου μεταξύ των εξωνίων 8 και 9 του γονιδίου *DBY* (DEAD box *Y*-linked), οι οποίοι δεν

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εμφανίζουν κανένα ανασυνδιασμό (Gubbay *et al*., 1990, Sinclair *et al*., 1990, Hellborg και Ellegren, 2004).

Η πληθυσμιακή ανάλυση αποκάλυψε τρεις διαφορετικές αλληλουχίες για το εξώνιο *SRY* (EX-A, EX-B, EX-C) και δύο αλληλουχίες για το ιντρόνιο *DBY* (IN-A, IN-B). Από την ανάλυση του εξωνίου προέκυψε ότι η συντριπτική πλειονότητα των λαγών είχαν τον απλότυπο EX-A, τρεις λαγοί από το Νότιο Ισραήλ είχαν τον απλότυπο EX-B ενώ ένας λαγός από το Βόρειο Ισραήλ εμφάνισε τον απλότυπο EX-C. Όσον αφορά την πληθυσμιακή ανάλυση του ιντρονίου, ο απλότυπος IN-A βρέθηκε σε όλους τους λαγούς της Τουρκίας, του Ισραήλ, στο 27% των λαγών της Βουλγαρίας και στο 6,8% των λαγών της Ελλάδας, από τους οποίους οι οκτώ ανιχνεύθηκαν στη Βορειοανατολική Ελλάδα και τέσσερις στην Κεντρική Ελλάδα , ενώ ο απλότυπος IN-B βρέθηκε στην πλειονότητα των λαγών της Ευρώπης ενώ απουσίαζε εντελώς από την Τουρκία και το Ισραήλ. Τα δεδομένα της ανάλυσης αποκάλυψαν χαμηλά επίπεδα ενδοειδικού γενετικού πολυμορφισμού των δεικτών του χρωμοσώματος *Y*, κάτι που είχε αναφερθεί και σε προηγούμενη μελέτη του γονιδίου *SRY* στον ευρωπαϊκό λαγό (Putze *et al*., 2007).

Οι λαγοί που μελετήθηκαν στην εργασία αυτή, είχαν προηγουμένως αναλυθεί σε επίπεδο μιτοχονδριακού DNA (Stamatis *et al*., 2009), δίνοντας τη δυνατότητα για σύγκριση των αποτελεσμάτων των δύο δεικτών που κληρονομούνται μονογονεϊκά. Επίσης τα ίδια άτομα έχουν μελετηθεί ως προς την γενετική τους διαφοροποίηση σε επίπεδο πυρηνικού DNA, χρησιμοποιώντας δείκτες μικροδορυφόρων, αλλοενζύμων και RAPDs. Οι μελέτες αυτές, έδειξαν ένα γενικευμένο πρότυπο πληθυσμιακής ποικιλομορφίας και διαφοροποίησης, με σχετικά μεγάλο αριθμό μοναδικών αλληλομόρφων αλλά με ενδείξεις για παρουσία γενετικής ροής μεταξύ πληθυσμών που βρίσκονται σε γειτονικές περιοχές (Mamuris *et al*., 2002, Suchentrunk *et al*., 2003, Sert *et al*., 2005, Ben Slimen *et al*., 2008). Αντιθέτως, τα δεδομένα που προέκυψαν από την ανάλυση των δεικτών του χρωμοσώματος *Y* υποδηλώνουν την παρουσία δύο κύριων φυλογενετικών κλάδων, για τον ευρωπαϊκό λαγό, ανάμεσα στην Ευρώπη και την Ανατολή. Η νουκλεοτιδική διαφοροποίηση ανάμεσα στους δύο αυτούς απλοτύπους ήταν πολύ χαμηλή, με μόνο μία νουκλεοτιδική αντικατάσταση. Από τις προηγούμενες μελέτες στο μιτοχονδριακό DNA αποδείχθηκε η παρουσία μίας μεγάλης ζώνης επικάλυψης απλοτύπων στη Βουλγαρία και τη Βορειοανατολική Ελλάδα. Στην περιοχή αυτή

