Molecular evidence of Pleistocene bidirectional faunal exchange between Europe and the Near East: the case of the bicolored shrew (*Crocidura leucodon*, Soricidae)

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Abstract

We sequenced 1077 bp of the mitochondrial cytochrome *b* gene and 511 bp of the nuclear Apolipoprotein B gene in bicolored shrew (*Crocidura leucodon*, Soricidae) populations ranging from France to Georgia. The aims of the study were to identify the main genetic clades within this species and the influence of Pleistocene climatic variations on the respective clades. The mitochondrial analyses revealed a European clade distributed from France eastwards to northwestern Turkey and a Near East clade distributed from Georgia to Romania; the two clades separated during the Middle Pleistocene. We clearly identified a population expansion after a bottleneck for the European clade based on mitochondrial and nuclear sequencing data; this expansion was not observed for the eastern clade. We hypothesise that the western population was confined to a small Italo-Balkanic refugium, whereas the eastern population subsisted in several refugia along the southern coast of the Black Sea.

Keywords: Crocidura leucodon; Cytochrome *b* gene; Apolipoprotein B gene; Phylogeography.

Introduction

The impact of Pleistocene climatic fluctuations on European biota is well documented. Studies of mitochondrial DNA markers reveal general patterns involving southern refugia and northern recolonisation routes, as a modification of genetic diversity of terrestrial biota throughout the Holartic (Hewitt, 2000, 2004a,b). Populations were isolated in different glacial refugia by barriers such as mountains and seas, leading to various recolonisation patterns (e.g., Taberlet *et al.*, 1998; Hewitt, 1999). Widely accepted refugia include the Iberian Peninsula in the west and the Italo-Balkanic region in the east (e.g., Thorpe, 1984; Ferris *et al.*, 1993, 1998; Dumolin-Lapegue *et al.*, 1997; Santucci *et al.*, 1998; Taberlet *et al.*, 1998; Dubey *et al.*, 2006); however, a number of phylogeographical studies of various taxa have revealed an additional pattern of colonisation of Europe by populations originating from eastern areas such as the Caucasus, southern Urals, and western Asia (Cooper *et al.*, 1995; Bilton *et al.*, 1998; Nesbo *et al.*, 1999; Palme & Vendramin, 2002; Seddon *et al.*, 2002; Michaux *et al.*, 2004; Culling *et al.*, 2006; Dubey *et al.*, 2006). This pattern of post-glacial recolonisation appears to be more common than previously suspected and affected probably a large range of taxa.

With regard to population dynamics, few studies have detailed the impact of Pleistocene climatic oscillations, e.g., the dating of population expansions and identifying differences in patterns among clades of different geographic origin (Michaux *et al.*, 2004; Brändli *et al.*, 2005; Culling *et al.*, 2006; Dubey *et al.*, 2006; Dubey *et al.*, in press a; Koch *et al.*, 2006; Marmi *et al.*, 2006; Vörös *et al.*, 2006). In the same way, the majority of previous studies are based solely on mitochondrial DNA, whereas the inclusion of uniparentally and biparentally inherited markers present in nuclear genomes can enhance our understanding of population history (Brändli *et al.*, 2005; Dubey *et al.*, 2006).

In the present study, we focused on the bicolored shrew, *Crocidura leucodon* (Hermann, 1780), which is a strictly western Eurasian species, distributed from France to central and southern Europe to Turkey and Georgia. This species seems to be an interesting candidate for a phylogeographic study, as Vogel *et al.* (2003), based on only two samples, from Switzerland and Turkey, noted a substantial Kimura 2-paramters genetic distance of 3.8% between them for the Cytochrome *b* gene. In addition, Poulakakis *et al.* (2005) analysed three Greek samples (from Peloponnesus and Lesvos) obtained from owl pellets, based on the same gene, promoting such material as usable for phylogeographic studies, without giving any information concerning the distances between haplotypes. Nevertheless, the intraspecific genetic distances were considered as low.

We tested (i) in which way the Bosphorus strait has isolated the European and Near East populations, and (ii) in which way the Pleistocene climatic fluctuations have had different impacts on the western and eastern populations, such as bottleneck effects. To resolve these questions, we analysed sequence data from mitochondrial and nuclear markers (Cytochrome *b* and Apolipoprotein B) and considered the molecular clock.

Material and methods

Sampling

We analysed 60 samples of *Crocidura leucodon* collected from France to Georgia (Fig. 1 and Table 1), three other Eurasian crocidurine taxa, and as an out-group a soricine, *Sorex minutus*. This set of samples (Table 1) included material from the collections of Lausanne (IZEA), Switzerland; Prague (DZCU), Czech Republic; and Ljubljana (PMS), Slovenia. Some sequences were taken from Ohdachi *et al.* (2004), Fontanillas *et al.* (2005) and Dubey *et al.* (2006). We also used samples from Poulakakis *et al.* (2005) that were treated separately.

DNA extraction and amplification of Cytochrome *b* and Apolipoprotein B genes

Samples (livers) from IZEA collection were first frozen in the field in liquid nitrogen and kept for several years at –70 °C before being stored in ethanol until DNA extraction. Samples from the other collections were directly stored in ethanol. DNA extraction was carried out using the QIA Amp DNA Mini Kit (Qiagen). Double-stranded DNA amplifications of the mitochondrial cytochrome *b* gene (*cyt-b*) were performed with the primer pairs L14724/H15149, C1/C2, C3/H15915, and L14724/H15915 (Irwin *et al.*, 1991; Dubey *et al.*, 2006). Amplification of the Apolipoprotein B (*ApoB*) nuclear genes was performed using the primer pairs ApoBf /ApoBr (Dubey *et al.*, in press b). Amplification conditions for the *ApoB* consisted of 40 cycles of 45 s denaturation at 94 °C, 45 s annealing at 50 °C, and 90 s extension at 72 °C; for the *cyt-b*, the conditions of Dubey *et al.* (2006) were used.

