

Short Communication

# Molecular phylogenetics reveals Messinian, Pliocene, and Pleistocene colonizations of islands by North African shrews

Sylvain Dubey<sup>a,b,\*</sup>, Kazuhiro Koyasu<sup>a,c</sup>, Roumen Parapanov<sup>a</sup>, Mohammed Ribi<sup>c,d</sup>,  
Rainer Hutterer<sup>e</sup>, Peter Vogel<sup>a</sup>

<sup>a</sup> Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland

<sup>b</sup> Shine Laboratory, School of Biological Sciences, University of Sydney, Hydon-Laurence Building A08, Sydney, NSW 2006, Australia

<sup>c</sup> School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan

<sup>d</sup> Haut Commissariat aux Eaux et Forêts, Agdal-Rabat, Morocco

<sup>e</sup> Zoologisches Forschungsmuseum Alexander Koenig, Adenaueralle 160, D-53113 Bonn, Germany

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## 1. Introduction

Since the seminal work of McArthur and Wilson (1967) on the equilibrium theory of island faunas, colonization and extinction events have remained a focus of research; however, these dynamic events are clearly related to continental islands where the colonization probability and species turnover rates are high (Diamond, 1969). On oceanic islands, and even shelf islands, natural colonization events by terrestrial mammals are so exceptional that local evolution and speciation is more rapid than the arrival of new species (Williamson, 1981). This was the case until at least the end of the last Ice Age, before the arrival of humans. This model is also true for most islands in the Mediterranean basin, as well as the Canary Islands (Juan et al., 2000), situated within the Atlantic Ocean approximately 100 km off the coast of Morocco. Introductions occurred either during periods of low sea level that led to the formation of filter bridges that allowed the passage of certain organisms, or by sweepstake routes that enabled a small number of organisms to cross by rafting (Simpson, 1940). Such jump dispersals, followed by bottlenecks, genetic drift, and new selective conditions, led to pronounced morphological changes exemplified by dwarf elephants, dwarf hippos, and giant rodents (Sondaar, 1977). All of these mammals became extinct during periods of climatic change or following the immigration of newcomers, but the apoc-

alyptic end of most island endemics began with the arrival of humans during the early Holocene (Diamond, 1992). According to Sondaar (1977), the famous dwarfing and gigantism on these islands are in part explained by a predator-free environment.

To our knowledge, in regard to mammals, only four Pleistocene island endemic taxa survived the extinction events in the Mediterranean basin: the Cypriot mouse *Mus cypriacus* (Cucchi et al., 2006), the “Cyprian shrew” *Crocidura suaveolens cypriacus* (Dubey et al., 2007a), the Cretan shrew *C. zimmermanni* (Vogel et al., 1986), and possibly the Sicilian shrew *C. sicula* on the Siculo–Maltese archipelago (Vogel et al., 1990). Interestingly, these small mammals have no morphological peculiarities and were therefore regarded as continental forms introduced by humans. The endemic character of the mammals from Cyprus was discovered only very recently thanks to molecular analyses and dating of the split from continental forms by molecular clock analyses (Cucchi et al., 2006; Dubey et al., 2007a). The Cretan shrew was discovered thanks to the derived karyotype of  $2n = 34$ , not found outside of this island (Vogel, 1986).

In the present study, we focus on the Sicilian shrew *C. sicula* and its relatives on other islands as well as continental Africa. These taxa have a complex history that exemplifies the problem of species assignment on islands. In the 1980s, Kotsakis (1984) discovered a fossil shrew, *Crocidura esuae*, in the Spinagallo cave in Sicily that was considered to be identical to the fossil shrew found in Malta’s Ghar Dalam cave, in both cases associated with dwarf elephants. At that time, the extant shrew of Sicily was assigned by Corbet (1978) to the Greater white-toothed shrews *C. rus-*

\* Corresponding author. Address: Shine Laboratory, School of Biological Sciences, University of Sydney, Hydon-Laurence Building A08, Sydney, NSW 2006, Australia. Fax: +61 2 9351 5609.