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παρατηρείται, επίσης, και η παρουσία των δύο απλότυπων που προέκυψαν από την ανάλυση των δεικτών του χρωμοσώματος *Y*, του ευρωπαϊκού και του ανατολικού απλοτύπου, γεγονός που ενισχύει την θεωρία περί ύπαρξης γονιδιακής ροής από περιοχές της Ανατολής προς την Ευρώπη, μέσω μίας ζώνης ξηράς στην περιοχή του Βοσπόρου την Τελευταία Περίοδο του Πλειστόκαινου, η οποία εξαφανίστηκε, λόγω της αύξησης της στάθμης της θάλασσας, 8000 χρόνια π.Χ. (Geoffrey και Hosey, 1982, Gökaşan *et al*., 1997). Σε αντίθεση με το μιτοχονδριακό DNA, βρέθηκαν λαγοί στην Κεντρική Ελλάδα οι οποίοι είχαν ανατολικού τύπου *Y*-DNA. Είναι πιθανόν η τάση των θηλυκών λαγών για φιλοπατρία να καθιστά πιο δύσκολη τη διασπορά του mtDNA σε σχέση με το DNA του *Y* χρωμοσώματος, όπως επίσης, υπάρχει η πιθανότητα οι λαγοί αυτοί να προέρχονται από προγενέστερες απελευθερώσεις λαγών, οι οποίοι εισήχθησαν από περιοχές της Βουλγαρίας (Stamatis *et al*., 2007). Το σύνολο των πολυμορφισμών στο *Y* χρωμόσωμα εντοπίστηκε στους πληθυσμούς της Ανατολής, παρά το σχετικά μικρότερο αριθμό ατόμων που αναλύθηκαν σε αυτή την περιοχή (n=38), σε σύγκριση με τους πληθυσμούς της Ευρώπης (n=407). Όλες οι νουκλεοτιδικές αντικαταστάσεις που ανιχνεύτηκαν στο εξώνιο του χρωμοσώματος *Y*, εντοπίστηκαν σε τρεις λαγούς από το Ισραήλ, διαχωρίζοντας τους λαγούς αυτούς από τους υπόλοιπους λαγούς της Τουρκίας και του Ισραήλ. Οι πολυμορφισμοί αυτοί είναι, πιθανόν, αποτέλεσμα συνεχόμενης γενετικής ροής και γονιδιακής διείσδυσης από γειτονικές περιοχές του Ισραήλ, λαμβάνοντας υπόψη τον εκτεταμένο διαειδικό υβριδισμό μέσα στο γένος *Lepus*, όπως έχει αναφερθεί σε πολλές περιπτώσεις (Thulin *et al*., 1997, Melo-Ferreira *et al*., 2005, 2007, Alves *et al*., 2006, Ben Slimen *et al*., 2007).

3.3 Ανθρωπογενείς δραστηριότητες, εκτροφές και απελευθερώσεις ευρωπαϊκών λαγών

Ο ευρωπαϊκός λαγός αποτελεί ένα από πιο σημαντικά θηρεύσιμα είδη στην Ελλάδα αλλά και σε πολλές ευρωπαϊκές χώρες (Pielowski, 1976). Τα τελευταία χρόνια έχει παρατηρηθεί κατακόρυφη μείωση των πληθυσμών του ευρωπαϊκού λαγού εξαιτίας διαφόρων γεωργικών δραστηριοτήτων, που μεταβάλλουν τις περιοχές διαβίωσης και της έντονης πίεσης που ασκείται στους πληθυσμούς από τη θήρευση και από διάφορες

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ασθένειες. Βάσει ιστορικών δεδομένων, έχουν πραγματοποιηθεί αρκετές μεταφορές πληθυσμών ευρωπαϊκού λαγού, οι οποίες, ενδεχομένως, επηρέασαν το φυλογεωγραφικό πρότυπο του μιτοχονδριακού DNA του ευρωπαϊκού λαγού, στις περιοχές που έχουν μελετηθεί (Kasapidis *et al*., 2005, Suchentrunk *et al*., 2006). Τις τελευταίες δεκαετίες, πραγματοποιήθηκαν αρκετά προγράμματα εμπλουτισμού σε πολλές περιοχές που παρατηρήθηκε μείωση του πληθυσμών του λαγού, εισάγοντας αλλόχθονα άτομα του είδους από διάφορες ευρωπαϊκές χώρες, με αποτέλεσμα να έχει επηρεαστεί η γενετική δομή των γηγενών πληθυσμών του είδους ή συγγενικών ειδών (Flux, 1983, Pierpaoli *et al*., 1999). Μεγάλος αριθμός εκτροφείων σε χώρες όπως η Βουλγαρία, η Πολωνία, η Σλοβακία και η Ουγγαρία, προμήθευαν τις κυνηγετικές οργανώσεις διαφόρων περιοχών της Δυτικής και της Κεντρικής Ευρώπης, με σκοπό τον εμπλουτισμό των περιοχών αυτών με πληθυσμούς λαγών.

Στην Ελλάδα οι απελευθερώσεις λαγών απαγορεύτηκαν το 2001, μετά από απόφαση του Υπουργείου Γεωργίας. Σύμφωνα με τα στοιχεία του Υπουργείου, περισσότεροι από 2000 λαγοί, προερχόμενοι από ιδιωτικά εκτροφεία, αφού προηγουμένως είχαν εισαχθεί από χώρες όπως η Βουλγαρία, η Γιουγκοσλαβία και η Ιταλία, απελευθερώθηκαν μόνο στην κεντρική Ελλάδα από Κυνηγετικές Οργανώσεις κατά τη δεκαετία 1991-2001. Ωστόσο, μέχρι το 1998, τα προγράμματα εμπλουτισμού στην Ελλάδα παρέμεναν ανεξέλεγκτα καθώς δεν υπήρχε καμία παρακολούθηση των απελευθερωμένων λαγών.