PCR products were checked on a 1% agarose gel and then purified using the QIAquick PCR Purification Kit (QIAgen) following the manufacturer's instructions. DNA sequencing was performed in a total volume of 10 µl containing 1-3 µl of amplified PCR product, 1 µl of 10 µM primer, and 4 µl of ABI PRISM^{TM} Dye Terminator 1 (Perkin–Elmer). Sequence reactions were visualised on an ABI 3100 genetic analyser (Applied Biosystems, USA).

Phylogenetic methods

Nucleotide sequences of *cyt-b* and *ApoB* genes were edited with Sequence Navigator (Parker, 1997) and manually aligned. Two methods of phylogenetic analyses were carried out for *cyt-b*, using PAUP*version 4.0b10 PPC (Swofford, 1998). Tests were conducted on the complete fragment (1077 bp), all codon positions were used, and trees were rooted using sequences of *Sorex minutus* (DQ630379). A Neighbour Joining (NJ) tree was constructed using the general time reversible (GTR; Rodriguez *et al.*, 1990) genetic distance, which was selected previously using Modeltest 3.06 according to the protocol of Posada & Crandall (1998). The Parsimony analyses (MP) were performed using the following options: heuristic

search, stepwise-addition of sequences, 10 replicates of random addition of taxa, and TBR branch swapping (Swofford, 1998); all codon positions were equally weighted. Bootstrap support values were obtained with 1000 pseudo-replicates and 10 random replicates of stepwise-addition sequences. Fast maximum likelihood (ML) heuristic searches and bootstrap analyses (1000 replicates) were performed using PHYML (Guindon & Gascuel, 2003) with a GTR model.

Nucleotide diversity and genetic structure

The nucleotide diversities (π) of *cyt-b* and *ApoB* and the population genetic structure for *cyt-b* (analysis of molecular variance, AMOVA) were estimated using Arlequin version 2.0 Software (Schneider, 2000). AMOVA was performed at two different hierarchical levels: among clades and within clades.

Molecular clock

The molecular clock hypothesis was tested following the method of Posada and Crandall (1998). Estimation of divergence time from the molecular data was performed according to the calibration developed for *cyt-b* in Soricidae by Fumagalli *et al.* (1999), based on an estimate of 20 Myr for the split between the Soricinae and the Crocidurinae and on the number of third-position transversions observed. Nevertheless, it could not be used directly because of the low number of third-position transversions observed at a specific level within the family. Thus, we used the calibration of Dubey *et al.* (2006), based on the previous study that allowed a better estimated, due to the use of a ML distance including all the codon positions with a divergence rate of 0.057 ML distance/Myr (95% CI: 0.044-0.070).

Isolation with migration

We used the ‘‘isolation with migration’’ (or IM, Nielsen and Wakeley 2001) model to date the divergence between populations of both side of the Marmara Sea within each clade. As suggested by Hey (2007), we started the simulations using a burn-in of 100,000 steps

followed by a ½ hour run with maximum values for theta, m and t arbitrary set to 10 and the –J1 run option. From there, appropriate values for priors were chosen. In each following runs, plots of parameter trend lines were systematically consulted for assessing how well the Markov chain was exploring the parameter space. Convergence by the Markov chain simulations was assessed by monitoring three independent chains, and by assessing the autocorrelation of parameter values over the course of each run. Individual simulations were run for 10 million updates or more. For each of the demographic parameters, we recorded the marginal density. The peaks of the resulting distributions were taken as estimates of the parameters and the 90% highest posterior density (HPD) interval was taken for the credibility intervals. Estimates with IM are scaled by the overall neutral mutation rate per gene per generation. We assigned an inheritance scalar of 0.25 as usual for mtDNA and assumed 1 year per generation. To convert parameter estimates to time scale units, we used the average rate of evolution for the *cyt-b* gene in *Crocidura* species from Dubey et *al.* (2006), corresponding to $3.07E^{-5}$ (95% CI: $2.37E^{-5}$-$3.77E^{-5}$) mutation events/locus/year.

Expansion time

To test the hypothesis of recent population growth from a low-diversity founder population within the different clades, several tests were performed for *cyt-b*. We used three methods implemented in Arlequin version 2.0 (Schneider, 2000). The first method, Fu's (1997) F_S statistic, tests the probability of having no fewer than the number of observed alleles in the sample given that θ (heterozygosity per sites) = π. This statistic tends to be negative when there is an excess of recent mutations (or rare alleles). The second method, Tajima's (1989) *D* statistic, tests the null hypothesis that two estimates of the neutral mutation parameter, one derived from the average number of pairwise nucleotide differences and the other based on the number of segregating sites in the sample, are equal. In the third test, pairwise mismatch distributions among individuals were plotted and tested for goodness-of-fit

to a model of sudden expansion using parametric bootstrapping with 1000 replicates (Schneider & Excoffier, 1999). Expansion time after the bottleneck was estimated from the mismatch distribution (τ) (Rogers, 1995) and uncorrected distances (p). Evolutionary rate for uncorrected (p) distance was estimated using the molecular clock developed by Fumagalli *et al.* (1999) with a divergence rate of 0.061 uncorrected distance (p)/Myr (95% CI: 0.054-0.069).

Results

Cytochrome *b* gene

The 60 *C. leucodon* samples showed 41 different haplotypes of 1077 bp and contained 366 variable sites, of which 218 were parsimony-informative. No insertions or deletions were observed. As the three phylogenetic methods gave identical arrangements of the main branches, the relationship between haplotypes is given only for the ML analysis in Fig. 2.

The *Crocidura leucodon* samples formed a monophyletic unit (all bootstrap values of 100%), well differentiated from the other Eurasian species (Fig. 2). Within this unit, two major and strongly supported clades were found (all bootstrap values ≥80%).