E-mail address: [sylvain.dubey@bio.usyd.edu.au](mailto:sylvain.dubey@bio.usyd.edu.au) (S. Dubey).

*sula*, whereas the only extant species on Malta, now restricted to the small island of Gozo, was assigned by Schembri and Schembri (1979) to the lesser white-toothed shrew *C. suaveolens*. The extant shrews were thought to have originated either from Italy or Africa and been introduced by human settlers in the Holocene, as summarized in Fig. 1A.

This view changed when chromosome studies of the extant shrew of Sicily revealed a karyotype of  $2n = 36$ , previously unknown from the Mediterranean region but identical to that of the shrew from Gozo (Vogel et al., 1990). For this taxon, the oldest available name, *Crocicidura sicula* Miller, 1900, was applied, and Hutterer (1990) suggested conspecificity with the fossil *C. esuae*.

The interpretation of an endemic Siculo–Maltese shrew species was subsequently challenged by Sarà (1995). Based on a morphometric study of these shrews and the Canary shrew *C. canariensis*, which all share the same karyotype

(Hutterer et al., 1987), Sarà (1995) concluded that all should be assigned to the Sicilian shrew *C. sicula*, except for the fossil *C. esuae*. This led to a new hypothesis (Fig. 1B) of a late Pleistocene dispersal to the Canary Islands and Sicily over land bridges from an unknown African *C. sicula* population.

The first molecular investigation to be conducted (Vogel et al., 2003) revealed a genetic divergence of Kimura two-parameter distance (K2P) of 14.3% between the shrews of Sicily and those of the Canary Islands, clearly higher than the level of intraspecific populations (Bradley and Baker, 2001). This result led us to suggest a third hypothesis concerning the origin of these island shrews; namely, an African ancestor of both species that is either extinct or survives in an extant species (Fig. 1C). Such a potential ancestor was recently found (Vogel et al., 2006) when a cytotoxic analysis of the Saharan shrew *C. tarfayaensis* revealed an identical karyotype of  $2n = 36$ . The discovery of this shrew opened the way to a new molecular investigation to test not only the hypothesis of a common African ancestor, but also to determine the period of colonization using the molecular clock approach and to develop a plausible scenario of colonization events.

## 2. Materials and methods

### 2.1. Sampling, extraction, and DNA amplification

We analyzed 17 samples of *Crocicidura* from nine different species distributed around the Mediterranean basin and the Canary Islands (Table 1), a more distant crocidurine shrew, *Suncus murinus* was used as outgroup. Most of the shrews analyzed in this study are from the IZEA collection (University of Lausanne, Switzerland). The two sequences of *Crocicidura suaveolens* were taken from Dubey et al. (2006).

DNA extraction was carried out using a QIAamp DNA Mini Kit (Qiagen). Double-stranded DNA amplifications of cytochrome *b* gene were performed with the primer pairs L14841/C4, C1/C2, C3/H15915, and L14841/H15915 (Irwin et al., 1991; Dubey et al., 2006). Amplification conditions consisted of 35 thermal cycles of 30 s denaturation (60 s for the set L14841/H15915) at 94 °C, 45 s annealing (60 s, L14841/H15915) at 50 °C, and 60 s (120 s, L14841/H15915) extension at 72 °C. For details on DNA purification and cycle sequencing see Dubey et al. (2007a).

### 2.2. Phylogenetic analyses

Nucleotide sequences of *cyt-b* gene were edited with Sequence Navigator (Parker, 1997) and aligned by eye. Three methods of phylogenetic analyses were carried out using PAUP\* version 4.0b10 PPC (Swofford, 1998). Tests were conducted on the total fragments (985 bp), all codon positions were used, and trees were rooted using sequence of *S. murinus*. A neighbour-joining (NJ) tree was constructed using Kimura two-parameter genetic distances (Kimura, 1980). This distance was chosen because it allows