Στην εργασία των Mamuris *et al*., (2001) μελετήθηκαν λαγοί που είχαν συλλεχθεί κατά τη διάρκεια δύο κυνηγετικών περιόδων (1998 και 1999) καθώς και λαγοί που προέρχονταν από δύο ελληνικά εκτροφεία. Από την ανάλυση με τη μέθοδο RFLP προέκυψαν τρεις ομάδες λαγών: ο άγριος πληθυσμός λαγών, ο εκτρεφόμενος πληθυσμός και ένας άγριος πληθυσμός με μιτοχονδριακό DNA ελάχιστα διαφοροποιημένο με αυτό που παρατηρήθηκε στον εκτρεφόμενο πληθυσμό. Για να ελεγχθεί ο βαθμός της γονιδιακής διείσδυσης των εκτρεφόμενων-απελευθερωμένων λαγών στους φυσικούς πληθυσμούς και να ανιχνευτεί η καταγωγή των λαγών αυτών, προσδιορίστηκαν κατάλληλοι διαγνωστικοί δείκτες στο mtDNA (Stamatis *et al*., 2007). Οι δείκτες αυτοί εφαρμόστηκαν σε ένα μεγάλο αριθμό λαγών της Ελλάδας (συλλογή δειγμάτων από 1999 έως 2004), της Τουρκίας, του Ισραήλ, διαφόρων ευρωπαϊκών χωρών καθώς και στους

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πληθυσμούς των εκτρεφόμενων λαγών και διέκριναν αποτελεσματικά τις τρεις ομάδες λαγών, που είχαν προκύψει από τη μελέτη των Mamuris *et al*., (2001). Η ανάλυση των δειγμάτων με τα διαγνωστικά ένζυμα έδωσε τέσσερα διαφορετικά πρότυπα mtDNA. Το πρότυπο Α βρέθηκε σε όλους τους λαγούς της Τουρκίας , του Ισραήλ καθώς και σε ένα ποσοστό λαγών από την Βορειοανατολική Ελλάδα (15,4%) και τη Βουλγαρία (21,8%). Το πρότυπο Β εμφανίστηκε αποκλειστικά στην Ελλάδα και τη Βουλγαρία, όπως και το πρότυπο C2. Οι λαγοί που προέρχονταν από τη Γερμανία, την Ολλανδία, την Πολωνία, την Αυστρία, την Ελβετία, τη Σερβία και ένα ποσοστό από των λαγών της Βουλγαρίας, καθώς και οι εκτρεφόμενοι λαγοί, εμφάνισαν το πρότυπο C1.

Από τα στοιχεία της μελέτης, σε συνδυασμό και με τα αποτελέσματα των Mamuris *et al*., (2001), μέχρι το 2003, αφού είχαν αναλυθεί περισσότεροι από 400 λαγοί δεν είχε βρεθεί κανένας με πρότυπο C1 (ευρωπαϊκό ή εκτροφείου) στους φυσικούς πληθυσμούς της Ελλάδας. Ωστόσο, αναλύοντας δείγματα που συλλέχθηκαν τη διετία 2003-2004, ανιχνεύτηκαν εννέα λαγοί (επτά από την Κεντρική και δύο από τη Βόρεια Ελλάδα) με το πρότυπο C1. Δεδομένου ότι οι απελευθερώσεις απαγορεύτηκαν στην Ελλάδα το 2001 και επίσης, κανένας λαγός με πρότυπο C1 δεν είχε βρεθεί στους ελληνικούς πληθυσμούς μέχρι το 2003, η παρουσία των εννέα ατόμων με το πρότυπο C1 αποδεικνύει ότι ένα ποσοστό των απελευθερωμένων λαγών επιβίωσε για αρκετό χρονικό διάστημα, ώστε να έχουν τουλάχιστον έναν αναπαραγωγικό κύκλο και να μεταβιβάσουν το γονιδίωμα τους.

Τα προγράμματα εμπλουτισμού αποσκοπούν στο να προλαμβάνουν τη μείωση ή ακόμα και την εξάλειψη των τοπικών πληθυσμών λαγού και να αυξάνουν τη γενετική ποικιλότητα, μειώνοντας το βαθμό συγγένειας και αιμομιξίας μέσα στους πληθυσμούς αυτούς. Η πυκνότητα των πληθυσμών του λαγού στην Κεντρική και τη Δυτική Ελλάδα κυμαίνεται από 1,1 έως 2,4 άτομα ανά 100 εκτάρια, τιμές αρκετά χαμηλότερες από αυτές που παρατηρούνται σε άλλες ευρωπαϊκές χώρες (Smith *et al*., 2005). Επιπλέον, από στοιχεία του Υπουργείου Γεωργίας για την περίοδο 1986 έως 1990, παρατηρήθηκε αυξημένη θνησιμότητα στους πληθυσμούς του λαγού εξαιτίας του συνδρόμου του ευρωπαϊκού λαγού. Σε αυτές τις περιπτώσεις, η ελεγχόμενη εκτροφή και τα προγράμματα εμπλουτισμού, ακολουθούμενα από αυστηρό γενετικό έλεγχο, πρέπει να λαμβάνονται υπόψη και να αξιολογούνται ανάλογα.