Clade I (western clade) included European samples from Czech Republic, France, Germany, Greece (Lesvos), Italy, Serbia, Slovakia, Slovenia, Switzerland, and samples from northwestern Anatolia, Turkey. Clade II (eastern clade) included the European samples from Bulgaria and Romania and the samples from Georgia and Anatolia (with the exception of westernmost Anatolia).

AMOVA showed that the majority of mtDNA variation (84.09%) is distributed between the two clades of *C. leucodon*; only a small percentage of this variation (15.91%) is observed within clades. Mean pair-wise GTR distance between clades is 3.94%. The mean

pairwise GTR distances within clades and nucleotide diversities were 0.65% and 0.057 for the western clade, respectively, and 0.71% and 0.059 for the eastern clade.

Three additional sequences from Lesvos, Stymfalia, and Parnitha (Greece) described by Poulakakis *et al.* (2005) belong to the western clade, but were not included in our phylogenetic analyses. The analysis of these data (AY452166, AY452176, AY452165) revealed an unexpected result. The mean GTR distance between our samples and the Poulakakis *et al*. samples from Lesvos was 2.8%. In comparing the sequences, we observed a large number of mutations at the beginning and end of their *cyt-b* sequence. When the last 13 bp of their sequence were deleted, the mean GTR distance fell to 0.89%, and this was further reduced to 0.42% by deleting the first 48 bp of their sequence (see Table 2 for the details of the mutations observed at the beginning and the end of the sequences of Poulakakis *et al.*). In addition, the mean GTR distance between the Poulakakis *et al*. samples from Lesvos and those from continental Greece was 2.15%, whereas the mean distance between our samples from Lesvos and the Poulakakis *et al*. samples was 6.22%; this value is still much greater than the mean GTR distance between all of our sequences from this clade (0.65%) and the mean GTR distance between the two major clades (western and eastern) obtained in our analyses (3.94%, Fig. 2).

A likelihood ratio test led to the acceptance of the molecular clock hypothesis for the whole sample ($df = 43$, $\chi^2 = 53.44$, $P = 0.13$). – Ln Likelihood values with and without the molecular clock assumption are 4169.79 and 4143.07, respectively, for the best trees. On the basis of the calibration of Fumagalli *et al.* (1999) and Dubey *et al.* (2006), we estimated the divergence time between the western and eastern clade to be 0.691 Myr (95% CI: 0.510-0.980; Fig. 2).

The IM model has six demographic parameters but we were particularly interested in three of them: t, the time of population splitting (in generation) in the past, and m1 and m2,

the gene-flow rates per gene copy per generation. Once priors were correctly defined, we ran three runs for each clade. The different runs revealed unambiguous marginal posterior probability distributions of the parameters in each clade. The migration parameters revealed a peak at the lower limit of resolution in both directions (from East to West and vice versa) in both clades, as expected given that the Marmara Sea is a nonnegotiable barrier to dispersal once field with water (during interglacials). We hereafter interpret the locations of these peaks as being at zero and then simulated three more runs for each clade fixing m1 and m2 to zero for estimating the time of the splits. The marginal posterior probability distribution of t revealed a sharp peak at 0.525 and 1.785 for the Western and the Eastern clade respectively (Fig. 3). When converted to a scale of years, the divergence times between both sides of the Marmara Sea were estimated to be 17,100 yr (90% HPD interval: 1,700-40,600) for the Western clade, and 58,200 yr (90% HPD interval: 16,100-109,000) for the Eastern one.

We observed a non-significant *P*-value for the mismatch distribution test of goodness-of-fit for the western clade (*Harpending's Raggedness index* = 0.02, $P = 0.28$) and a significant *P*-value for the eastern clade (*Harpending's Raggedness index* = 0.12, $P = 0.03$; Table 2). The frequency of the mean pairwise difference between haplotypes showed a bell-shaped distribution for the western clade, contrasting with the more complex distribution obtained for the eastern clade (Fig. 1). Fu's F_S statistics and Tajima's *D* were significant ($Fs = -10.68$, $P < 0.001$; $D = -1.48$, $P = 0.04$; Table 3) for the western clade but not significant for the eastern ($Fs = -2.02$, $P = 0.16$; $D = -0.67$, $P = 0.26$); consequently, we inferred a scenario of expansion for the western population and non-expansion for the eastern population.

The timing of expansions was estimated from the mismatch distribution according to the method proposed by Rogers (1995; Fig. 1B, C). The τ value for the western population was 6.71 (95% CI: 4.19-8.39). Assuming no saturation of uncorrected distances (p), as shown in Dubey *et al*. (2006), distance was 0.0612 per million years (95% CI: 0.054-0.069). With a

generation time of 1 year, the population expansion time was estimated to be 51,900 years (95% CI: 28,100-72,100).

Apolipoprotein B gene

The 44 analysed samples showed four different *ApoB* alleles of 511 bp, named A1 to A4 (Genbank accession: EF011555-EF011558), all the alleles differing from each others by only one mutation at the position 62, 96 and 144 of our sequence alignment. Consequently, the different alleles of heterozygous samples were easily determined, as only one mutation was observed between alleles. The western mitochondrial clade samples were all homozygous for the allele A1 (Table 1). In contrast, thirteen A1 homozygotes, two A1/A2 compound heterozygotes, one A1/A3 compound heterozygote, one A1/A4 compound heterozygote, and one A4 homozygote were found in the eastern mitochondrial clade. Consequently, nucleotide diversities were 0.0000 for the western clade and 0.0006 for the eastern clade.