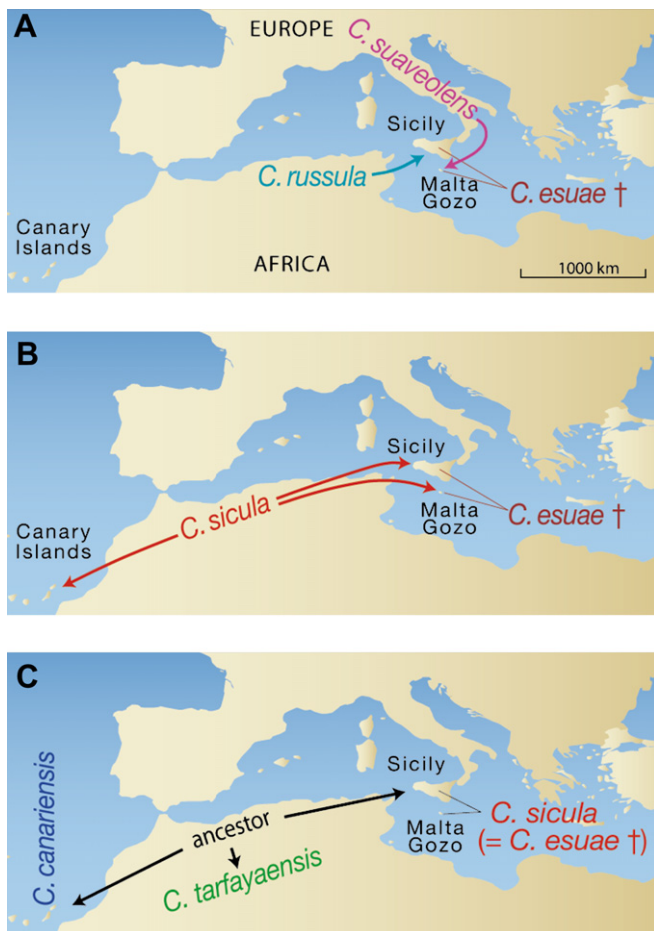


Fig. 1. Hypotheses of the origin of shrews from Sicily, Malta/Gozo, and the Canary Islands. (A) Hypothesis of Kotsakis (1984): the fossil species *C. esuae* was replaced by two introduced species (*C. russula* and *C. suaveolens*). (B) Hypothesis of Sarà (1995): *C. sicula* migrated to the Canary Islands and Sicily from an unknown population in North Africa, replacing the fossil *C. esuae*. (C) Hypothesis of Vogel et al. (2006): *C. sicula* (= *C. esuae*) on Sicily and *C. canariensis* on the Canary Islands are endemic species that evolved from a dispersing North African ancestor that may be related to the recent Saharan shrew *C. tarfayaensis*.

Table 1

Species sequenced and used in the present study (identification number on Fig. 2), collection code, geographic origin of samples, and GenBank accession number

Species	Coll. code	Locality	GenBank
<i>Crocidura canariensis</i> (1)	IZEA 4285	Fuerteventura, Canary Islands, Spain	DQ521042
<i>Crocidura canariensis</i> (2)	LJCC3	Lanzarote, Canary Islands, Spain	DQ975247
<i>Crocidura ichnusae</i>	IZEA 4011	Tunisia	DQ975240
<i>Crocidura leucodon</i>	IZEA 6038	Turkey	DQ975249
<i>Crocidura olivieri</i>	IZEA 3077	Central African Republic	DQ975250
<i>Crocidura russula</i>	IZEA 5936	Spain	DQ975238
<i>Crocidura russula</i>	IZEA 2641	Morocco	DQ975239
<i>Crocidura sicula</i>	IZEA 2943	Sicily, Italy	DQ521040
<i>Crocidura sicula</i>	IZEA 4000	Gozo, Malta	DQ975241
<i>Crocidura suaveolens</i>	IZEA 3191	Spain	AY843491
<i>Crocidura suaveolens</i>	IZEA 7945	Italy	AY843459
<i>Crocidura tarfayensis</i> (1)	IZEA 7807	Morocco	DQ975242
<i>Crocidura tarfayensis</i> (2)	IZEA 7808	Morocco	DQ975243
<i>Crocidura tarfayensis</i> (3)	IZEA 7811	Morocco	DQ975244
<i>Crocidura tarfayensis</i> (4)	IZEA 7809	Morocco	DQ975245
<i>Crocidura tarfayensis</i> (5)	ZFMK 2000.039	Morocco	DQ975246
<i>Crocidura zimmermanni</i>	IZEA 2053	Crete, Greece	DQ521041
<i>Suncus murinus</i>	IZEA V546	India	DQ630386