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Σε αντίθεση με τους φυσικούς πληθυσμούς, οι εκτρεφόμενοι πληθυσμοί εμφάνισαν σχετικά μικρή γενετική ποικιλότητα (Mamuris *et al*., 2001, 2002). Στο μοναδικό τομέα που θα μπορούσαν, οι λαγοί αυτοί, να συνεισφέρουν είναι στον εμπλουτισμό του μιτοχονδριακού DNA των ελληνικών πληθυσμών με τους απλοτύπους του mtDNA των εκτρεφόμενων λαγών. Από τις μελέτες προκύπτει ότι η Βουλγαρία και η Βορειοανατολική Ελλάδα είναι οι μοναδικές περιοχές της Ευρώπης, στις οποίες οι πληθυσμοί λαγών εμφανίζουν και τα τέσσερα πρότυπα mtDNA, το οποίο πιθανόν, έως ένα βαθμό, να οφείλεται σε απελευθερώσεις λαγών με διαφορετικού τύπου μιτοχονδριακό DNA. Επίσης, η αυξημένη γενετική ποικιλομορφία των πληθυσμών του λαγού στην Ελλάδα σε σχέση με τις υπόλοιπες ευρωπαϊκές χώρες (Mamuris *et al*., 2001, 2002, Suchentrunk *et al*., 2003), καθώς και η αλλαγή που ανιχνεύτηκε στη γενετική δομή των ελληνικών πληθυσμών, ως αποτέλεσμα των απελευθερώσεων, οδήγησαν στην απαγόρευση των προγραμμάτων εμπλουτισμού στην ελληνική επικράτεια και στον επαναπροσδιορισμό των προγραμμάτων διαχείρισης των φυσικών ζωικών πληθυσμών, με ταυτοποίηση και γενετικό έλεγχο των εκτρεφόμενων πληθυσμών ώστε να διαπιστωθεί ο βαθμός γενετικής συγγένειας με τους φυσικούς πληθυσμούς.

3.4 Συμπεράσματα

- Ο εποικισμός με ευρωπαϊκούς λαγούς σε μεγάλες περιοχές της Ευρώπης άρχισε, στο τέλος της τελευταίας παγετωνικής περιόδου και στην αρχή του Ολόκαινου, από έναν μόνο αρχικό πληθυσμό των κεντρικών ή νοτιο-κεντρικών Βαλκανίων και υπήρξε σχετικά ταχύτατος. Αυτό έρχεται σε αντίθεση με την υπόθεση εποικισμού της Ευρώπης από μερικά μικρά θηλαστικά, κατά το τέλος του Πλειστόκαινου, από μερικά καταφύγια της ανατολικής Ευρώπης και της δυτικής Σιβηρίας.

- Οι μέχρι τώρα αναλύσεις έδειξαν ότι στην Ιβηρική χερσόνησο τα μη υβριδισμένα άτομα ευρωπαϊκού λαγού έχουν τους κλασσικούς απλότυπους mtDNA, γεγονός που αποκλείει την περιοχή αυτή από το να έπαιξε το ρόλο καταφυγίου για το *L. europaeus* κατά τον Πλειστόκαινο.

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- Στη Βουλγαρία και τη βορειοανατολική Ελλάδα είναι παρούσες όλες οι απλοομάδες, δημιουργώντας μια εκτεταμένη περιοχή επικάλυψης όλων των απλοτύπων.

- Ανιχνεύθηκε γονιδιακή ροή από την Ανατολία προς την Ευρώπη, πιθανότατα κατά τη διάρκεια της τελευταίας περιόδου του Πλειστόκαινου, μέσω της γέφυρας του Βοσπόρου.

- Οι ευρωπαϊκοί λαγοί από τη Μ. Βρετανία εμφάνισαν πολύ χαμηλό πολυμορφισμό, αλλά δεν αποτελούν ένα ξεχωριστό υποείδος (*L. e. occidentalis*), όπως είχε θεωρηθεί παλαιότερα. Η βόρεια Γερμανία θα μπορούσε να είναι η περιοχή προέλευσης των σύγχρονων πληθυσμών της Βρετανίας.

- Η υπερθήρευση και οι συνεχείς απελευθερώσεις αλλόχθονων λαγών θα μπορούσαν να εξηγήσουν τη μαζική παρουσία των απλοτύπων της ευρωπαϊκής ομάδας EU-A στην κεντρική και βόρεια Ιταλία. Η πρακτική αυτή ίσως συνέβαλε στην αντικατάσταση των γηγενών απλοτύπων SEE που πιθανώς ήταν διαδεδομένοι αρχικά. Τα δεδομένα μας απορρίπτουν την ύπαρξη ενός διαφορετικού υποείδους (*L. e. meridiei*) στην Ιταλική χερσόνησο.

- Αν και οι πολλές επιχειρήσεις εμπλουτισμού και ανθρωπογενών απελευθερώσεων ευρωπαϊκού λαγού που έχουν πραγματοποιηθεί θα μπορούσαν να εξηγήσουν την παρουσία μη αναμενόμενων απλότυπων σε ορισμένες περιοχές, εντούτοις ανιχνεύθηκε ένα έντονο φυλογεωγραφικό σήμα σε όλες τις περιοχές που μελετήθηκαν.