Discussion

Quality of samples

The integration of our *cyt-b* sequences with those of Ohdachi *et al.* (2004), Fontanillas *et al.* (2005) and Dubey *et al.* (2006), analysed from preserved tissues, showed expected genetic distances between samples. In contrast, the material of *C. leucodon* from owl pellets analysed by Poulakakis *et al.* (2005) showed GTR distances with our *C. leucodon* samples much higher than expected, even between samples from the same island (Lesvos; mean GTR distance: 2.8%). A distance that fell to 0.42% by deleting the first and the last bp of their sequence from Lesvos. Moreover, no non-synonymous mutations were observed in the *cyt-b* gene for our *C. leucodon* samples, whereas mutations at the end of the Poulakakis *et al.* (2005) sequences resulted in two amino-acid substitutions; thus, these mutations appear to be highly suspect. These mutations may in fact be artefacts of DNA sequencing as a result of the poor template

quality obtained from the owl pellets (Taberlet and Fumagalli, 1996; Waits and Paetkau, 2005). This reinforces the value of repeating the DNA extraction and/or the analysis for each non-invasive sample several times in order to validate the sequencing result (Waits and Paetkau, 2005). Consequently, the affirmation of Poulakakis *et al.* (2005), concerning the use of their samples for phylogenetic and phylogeographical studies of small mammals, has to be questioned.

Biogeography

Based on our mitochondrial phylogenetic analyses, *Crocidura leucodon* populations are divided in two main mitochondrial clades (mean GTR distance between clades: 3.94%; Fig. 1A & 2). The first clade (western clade) includes samples from western and central Europe, and unexpectedly, two samples from Lesvos Island and three from northwestern Anatolia, to which the Greek samples (Lesvos, Parnitha, and Stymfalia) from Poulakakis *et al.* (2005; results not shown) should be added. The second clade (eastern clade) includes samples from Bulgaria, Romania, Anatolia (with the exception of westernmost Anatolia) and Georgia. These two clades could represent the chromosomal differences observed between the Georgian samples and those from Czech Republic and Lesvos that were detected in the karyotype analyses undertaken by Biltueva *et al.* (2001).

The separation between these two mitochondrial lineages of *C. leucodon* occurred in the Middle Pleistocene, 0.691 Myr (95% CI: 0.510-0.980), the period immediately following the Günz glacial events (790,000-950,000 years BP). This suggests the isolation of populations by submergence of the Bosphorus Strait (between the Black Sea and the Marmara Sea) with increasing sea levels following the glacial period.

Sedimentological and palaeontological evidence reveals that the Bosphorus Strait has alternatively submerged and emerged since the Middle Pleistocene before being completely submerged from the Mid-Late Holocene (Kerey *et al.*, 2004). Consequently, the fact that we

observed a lack of clear structure between samples situated on either side of the Bosphorus Strait indicates that south-eastern Europe was probably colonised by the eastern mitochondrial lineages during a recent land bridge connection between Europe and the Near East (Late Pleistocene). A hypothesis confirmed by the migration analyses that estimated this event of vicariance to the Upper Pleistocene (58,200 years BP; 90% HPD: 16,100 to 109,000). Based on similar results, the colonisation of western Anatolia by the western mitochondrial lineage occurred during the same period (17,100 years BP; 90% HPD: 1,700 to 40,600; Late Pleistocene-Holocene). Thus, this strait appears to be a permeable biogeographic barrier for *C. leucodon*. This permeability has already been demonstrated in classical zoogeography for various species (e.g., Hosey, 1982) on the basis of unidirectional migrations from Europe to Anatolia (Hosey, 1982; Filippucci & Simson, 1996; Kryštufek, 2002). However, our study demonstrate for the first time a Late Pleistocene-Holocene bidirectional exchange of two different conspecific lineages between Europe and the Near East, whereas previous studies have only revealed unidirectional colonisation.

The last climatic fluctuations of the Upper Pleistocene (126,000-11,500 years BP; Ogg, 2004) had contrasting impacts on the western and eastern mitochondrial clades. For the western clade, the bell-shaped curve of the mismatch distributions (Fig. 1B) of the *cyt-b* gene indicates an expansion following a bottleneck 50,800 years ago (95% CI: 28,100-72,100). This finding reveals that the last glacial maximum of the Pleistocene (22,000 years BP) had a moderate impact on these small mammal populations. Conversely, no sign of expansion was detected for the eastern mitochondrial clade of *C. leucodon*, which is consistent with the absence of nuclear polymorphism in the western clade (π: 0.0000), whereas four alleles are present in the eastern (π: 0.0006).

Thus, the European population appears to have been reduced to a small number of individuals during the last glaciations, probably confined to within a small Italo-Balkanic

refugium. Conversely, the eastern clade appears to have persisted with a greater population size and probably in several refugia around the Black Sea. This pattern has also been suggested for mammals and plants (Michaux *et al.*, 2004; Heuertz *et al.*, 2006; Kučera *et al.*, 2006), based on a higher genetic diversity observed in Anatolian populations compared to European ones. Moreover, these results are also supported by palynological data that indicate that open wooded cover in this area persisted through the full glacial condition of the Pleistocene (Tarasov *et al.*, 2000).

Conclusion

Based on molecular data, we confirmed that the Bosphorus Strait was a permeable barrier. This finding has been suggested previously by the colonisation of western Anatolia by European populations of the lesser white-toothed shrew in the Lower Pleistocene (*C. suaveolens*; Dubey *et al.*, 2006) and more recently by the hedgehog (Filippucci & Simson, 1996; Kryštufek, 2002). Moreover, we provide evidence that European populations of the bicolored shrew may be the source of recent West Anatolian populations.

We also found a marked difference in population history between the two divergent mitochondrial lineages of bicolored shrew, suggesting that Pleistocene climatic variations have more strongly reduced the genetic diversity of the European population than that of the Near East population. We hypothesise that this could be a general pattern for fauna and flora, as other studies comparing these geographical areas have reported similar differences (Michaux *et al*., 2004; Heuertz *et al*., 2006; Kučera *et al*., 2006).