us to compare our results with others studies. 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. For ML analyses, likelihood ratio tests, implemented with the computer program Modeltest 3.06, were first used to choose the mutation model that best fit the data (36). The GTR+I+G model was selected with base frequencies ( $A = 0.3024$ ,  $C = 0.2978$ ,  $G = 0.1195$ ,  $T = 0.2804$ ) estimated from the data, an unequal distribution of rates at variable sites ( $\alpha = 1.9972$ ), and six different substitution types (rate [A–C] = 1.8238, rate [A–G] = 12.4629, rate [C–T] = 28.7198, rate [A–T] = 2.0043, rate [C–G] = 0.4372, rate [G–T] = 1.0000). Maximum likelihood analyses were then performed, assuming this model, using the heuristic search option with a stepwise addition of sequences. NJ, MP, and ML results were compared for congruence of tree topologies. Bootstrap support values were obtained with 1000 pseudoreplicates for MP, NJ, and ML analyses, and 10 random replicates of stepwise-addition sequences.

### 2.3. Molecular clock analyses

The molecular clock hypothesis was tested following Posada and Crandall (1998) calculating the log likelihood score with molecular clock enforced and comparing it with the log likelihood previously obtained without enforcing the molecular clock. In addition, to identify whether there was heterogeneity in the rates of *cyt-b* substitutions among species, relative-rate tests were conducted between each of them, using RR TREE, version 1.0 (Robinson et al., 1998), which improves the test of Wu and Li (1985). Relative-rate tests were performed on the proportions of synonymous

( $K_s$ ), non-synonymous ( $K_a$ ) substitutions. The Bonferroni correction (Rice, 1989) was applied to deal with statistical artifacts created by multiple comparisons.

Estimation of the divergence time from the molecular data was performed according to a calibration developed previously for the Soricidae by Fumagalli et al. (1999), and that allowed fully coherent results in a biogeographic point of view (see, e.g., Vogel et al., 2003; Brändli et al., 2006; Cosson et al., 2005; Dubey et al., 2006, 2007a,b). This calibration is based on an estimate of 20 million years for the split between Crocidurinae and Soricinae shrews, and it was developed considering the cytochrome *b* sequence divergences based on third position transversions, with a rate of 1.36%/Myr (95% confidence interval: 1.08–1.64%/Myr).

### 3. Results and discussion

For this investigation, based on the sequence of 998 bp of the cytochrome *b* gene, we analyzed the Saharan shrew *C. tarfayaensis* from Morocco, the Sicilian shrew *C. sicula* from Sicily and Gozo, and the Canarian shrew *C. canariensis* from Fuerteventura and Lanzarote. Several other African and three continental European shrew species were included to test possible relationships (see Section 2). The results of this analysis are shown in Fig. 2. The maximum likelihood tree clearly reveals that the  $2n = 36$  chromosome group is an independent clade, separate from all other European species.

Relative-rate tests revealed that there were no significant divergences in evolutionary rate between species; and in addition the likelihood ratio test led to acceptance of the molecular clock hypothesis ( $\chi^2 = 17.62$ ,  $ddl = 15$ ,  $p = 0.28$ ).

The first split separated the Sicilian shrew *C. sicula* from the North African ancestor when the species reached the





of the greater white-toothed shrew *C. russula* (originally described as *C. osorio* from Gran Canaria) was possibly introduced from Spain by the Spanish conquistadors (Vogel et al., 2003). In conclusion, shrews represent a highly interesting model organism in terms of illustrating different processes of island colonization.