- Τα φυλογενετικά δένδρα που προέκυψαν από την ανάλυση των γονιδίων tRNA του mtDNA ήταν συγκρίσιμα με αυτά που έδωσαν τα υπόλοιπα μιτοχονδριακά τμήματα. Είναι πολύ πιθανό ότι τα γονίδια tRNA που αναλύθηκαν, συσσώρευσαν μεταλλάξεις σε θέσεις οι οποίες δεν επηρεάζουν το ρόλο τους στη μιτοχονδριακή σύνθεση των πρωτεϊνών.

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- Οι μοριακές αναλύσεις αυτών των γονιδίων tRNA μπορεί να χρησιμοποιηθούν ως πολύτιμα βοηθητικά εργαλεία για την σύνδεση της ακριβούς βιοχημικής λειτουργίας των μιτοχονδρίων με τις εξελικτικές και φυλογενετικές μελέτες.

- Η μελέτη των tRNAs κατέγραψε εξελικτικές αλλαγές που μπορούν να συνδεθούν άμεσα με τη διακριτή φυλογεωγραφική κατανομή του *L. europaeus*. Το γεγονός ότι οι νουκλεοτιδικές αντικαταστάσεις που ανιχνεύθηκαν εμφανίζονται σε ένα μεγάλο ποσοστό των ατόμων του *L. europaeus* υποδηλώνει ότι είναι ανεκτές από την πίεση της φυσικής επιλογής.

- Σε συμφωνία με τα υπόλοιπα φυλογενετικά δεδομένα του mtDNA, η ανάλυση του γονιδίου Cyt*b* επιβεβαιώνει την ύπαρξη τουλάχιστον τεσσάρων διαφορετικών απλοομάδων με πολύ καλά προσδιορισμένη κατανομή στην Ευρώπη και την Ανατολία.

- Η ανάλυση του Cyt*b* υποστηρίζει επίσης το βαθύ διαχωρισμό των πληθυσμών του *L. europaeus* ανάμεσα στην Ανατολία (Τουρκία και Ισραήλ) και την Ευρώπη σε επίπεδο mtDNA.

- Σε αντίθεση με τους διγονεϊκούς πυρηνικούς μοριακούς δείκτες, τα δεδομένα του *Y*-DNA υποστηρίζουν την ύπαρξη δύο βασικών φυλογενετικών κλάδων για το είδος *L. europaeus* ανάμεσα στην Ευρώπη και την Ανατολία.

- Σε αντίθεση με το mtDNA, ο τύπος του *Y*-DNA της Ανατολίας εντοπίστηκε και σε μερικά άτομα της κεντρικής Ελλάδας. Είναι πολύ πιθανό ότι η τάση για φιλοπατρία των θηλυκών ευρωπαϊκών λαγών, καθιστά την εισδοχή του mtDNA πιο δύσκολη σε σχέση με το *Y*-DNA και καταλήγει σε διαφορετικά πρότυπα κατανομής.

- Οι ελληνικοί πληθυσμοί του *L. europaeus* εμφανίζουν πολύ μεγαλύτερη γενετική ποικιλότητα σε σχέση με τους εκτρεφόμενους αλλά και τους κεντροευρωπαϊκούς πληθυσμούς, όπως συνάγεται από τα δεδομένα του mtDNA και των RAPDs.

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Επομένως, θα ήταν άστοχο να χρησιμοποιηθούν απελευθερώσεις εκτρεφόμενων ατόμων για να εμπλουτιστεί η ήδη πλούσια γενετική δεξαμενή των ελληνικών πληθυσμών.

- Το επόμενο διαχειριστικό ερώτημα είναι εάν θα υπάρξει το φαινόμενο της γενετικής κατάπτωσης μετά από ανάμιξη των τοπικών γονιδιακών δεξαμενών με μη προσαρμοσμένα γονίδια εισαγόμενων ατόμων. Η Βουλγαρία και πλέον, μετά τις απελευθερώσεις, η βορειοανατολική Ελλάδα είναι οι μόνες περιοχές της Ευρώπης όπου συνυπάρχουν και οι τέσσερεις απλοομάδες. Και στις δύο περιπτώσεις δεν υπάρχουν τα κατάλληλα δεδομένα για αποφανθούμε ως προς τις πιθανές αλληλεπιδράσεις μεταξύ των διαφόρων απλοομάδων. Εάν μακροχρόνια τα εισαγόμενα ξένα γονίδια επιβιώσουν, δημιουργώντας νέους γενότυπους με τα ήδη υπάρχοντα, θα αποδειχθεί ότι αυτά μπορούν να προσαρμοστούν. Καθώς τα πυρηνικά γονίδια δεν είναι και τόσο διαφοροποιημένα μεταξύ της Ελλάδας και της υπόλοιπης Ευρώπης, τα ξένα πυρηνικά γονίδια δεν θα αποτελέσουν ένα σοβαρό μειονέκτημα. Επομένως, υπό τις συγκεκριμένες περιπτώσεις, τα προγράμματα απελευθερώσεων θα μπορούσαν να ενταχθούν σε ένα διαχειριστικό σχήμα, με την προϋπόθεση ότι και όλες οι άλλες μη γενετικές παράμετροι λαμβάνονται υπόψη και ελέγχονται αυστηρά.