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Fig. 1 (A) Location of analysed samples, tentative distribution of mitochondrial clades (grey areas), allelic frequencies of nuclear genes (cheese), and nucleotide diversity of mitochondrial and nuclear gene; (B, C) Observed (solid line) and expected (dotyed-line) mismatch distributions for a sudden expansion of the western and eastern clades, respectively.

Fig. 2 Phylogeny of the 1077bp *cyt-b* fragment analysed with maximum likelihood, using the GTR model of substitution. Values in branches are indices of support for the major branches for maximum likelihood (ML), maximum parsimony (MP) and distance (NJ) analyses (percentage of 1000 replications). Codes are as in Table 1.

Fig. 3 Marginal posterior probability (p) distribution of the divergence time parameter (t) in year, from the isolation with migration model, between populations situated western and eastern of the Bosphorus Strait within the Western and Eastern Clade, and estimated time for the splits with the 90% highest posterior density interval (HPD).

Table 1 Details of samples used in this study: Species, samples location, collection, location on map (Fig. 1), *cyt-b* Id. Code, accession number of *cyt-b* sequences, and *ApoB* alleles. Abbreviations of countries are: Bulgaria (BG), Czech Republic (CZ), Germany (DE), France (FR), Georgia (GE), Greece (GR), Hungary (HU), Italy (IT), Russia (RU), Serbia (SE), Slovak Republic (SK), Slovenia (SI), Switzerland (CH), and Turkey (TR).

Species	Samples location	Coll. code	Number on the map	*Cyt-b* Id. code	Accession (*cyt-b*) and Apob allele
Sorex minutus	Champmartin, CH	IZEA 7622	/	/	DQ630379/-
Crocidura suaveolens	Fulophasa, HU	Dubey et al. (2006)	/	/	AY843451/-
Crocidura russula	Vaud, CH	Fontanillas et al. (2005)	/	/	AY769264/-
Crocidura lasiura	Ussurisk, RU	Ohdachi et al. (2004)	/	/	AB077071/-
Crocidura leucodon	Visp, CH	IZEA 7553	1	CH1	DQ994744/A1
Crocidura leucodon	Réchy, CH	IZEA 7552	1	CH2	DQ994745/A1
Crocidura leucodon	Brigerbad, CH	IZEA 2951	1	CH3	DQ994747/-
Crocidura leucodon	Raron, CH	IZEA 7532	1	CH4	DQ994746/-
Crocidura leucodon	Réchy, CH	IZEA 5590	1	CH9	DQ994794/-
Crocidura leucodon	Brigerbad, CH	IZEA 7526	1	/	-/A1
Crocidura leucodon	Gordevio, CH	IZEA 5965	2	CH5	DQ994748/-
Crocidura leucodon	Gordevio, CH	IZEA 5963	2	CH6	DQ994749/A1
Crocidura leucodon	Quartino, CH	IZEA 5455	2	CH7	DQ994750/A1
Crocidura leucodon	Grison, CH	IZEA 9013	3	CH8	DQ994785/A1
Crocidura leucodon	Karlsruhe, DE	IZEA 5441	4	DE5	DQ994795/A1
Crocidura leucodon	Rendsburg, DE	IZEA 7835	5	DE1	DQ994761/-
Crocidura leucodon	Rendsburg, DE	IZEA 7836	5	DE2	DQ994762/-
Crocidura leucodon	Rendsburg, DE	IZEA 7837	5	DE3	DQ994763/A1
Crocidura leucodon	Rendsburg, DE	IZEA 7838	5	DE4	DQ994764/-
Crocidura leucodon	Mignouillard, FR	IZEA 9006	6	FR4	DQ994780/A1
Crocidura leucodon	Mignouillard, FR	IZEA 9002	6	FR5	DQ994781/A1
Crocidura leucodon	Chapelle, FR	IZEA 9003	7	FR2	DQ994779/A1
Crocidura leucodon	Chapelle, FR	IZEA 9005	7	FR3	DQ994782/A1
Crocidura leucodon	St-Martin, FR	IZEA 9008	8	FR1	DQ994783/A1
Crocidura leucodon	St-Etienne, FR	IZEA 9009	9	FR6	DQ994784/A1
Crocidura leucodon	Vercelli, IT	IZEA 7948	10	IT1	DQ994767/-
Crocidura leucodon	Vercelli, IT	IZEA 7946	10	IT2	DQ994768/-
Crocidura leucodon	Vercelli, IT	IZEA 7952	10	IT3	DQ994769/-
Crocidura leucodon	Serramazoni, IT	IZEA 5662	11	IT4	DQ994770/A1
Crocidura leucodon	Serramazoni, IT	IZEA 5649	11	IT5	DQ994771/-
Crocidura leucodon	Serramazoni, IT	IZEA 5663	11	IT6	DQ994772/A1
Crocidura leucodon	Piacenza, IT	IZEA 7517	12	IT7	DQ994773/A1
Crocidura leucodon	Bratislava, SK	IZEA 5728	13	SK1	DQ994765/-
Crocidura leucodon	Bratislava, SK	IZEA 5723	13	SK2	DQ994766/A1
Crocidura leucodon	Lesbos, GR	IZEA 4153	14	GR1	DQ994777/A1
Crocidura leucodon	Lesbos, GR	IZEA 3929	14	GR2	DQ994778/A1
Crocidura leucodon	Lesbos, GR	Poulakakis et al., 2005	14	-	AY452165/-
Crocidura leucodon	Burgas, BG	IZEA 8059	15	BG1	DQ994774/A1/A2
Crocidura leucodon	Burgas, BG	IZEA 8060	15	BG2	DQ994775/A1
Crocidura leucodon	Burgas, BG	IZEA 8063	15	BG3	DQ994776/A1
Crocidura leucodon	Burgas, BG	IZEA 8059	15	-	-/A1/A2
Crocidura leucodon	Alazani, GE	IZEA 23629	16	GE1	DQ994756/A1/A4
Crocidura leucodon	Alazani, GE	IZEA 23635	16	GE2	DQ994757/A4
Crocidura leucodon	Alazani, GE	IZEA 23613	16	GE3	DQ994758/A1
Crocidura leucodon	Dusheti, GE	IZEA 2880	17	GE4	DQ994760/A1/A3
Crocidura leucodon	Cakalli, TR	IZEA 6064	18	TR1	DQ994751/A1