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## References

- Bradley, R.D., Baker, R.J., 2001. A test of the genetic species concept: cytochrome-b sequences and mammals. *J. Mammal.* 82, 960–973.
- Brandli, L., Handley, L.-J., Vogel, P., Perrin, N., 2006. Evolutionary history of the greater white-toothed shrew (*Crocidura russula*) inferred from analysis of mtDNA, Y, and X chromosome markers. *Mol. Phylogenet. Evol.* 37, 832–844.
- Censky, E.J., Hodge, K., Dudley, J., 1998. Over-water dispersal of lizards due to hurricanes. *Nature* 395, 556.
- Corbet, G.B., 1978. The Mammals of the Palaearctic Region: A Taxonomic Review. Cornell University Press, British Museum (Natural History), p. 314.
- Cosson, J.F., Hutterer, R., Libois, R., Sara, M., Taberlet, P., Vogel, P., 2005. Phylogeographic footprints of the Strait of Gibraltar and Quaternary climatic fluctuations in the western Mediterranean: a case study with the greater white-toothed shrew *Crocidura russula* (Mammalia: Soricidae). *Mol. Ecol.* 14, 1151–1152.
- Cucchi, T., Orth, A., Auffray, J.C., Renaud, S., Fabre, L., Catalan, J., Hadjisterkotis, E., Bonhomme, F., Vigne, J.D., 2006. A new endemic species of the subgenus *Mus* (Rodentia, Mammalia) on the Island of Cyprus. *Zootaxa* 1241, 1–36.
- Diamond, J.M., 1969. Avifaunal equilibria and species turnover rates on the Channel islands off California. *Proc. Nat. Acad. Sci. USA* 64, 57–63.
- Diamond, J.M., 1992. Twilight of the pygmy hippo. *Nature* 359, 15.
- Dubey, S., Cosson, J.-F., Magnanou, E., Vohralik, V., Benda, P., Frynta, R., Hutterer, R., Vogel, P., 2007a. Mediterranean populations of the Lesser white-toothed shrew (*Crocidura suaveolens* group): an unexpected puzzle of Pleistocene survivors and prehistoric introductions. *Mol. Ecol.* 16, 3438–3452.
- Dubey, S., Cosson, J.-F., Vohralik, V., Krystufek, B., Diker, E., Vogel, P., 2007b. Molecular evidence of Pleistocene bidirectional faunal exchange between Europe and the Near East: the case of the bicolored shrew (*Crocidura leucodon*, Soricidae). *J. Evol. Biol.* 20, 1799–1808.
- Dubey, S., Zaitsev, M., Cosson, J.-F., Abdoukadi, A., Vogel, P., 2006. Pliocene and Pleistocene diversification and multiple refugia in a Eurasian shrew (*Crocidura suaveolens* group). *Mol. Phylogenet. Evol.* 38, 635–647.
- Fumagalli, L., Taberlet, P., Stewart, D.T., Gielli, L., Hausser, J., Vogel, P., 1999. Molecular phylogeny and evolution of Sorex shrews (Soricidae: Insectivora) inferred from mitochondrial DNA sequence data. *Mol. Phylogenet. Evol.* 11, 222–235.
- Hunt, C.O., Schembri, P.J., 1999. Quaternary environments and biogeography of the Maltese Islands. In: Mifsud, A., Savona Ventura, C. (Eds.), Facets of Maltese Prehistory. The Prehistoric Society of Malta, Malta, pp. 41–75, vii + pp. 243.
- Hutterer, R., 1990. Temporal and geographical variation of shrews of the Sicilian-Maltese archipelago since the Pleistocene. *Vie Milieu* 40, 213–217.
- Hutterer, R., Lopez Jurado, L.F., Vogel, P., 1987. The shrews of the eastern Canary Islands: a new species (Mammalia, Soricidae). *J. Nat. Hist.* 21, 1347–1357.
- Irwin, D.M., Kocher, T.D., Wilson, A.C., 1991. Evolution of the cytochrome *b* gene of mammals. *J. Mol. Evol.* 32, 128–144.
- Juan, C., Emerson, B.C., Oromı, P., Hewitt, G.M., 2000. Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. *Trends Ecol. Evol.* 15, 104–109.
- Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120.
- Kotsakis, T., 1984. *Crocidura esui* n. sp. (Soricidae, Insectivora) du Pleistocene de Spinagallo (Sicilie orientale, Italie). *Geol. Rom.* 23, 51–64.
- Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400, 652–655.
- McArthur, R.H., Wilson, E.O., 1967. The Theory of Island Biogeography. Princeton Univ. Press, Princeton, NJ.
- Nagel, A., 1977. Torpor in the European white-toothed shrews. *Experientia* 33, 1455–1456.
- Parker, S.R., 1997. Sequence Navigator. Multiple sequence alignment software. *Methods Mol. Biol.* 70, 145–154.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Rice, W.R., 1989. Analyzing tables of statistical tests. *Evolution* 43, 223–225.
- Robinson, M., Gouy, M., Gautier, C., Mouchiroud, D., 1998. Sensitivity of the relative-rate test to taxonomic sampling. *Mol. Biol. Evol.* 15, 1091–1098.
- Sara, M., 1995. The Sicilian (*Crocidura sicula*) and the Canary (*C. canariensis*) shrew (Mammalia, Soricidae): peripheral isolate formation and geographic variation. *Boll. Zool.* 62, 173–182.
- Schembri, P.J., Schembri, S.P., 1979. On the occurrence of *Crocidura suaveolens* Pallas, (Mammalia, Insectivora) in the Maltese Islands with notes on other Maltese shrews. *Cent. Medit. Nat.* 1, 18–21.
- Simpson, G.G., 1940. Mammals and land bridges. *J. Wash. Acad. Sci.* 30, 137–163.
- Sondaar, P.Y., 1977. In: Hecht, M.K., Goodey, P.C., Hecht, B.M. (Eds.), Major Patterns in Vertebrates Evolution. Plenum, New York, pp. 671–707.
- Sparti, A., 1990. Comparative temperature regulation of African and European shrews. *Comp. Biochem. Physiol.* 97A, 391–397.
- Steiner, C., Hobson, A., Favre, P., Stampfli, G.M., Hernandez, J., 1998. Mesozoic sequence of Fuerteventura (Canary Islands): witness of early Jurassic sea-floor spreading in the central Atlantic. *GSA Bull.* 10, 1304–1317.
- Swofford, D.L., 1998. PAUP\*. Phylogenetic Analysis Using Parsimony (\* and other Methods). Version 4.0b1. Sinauer Associates, Sunderland, MA.
- Vogel, P., 1976. Energy consumption of European and African shrews. *Acta Theriol.* 21, 195–206.
- Vogel, P., 1986. Der Karyotyp der Kretaspitzenmaus, *Crocidura zimmermanni* Wettstein, 1953 (Mammalia, Insectivora). *Bonn. Zool. Beitr.* 37, 35–38.
- Vogel, P., Maddalena, T., Catzeflis, F., 1986. A contribution to taxonomy and ecology of shrews from Crete and Turkey (*Crocidura zimmermanni* and *C. suaveolens*). *Acta Theriol.* 39, 537–545.
- Vogel, P., Cosson, J.-F., Lopez Jurado, L.F., 2003. Taxonomic status and origin of the shrews (Soricidae) from the Canary Islands inferred from a mtDNA comparison with the European *Crocidura* species. *Mol. Phylogenet. Evol.* 27, 271–282.
- Vogel, P., Mehmeti, A.-M., Dubey, S., Vogel-Gerber, C., Koyasu, K., Ribı, M., 2006. Habitat. Morphology and karyotype of the Saharan

- shrew *Crocidura tarfayaensis* (Mammalia: Soricidae). *Acta Theriol.* 51, 353–361.
- Vogel, P., Schembri, P.J., Borg, M., Sultana, J., 1990. The shrew (*Crocidura* sp.) of Gozo, a probable survivor of the Pleistocene fauna of Mediterranean Islands. *Z. Säugetierkunde* 55, 357–359.
- Williamson, M., 1981. *Island Populations*. Oxford Univ. Press, Oxford, p. 286.
- Wu, C.I., Li, W.H., 1985. Evidence for higher rates of nucleotide substitutions in rodents than in man. *Proc. Natl. Acad. Sci. USA* 82, 1741–1745.