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4. ΒΙΒΛΙΟΓΡΑΦΙΑ

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J., Staden, R. and Young, I.G. (1981) Sequence and organization of the human mitochondrial genome. Nature, 290:457-465.

Arnason, U., Adegoke, J.A., Bodin, K., Born, E.W., Esa, Y.B., Gullberg, A., Nilsson, M., Short, R.V., Xu, X., Janke, A. (2002) Mammalian mitogenomic relationships and the root of the eutherian tree. Proc Natl Acad Sci U S A.99(12): 8151-6.

Arnold, H.R. (1993) Atlas of mammals in Britain. ITE research publ. No. 6. London, HMSO pp. 1-145.

Avise, J. C. (1986) Mitochondrial DNA and the evolutionary genetics of higher animals. Phil. Trans. R. Soc. Lond. B312: 325-42.

Avise, J.C., Arnold, J., Ball, R.M., Berminham, E., Lamp, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., (1987) INTRASPECIFIC PHYLOGEOGRAPHY: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. Ann. Rev. Ecol. Syst. 18: 489-522.

Avise, J.C. (2004) Molecular Markers, Natural History, and Evolution. Sinauer Associates, Sunderland, MA.

Awadalla, P., Eyre-Walker, A., Maynard Smith, J. (1999) Linkage Disequilibrium and Recombination in Hominid Mitochondrial DNA. Science. 286 (5449): 2524–2525.

Bansfield, A. (1974) Mammals of Canada. Toronto: University of Toronto Press

Ben Slimen, H., Suchentrunk, F., Shahin, A.B. & Ben Ammar Elgaaied, A. (2007) Phylogenetic analysis of mtCR-1 sequences of Tunisian and Egyptian hares (*Lepus sp*. or *spp*., Lagomorpha) with different coat colours. Mammalian Biology, 72: 224–239.

Ben Slimen, H., Suchentrunk, F., Stamatis, C., Mamuris, Z., Sert, H., Alves, P.C., Kryger, U., Shahin, A.B., Ben Ammar Elgaaied, A., (2008) Population genetics of cape and brown hares (*Lepus capensis* and *L. europaeus*): a test of Petter's hypothesis of conspecificity. Biochem. Syst. Ecol. 36: 22–39.

Bilton, D.T., Mirol, P.M., Mascheretti, S., Fregda, K., Zima, J. & Searle, J.B. (1998) Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. Proceedings of the Royal Society B: Biological Sciences, 265: 1219–1226.

Bininda-Emonds, O. R. P.(2000) Factors influencing phylogenetic inference: A case study using the mammalian carnivores. Molecular Phylogenetics and Evolution, 16(1): 113-126.

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Bonhomme, F., Fernandez, J., Palacios, F., Catalan, L. and Machordon, A. (1986) Caractérisation biochimique du comples d'espéces du genre *Lepus* en Espagne. Mammalia, 50: 495-506.

Bonino, N., Montenegro, A. (1997) Reproduction of the European hare in Pantagonia, Argentina. Acta Theriologica, 42(1): 47-54.

Boore, L.J. (1999) Animal Mitochondrial Genomes. Nucleic Acids Research 27(8): 1767-1780.

Botstein, D., White, K.L., Skolnick, M. and Davis, R .W. (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32: 3 14-33

Broekhuizen, S., Maaskamp, F. (1980) Behaviour of does and leverets of the European hare (*Lepus europaeus*) whilst nursing. J. Zool. Lond. 191: 487-501.

Buburuzan, L., Gorgan, L.D., Băra, I.I. (2007) Types of DNA used in speciation and phylogeny studies. Analele Ştiinţifice ale Universităţii, Alexandru Ioan Cuza", Secţiunea Genetică şi Biologie Moleculară, TOM VIII.

Caillol, M., Meunier, M., Mondain-Monval, M., Simon, P. (1988) Seasonal variations in testis size, testosterone and LH basal levels, and pituitary response to luteinizing hormone releasing hormone in the brown hare, *Lepus europaeus*. Can. J. Zool., 67: 1626-1630.

Cheylan, G. (1991) Patterns of Pleistocene turnover, current distribution and speciation among Mediterranean mammals. Biogeography of Mediterranean invasions (ed. by R.H. Groves and F. Di Castri), pp. 227–262. Cambridge University Press, Cambridge.

Corbet, G.B. (1986) Relationships and origins of the European lagomorphs. Mammal Review, 16: 105–110.

Dolman, G., Phillips, B. (2004) Single copy nuclear DNA markers characterized for comparative phylogeography in Australian wet tropics rainforest skinks. Mol. Ecol. Notes 4: 185–187.

Downie, D.A., Gullan, P.J. (2004) Phylogenetic analysis of mealybugs (Hemiptera: Coccoidea: Pseudococcidae) based on DNA sequences from three nuclear genes, and a review of the higher classification. Syst. Entomol. 29: 238–259.