Crocidura leucodon	Rize, TR	IZEA 6045	19	TR2	DQ994752/-
Crocidura leucodon	Rize, TR	IZEA 6049	19	TR3	DQ994753/A1
Crocidura leucodon	Rize, TR	IZEA 6047	19	TR4	DQ994754/A1
Crocidura leucodon	Rize, TR	IZEA 6046	19	TR5	DQ994755/A1
Crocidura leucodon	Altindere, TR	IZEA 6039	20	TR6	DQ994759/A1
Crocidura leucodon	Altindere, TR	IZEA 6038	20	TR7	DQ994796/-
Crocidura leucodon	Altindere, TR	IZEA 6044	20	-	-/A1
Crocidura leucodon	Yellibeli, Kar., TR	DZCU TU-1179	23	TR8	DQ994787/A1
Crocidura leucodon	Çiğlikara, Ant., TR	DZCUTU-1195	24	TR9	DQ994786/A1
Crocidura leucodon	Karlovy Vary, CZ	DZCU 01400	25	-	-/A1
Crocidura leucodon	Karlovy Vary, CZ	DZCU 01399	25	CZ1	DQ994788/A1
Crocidura leucodon	Karlovy Vary, CZ	DZCU 01398	25	CZ2	DQ994789/A1
Crocidura leucodon	Mt. Cer, SE	PMS 7391	26	SE1	DQ994790/A1
Crocidura leucodon	Cataloi, RO	IZEA 8160	27	RO1	DQ994791/A1
Crocidura leucodon	Slobozia, RO	IZEA 8170	28	RO2	DQ994792/A1
Crocidura leucodon	Vrhnika, SI	PMS Slo 1	29	SI1	DQ994793/A1
Crocidura leucodon	Katranci-Biga, TR	2003.131	30	TR10	EF417543/A1
Crocidura leucodon	Özbek, İzmir, TR	2003.199	31	TR11	EF417544 /A1
Crocidura leucodon	Terzialan-Çan., TR	2003.217	32	TR12	EF417545/A1
Crocidura leucodon	Parnitha, GR	Poulakakis et al., 2005	21	-	AY452166/-
Crocidura leucodon	Stymfalia, GR	Poulakakis et al., 2005	22	-	AY452176/-

1

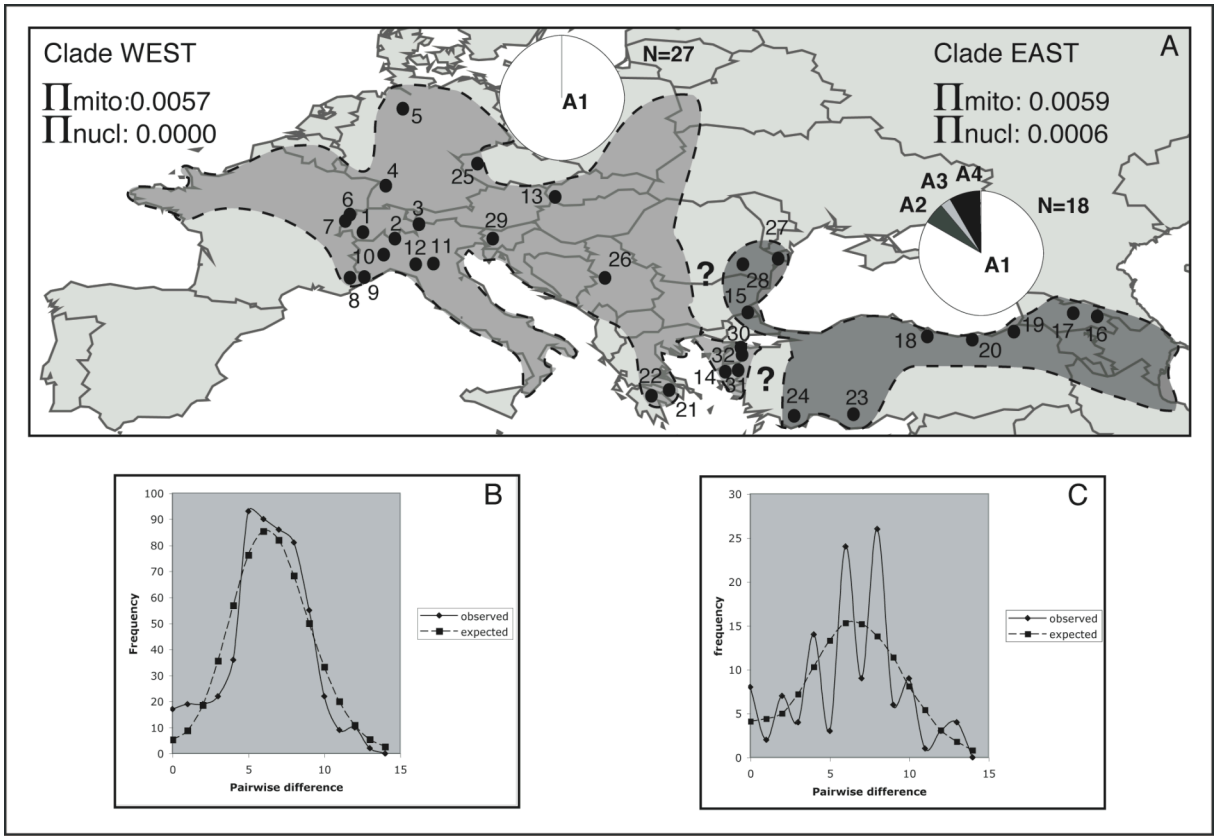
2

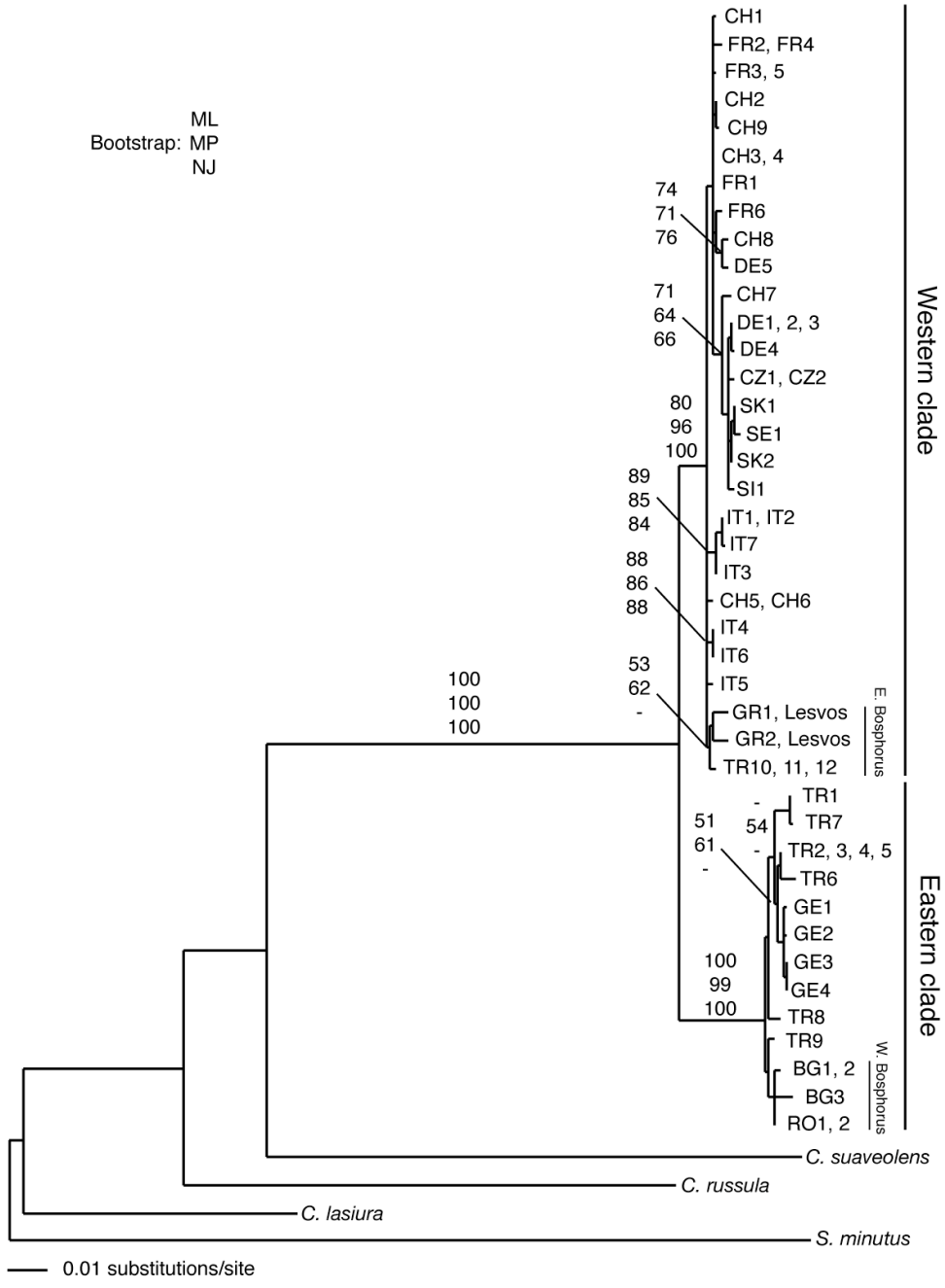
Table 2 Example of mutations observed between some of our samples and the samples of Poulakakis *et al*. (2005; *; Lesvos, AY452165; Parnitha, AY452166; Stymfalia, AY452176). Only the mutations including the first 107 bp and the last 52 bp of the Poulakakis sequences are shown. The samples SK2, IT5, TR10, GR1 and GR2 (this study) belong to the western mitochondrial clade, and the samples TR6 and GE1 from the eastern mitochondrial clade.

Samples	**Example of mutations observed between sequences**																		
SK2, Bratislava	TC	TGC	TTA	ATT	GCA	CAA	ATC	CTA	ACA	GGA	TTA	TTC	CTA	GCC	ATA	CAC	TAT	ACA	[53]
IT5, Serramazoni	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[53]
TR10, Katranci	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[53]
GR2, Lesvos	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	[53]
GR1, Lesvos	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[53]
*Lesvos	NN	NNN	NNN	NNN	NNN	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	. . T	[53]
*Parnitha	NN	NNN	NNN	NNN	NNN	. . .	. . .	. . G	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	. . .	[53]
*Stymfalia	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	C . .	. . T	. . .	. . .	. . .	. . .	. . .	. . .	[53]
TR6, Altindere	. .	. . .	. . .	. . .	. . .	. . .	. . .	T . .	. . .	. . .	C . .	. . .	T . .	. . .	. . .	. . .	. . .	. . .	[53]
GE1, Alazani	. .	. . .	. . .	. . .	. . .	. . .	. . .	T . .	. . .	. . .	C . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[53]
SK2, Bratislava	TCT	GAT	ACT	ACA	ACA	GCT	TTC	TCC	TCC	GTA	ACC	CAT	ATT	TGC	CGA	GAT	GTA	AAT	[107]
IT5, Serramazoni	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
TR10, Katranci	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
GR2, Lesvos	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
GR1, Lesvos	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
*Lesvos	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
*Parnitha	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
*Stymfalia	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	[107]
TR6, Altindere	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
GE1, Alazani	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	[107]
SK2, Bratislava	TA	GGA	TAT	GTT	CTT	CCC	TGA	GGT	CAA	ATA	TCA	TTT	TGA	GGT	GCA	ACA	GTA	AT	[332]
IT5, Serramazoni	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	[332]
TR10, Katranci	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	[332]
GR2, Lesvos	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	[332]
GR1, Lesvos	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	[332]
*Lesvos	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . G	. . T	G . .	NNN	NN	[332]
*Parnitha	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . G	. . T	G . .	NNN	NN	[332]
*Stymfalia	. .	. . .	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . G	. . T	G . .	AGT	T .	[332]
TR6, Altindere	. .	. . G	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	[332]
GE1, Alazani	. .	. . G	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	[332]