Dragg, A. (1974) Mammals of Ontario. Waterloo, Ontario: Otter Press

Fickel, J., Lieckfeldt, D., Pitra, C., (1999) Analysis of genetic diversity and tructure in neighboring populations of the European brown hare (*Lepus europaeus*, Pallas 1778). Zeitschrift für Jagdwissenschaft, 45: 230–237.

Fickel, J., Hauffe, H.C., Pecchioli, E., Soriguer, R., Vapa, L. and Pitra, C. (2008) Cladogenesis of the European brown hare (*Lepus europaeus* Pallas, 1778). Eur. J. Wildl. Res.54: 495-510

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Flux, J.E.C. (1983) Introduction to taxonomic problems in hares. Acta Zoologica Fennica, 174: 7–10.

Flux, J.E.C. and Angermann, R. (1990) The Hares and Jackrabbits. In: J.A., Chapman and J.E.C., Flux (eds.), Rabbits, Hares and Pikas. Status Survey and Conservation Action Plan, IUCN/SSC Lagomorph Specialist Group, Gland, Switzerland, pp 61-94.

Garrigan, D., Hammer, M.F., (2006) Reconstructing human origins in the genomic era. Nat. Rev. Genet. 7 (9): 669–80.

Geoffrey, R., Hosey, B., (1982) The Bosporus land-bridge and mammal distributions in Asia Minor and the Balkans. Saugetierkd. Mitt.30: 53–62.

Gokasan, E., Demirbag, E., Oktay, F.Y., Ecevitoglu, B., Simsek, M. & Yuce, H. (1997) On the origin of the Bosporus. Marine Geology, 140: 183–199.

Graves, J.A.M. (2006) Sex chromosome specialization and degeneration in mammals. Cell, 124: 901–914.

Gubbay, J., Collington, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., Lovell-Badge, R., (1990) A gene mapping to the sex- determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature, 346: 245–250.

Hall, E., Kelson, K. (1959) The Mammals of North America, vol. 2. New York: The Ronald Press Company.

Hamilton, W., Whitaker, J. (1943) Mammals of the Eastern United States. 2nd ed. Ithica, NY: Cornell University Press

Hartl, G.B., Suchentrunk, F., Nadlinger, K., Willing, R. (1993) An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. Acta Theriologica, 38: 33-57.

Hartl, G.B., Suchentrunk, F., Willing, R. and Petznek, R. (1995) Allozyme heterozygosity and fluctuating asymmetry in the brown hare (*Lepus europaeus*): A test of the developmental homeostasis hypothesis. Philosophical Transactions of the Royal Society of London, 350: 313-323.

Hellborg, L., Ellegren, H., (2004) Low levels of nucleotide diversity in mammalian Y chromosomes. Mol. Biol. Evol. 21: 158–163.

Hennig, W. (1966) Phylogenetic systematics. University of Illinois Press, Urbana.

Hewitt, G.M. (2000) The genetic legacy of the Quaternary ice ages. Nature, 405: 907–913.

Irimia, M., Roy, S.W. (2008) Spliceosomal introns as tools for genomic and evolutionary analysis. Nucleic Acids Res. 36 (5): 1703-12.

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Kasapidis, P., Suchentrunk, F., Magoulas, A., Kotoulas, G. (2005) The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations. Molecular Phylogenetic Evolution, 34: 55-66.

Krzywinski, J., Grushko, O., Besansky, N. (2006) Analysis of the complete mitochondrial DNA from Anopheles funestus : an improved dipteran mitochondrial genome annotation and a temporal dimension of mosquito evolution. Molecular phylogenetics and evolution, 39(2): 417-23

Lincoln, G. (1974) Reproduction and March madness in the Brown hare, *Lepus europaeus*. J. Zool. Lond. 174: 1-14.

Mamuris, Z., Sfougaris, A.I. and Stamatis, C. (2001) Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDBNA RFLP-PCR analysis: implications for conserving genetic diversity. Biological Conservation, 101: 187-196.

Mamuris, Z., Sfougaris, A.I., Stamatis, C., Suchentrunk, F. (2002) Assessment of genetic structure of Greek brown hare (*Lepus europaeus*) populations based on variation in random amplified polymorphic DNA (RAPD). Biochemical Genetics, 40: 323-38.

Markert, C.L. (1968) The molecular basis for isozymes Ann N Y Acad Sci.151(1): 14-40.

Melnick, D.J., Hoelzer, G.A., (1992) Differences in male and female macaque dispersal lead to contrasting distributions of nuclear and mitochondrial DNA variation. Int. J. Primatol. 13, 379–393.

Melo-Ferreira, J., Boursot, P., Suchentrunk, F., Ferrand, N. & Alves, P.C. (2005) Invasion from the cold: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. Molecular Ecology, 14: 2459–2464.

Melo-Ferreira, J., Boursot, P., Randi, E., Kryukov, A., Suchentrunk, F., Ferrand, N., Alves, P.C., (2007) The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. Mol. Ecol. 16: 605–618.