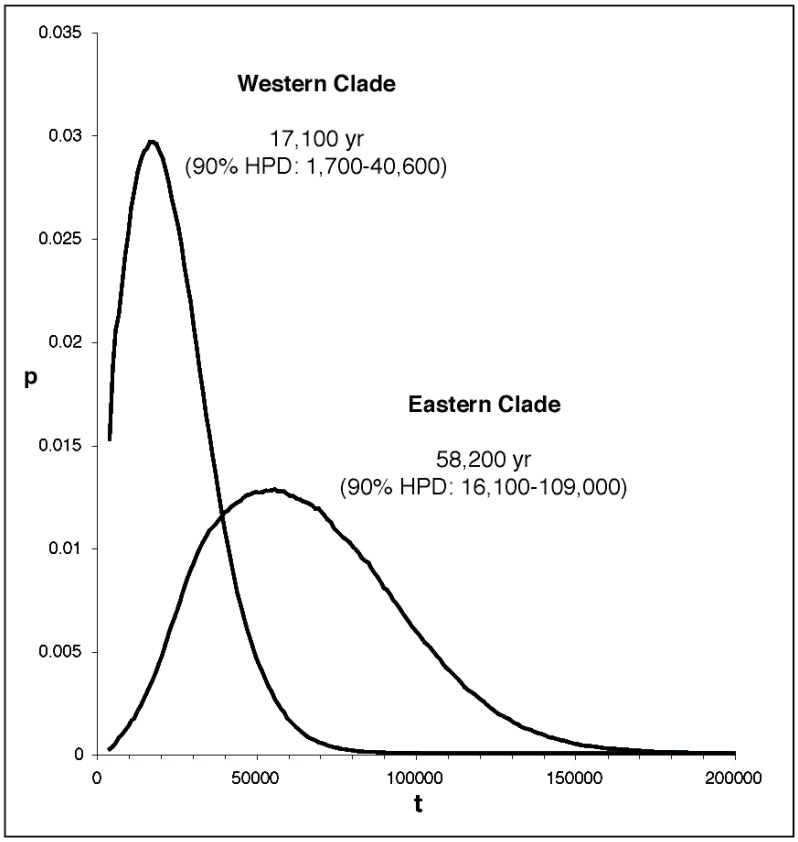
Table 3 Number of samples (Ns) and number of haplotypes (Nh) within the western and eastern mitochondrial bicolored shrew clades, genetic diversity, nucleotide diversity, mean pairwise differences between haplotypes, Goodness-of-fit test probability, Fu's *Fs* test probability, Tajima's *D* test probability, estimated τ value and expansion time for western clade.

Clade (Ns, Nh)	**Nucleotide div./Mean pairwise diff.**	**Goodness-of-fit test**		***Fu's Fs test***		***Tajima's test***		**τ**	**Expansion time**
		Rag. Index	*P*	Fs	*P*	Tajima's D	*P*		
Western (34, 23)	0.0057/6.21	0.02	= 0.28	–10.68	<0.001	–1.48	= 0.04	6.80 (95% CI: 4.19-8.39)	51,900 y BP (95% CI: 28,100–72,100)
Eastern (16, 11)	0.0059/6.31	0.12	= 0.03	–2.02	= 0.16	–0.67	= 0.26	7.47 (95% CI: 3.71-10.85)	/


Clade WEST
Πmito:0.0057
Πnucl: 0.0000
N=27
A1
Clade EAST
Πmito: 0.0059
Πnucl: 0.0006
A3
A4
A2
N=18
A1
A
B
Frequency
Pairwise difference
observed
expected
C
frequency
Pairwise difference
observed
expected


ML
Bootstrap: MP
NJ
CH1
FR2, FR4
FR3, 5
CH2
CH9
CH3, 4
FR1
FR6
CH8
DE5
CH7
DE1, 2, 3
DE4
CZ1, CZ2
SK1
SE1
SK2
SI1
IT1, IT2
IT7
IT3
CH5, CH6
IT4
IT6
IT5
GR1, Lesvos
GR2, Lesvos
TR10, 11, 12
TR1
TR7
TR2, 3, 4, 5
TR6
GE1
GE2
GE3
GE4
TR8
TR9
BG1, 2
BG3
RO1, 2
74
71
76
71
64
66
80
96
100
89
85
84
88
86
88
53
62
-
100
100
100
-
54
-
51
61
-
100
99
100
Western clade
Eastern clade
E. Bosphorus
W. Bosphorus
C. suaveolens
C. russula
C. lasiura
S. minutus
0.01 substitutions/site

1


Western Clade
17,100 yr
(90% HPD: 1,700-40,600)
Eastern Clade
58,200 yr
(90% HPD: 16,100-109,000)
p
t
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
50000
100000
150000
200000

2