Moore, J., Willmer, P. (1997) Convergent evolution in invertebrates. Biol. Rev.72: 1–60.

Olsen, G.J., Woese, C.R. (1993) Ribosomal RNA: a key to phylogeny. FASEB Journal 7: 113-123.

Perez-Suarez, G., Palacios, F., Boursot, P., (1994) Speciation and paraphyly in western mediterranean hares (*Lepus castroviejoi*, *L. europaeus*, *L. granatensis*, and *L. capensis*) revealed by mitochondrail DNA phylogeny. Biochemical Genetics, 32**:** 423-436.

Peterson, R. (1966) The Mammals of Eastern Canada. Oxford University Press.

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Pielowski, Z. (1976) The role of foxes in the reduction of the European hare population. In Ecology and Management of European Hare Populations (eds Z. Pielowski & Z. Pucek), pp. 135–148. Polish Hunting Association, Warsaw, Poland.

Pierpaoli, M., Riga, F., Trocchi, V. & Randi, E. (1999) Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. Molecular Ecology, 8: 1805–1817.

Poli, A., Nigro, M., Gallazi, D., Sironi, G., Lavazza, A. (1991) Acute hepatosis in the european brown hare (*Lepus europaeus*) in Italy. Journal of Wildlife Diseases, 27(4): 621-629.

Putze, M., Nurnberg, S., Fickel, J. (2007) Y-chromosomal markers for the European brown hare (*Lepus europaeus*, Pallas 1778). European Journal of Wildlife Research, 53: 257–264.

Queller, D.C., Strassman,,J.E., Hughes, C.R. (1993). Microsatellites and Kinship. Trends in Ecology and Evolution, **8**: 285–288

Russell, J.P. (2009) iGenetics: Μια Μεντελική Προσέγγιση. 2ος Τόμος

Schaffner, S.F., (2004) The X chromosome in population genetics. Nat. Rev. Genet. 5, 43–51.

Sert, H., Suchentrunk, F., Erdogan, A. (2005) Genetic diversity in brown hares (*Lepus europaeus* Pallas, 1778) from Anatolia and differentiation among Anatolian and European populations. Mammalian Biology 70:171–186.

Simon, C., Buckley, T.R., Frati, F., Stewart, J.B., Beckenbach, A.T. (2006) Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. Annu. Rev. Ecol. Evol. Syst. 37: 545–579.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., Goodfellow, P.N., (1990) A gene from the human sex-determining region encodes a protein with a homology to a conserved DNA-binding motif. Nature, 346: 240–244.

Smith, R.K., Vaughan Jennings, N. & Harris, S. (2005) A quantitative analysis of the abundance and demography of European hares *Lepus europaeus* in relation to habitat type, intensity of agriculture and climate. Mammal Review, 35: 1–24.

Smith, A.T. & Johnston, C.H. (2008) *Lepus europaeus*. In IUCN: Red List of Threatened Species. Version 2010.3

Suchentrunk, F., Hartl, G.B., Flux, J., Parkes, J. Haiden, A. and Tapper, S. (1998) Allozyme heterozygosity and fluctuating asymmetry in brown hares (*Lepus europaeus*) introduced to New Zealand: development homeostasis in populations with a bottleneck history. Acta Theriol. Suppl. 5: 35-52.

Institutional Repository - Library & Information Centre - University of Thessaly
18/05/2024 05:49:47 EEST - 18.227.105.135

Suchentrunk, F., Michailov, C., Markov, G., Haiden, A. (2000) Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoogeographical crossroads. Acta Theriologica, 45: 1-12.

Suchentrunk, F., Jaschke, C., Haiden, A. (2001) Little allozyme and mtDNA variability in brown hares (*Lepus europaeus*) from New Zealand and Britain - A legacy of bottlenecks? Mammalian Biology, 66: 48-59.

Suchentrunk, F., Mamuris, Z., Sfougaris, A. I. and Stamatis, C. (2003) Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. Biochem. Genet. 41: 127–140.

Suchentrunk, F., Ben Slimen, H., Stamatis, C., Sert, H., Scandura, M., Apollonio, M. & Mamuris, Z. (2006) Molecular approaches revealing prehistoric, historic, or recent translocations and introductions of hares (genus *Lepus*) by humans. Human Evolution, 21: 151–165.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G. & Cosson, J.F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. Molecular Ecology, 7: 453–464.

Thulin, C.G., Jaarola, M., Tegelström, H. (1997) The occurrence of mountain hare mitochondrial DNA in wild brown hares. Molecular Ecology, 6: 463-467.

Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acids Res. 18: 6531-6535.

Willows-Munro, S., Robinson T.J., Matthee, C.A. (2005) Utility of nuclear DNA intron markers at lower taxonomic levels: phylogenetic resolution among nine Tragelaphus spp.. Mol. Phylogenet. Evol. 35: 624–636.

Τριανταφυλλίδης Κ. (2001) Κλασική και Μοριακή Γενετική. Εκδοτικός Οίκος Αδελφών Κυριακίδη Α.Ε.

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18/05/2024 05:49:47 EEST - 18.227.105.135