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Euscorpius sicanus (Scorpiones: Euscorpiidae) from Tunisia: DNA barcoding confirms ancient disjunctions across the Mediterranean Sea

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Abstract

We used a DNA barcoding marker (mitochondrial *cox1*) to investigate the controversial natural occurrence of *Euscorpius sicanus* (C.L. Koch) in North Africa. We tested this hypothesis by comparing a sample collected from a mountain in Tunisia to disjunct populations in Sardinia, Malta, and Greece. Using these samples, and a few additional *Euscorpius* spp. from southern Europe as outgroups, we reconstructed the maternal phylogeny. We then used a molecular clock to place the phylogeny in a temporal context. The Tunisian sample grouped closest to a specimen from Sardinia, with both being more distantly related to *E. sicanus* from Malta, which is known to be genetically similar to samples from Sicily. Molecular clock estimates suggest an ancient disjunction across the Mediterranean Sea, with the divergence between samples from Sardinia and Tunisia estimated to have occurred between the Late Miocene and late Pliocene. The divergence date (mean = 5.56 Mya) closely corresponds with the timing of a sudden refilling of the Mediterranean Sea after it had evaporated during the Messinian salinity crisis. This rapid influx of water, in conjunction with tectonic activity, could have sundered connections between *Euscorpius* in North Africa and what is now the island of Sardinia. These results provide yet another case in which DNA barcodes have proven useful for more than just identifying and discovering species.

Keywords: Zanclean Flood, post-Messinian Flood, Messinian salinity crisis, molecular clock, *cox1*, mitochondrial DNA, barcode.

Introduction

Most of North Africa's rich scorpion fauna, which primarily consists of members of family Buthidae, is relatively well known (Vachon, 1952; Kovařík, 2006). However, species of the genus *Euscorpius* Thorell from North Africa have not been adequately characterized, even though records from the region date back to more than 100 years. Original reports documented "*E. carpathicus* (L.)" from isolated localities along the North African coast in Tunisia, Libya, and Egypt (Fet *et al.*, 2003). Many *Euscorpius* spp. are known to disperse with humans (Fet *et al.*, 2006), so the legitimacy of these reports has been controversial. Some introduced species, such as *E. italicus* (Herbst) in Yemen and Iraq, are even known to establish reproducing populations in non-native areas (Fet & Kovařík, 2003). Furthermore, some of the African populations of *Euscorpius* are represented by *E. flavicaudis* De Geer, a potential postglacial relict that presumably represents a recent introduction (Gantenbein *et al.*, 2001). As a result, when specimens identified as "*E. carpathicus sicanus* (C.L. Koch)" were reported from coastal regions of North Africa, it was brought into question whether the specimens were introduced from the northern Mediterranean, or if they represented an isolated relict population (Fet *et al.*, 2003).

Based on morphological and molecular characters, *E. carpathicus sicanus* was recently elevated to *E. sicanus* (C.L. Koch), and the degree of intraspecific genetic structure suggested that it might even represent a species complex (Fet *et al.*, 2003). With the type locality from Sicily, and other populations occupying portions of southern Italy, Sardinia, central and southern Greece, Malta, Madeira, and several North African localities, the geographic range of *E. sicanus* is highly fragmented by the Mediterranean Sea. Genetic samples (mitochondrial DNA) of *E. sicanus* were studied from a number of localities in Italy (including Sicily and Sardinia), Greece, and Malta (Fet *et al.*, 2003; Salomone *et al.*, 2007), but no African populations were analyzed.

In 2008, we (P. Stoev & N. Akkari) collected new *Euscorpius* specimens from North Africa that were identified in 2009 as *E. sicanus* (det. V. Fet). The scorpions were collected from Jebel Zaghouan (Fig. 1), a mountain range situated in northeastern Tunisia that reaches an elevation of 1,295 meters at Ras el Gossa. The mountain range is within the Semi-arid bioclimatic zone (Emberger, 1966) characterized by temperate winters and an average annual precipitation of 450–500 mm. Jebel Zaghouan lies in the major structural NE-SW lineament that was active since the Jurassic and is characterized by a predominance of red soils developed on Jurassic limestone. The vegetation near the summit is mostly dominated by *Quercus coccifera* L., the slopes are characterized by *Ceratonia siliqua* L., *Olea europaea* L. and *Pistacia lentiscus* L., and the shrub floor is composed mainly of *Tetraclinis articulata* (Vahl), *Phillyrea angustifolia* L., *Lavandula* sp. and *Thymus capitatus* (L.).

This non-desert habitat suggests that *Euscorpius* from Jebel Zaghouan could potentially represent native populations. We tested this hypothesis by comparing a DNA barcode (mitochondrial *cox1*) from one of the *E. sicanus* specimens (Fig. 2) collected from the Jebel Zaghouan of Tunisia with barcodes obtained from *E. sicanus* from Greece, Malta, and Sardinia, as well as outgroup congeneric species from southern Europe (Fig. 3). We used these data to investigate the matrilineal phylogeny, and to estimate divergence dates between mitochondrial lineages. If *E. sicanus* was recently introduced to North Africa, then we would expect the barcode from the Tunisian sample to be similar to that from Sardinia, Malta, or Greece. Alternatively, if the Tunisian specimen represents a relict population, then we would expect the barcode to be quite different than the *E. sicanus* barcodes from Greece, Sardinia, and Malta. Furthermore, molecular clock

estimates should indicate an ancient (Pre-Pleistocene) divergence between the sample from Tunisia, and those from Greece, Malta, and Sardinia.

Fig. 1. A view of Jebel Zaghouan Mts. in Tunisia where *Euscorpius sicanus* (C.L. Koch) was collected. Photo: N. Akkari.

Material and Methods

We analyzed 10 sequences obtained at Marshall University (V. Fet; two specimens from Greece) and the Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, Canada (G. Blagoev; all other specimens). Label data of the specimens used for DNA analysis are listed below. All sequence data were submitted to GenBank and can be accessed through BOLD (http://www.boldsystems.org, Ratnasingham & Hebert, 2007) under project "Scorpions of the Ancient Mediterranean 2 (AMSCO)". Voucher specimens are in a private collection of V. Fet and in the Biodiversity Institute of Ontario.

Material Examined: ***Euscorpius sicanus*** (C.L. Koch, 1837): GREECE, Thessaly, Mt. Pelion, Visitsa, 39°20'N, 23°08'E, 7 May 2001, leg. V. Fet, VF-0454 (JX414017); Thessaly, Mt. Ossa (Kissavos), Spilia, 39°47'45"N, 22°38'49"E, 9 May 2001, leg. V. Fet, VF-0455 (JX414018); ITALY, Sardinia, S. Niccolo Gerrei, near Grotta Saturru, 39.49816°N, 09.31503°E, 395 m, April 2006, leg. A. v.d. Mejden, VF-0789, AMSCO052-10 (JX133089). MALTA, Buskett Gardens, 35°51'41"N, 14°23'56"E, 17 September 2001, leg. P. Schembri, VF-0792, AMSCO053-10 (HM418288). TUNISIA, Zaghouan Governorate, Jebel Zaghouan Mts., along the trek, 36°22.423'N, 10°06'E to 36°22.924'N, 10°06.789'E, 650-780 m a.s.l., mixed forest, March 2008, leg. P. Stoev & N. Akkari, VF-0793, AMSCO054-10 (HM418289). ***Euscorpius carpathicus*** (L., 1767): ROMANIA, Caraş-Severin County, Băile Herculane, 44°52'43"N, 22°24'51"E, 4 June 2008 (F. Šťáhlavský), VF-0768, AMSCO044-10 (HM418284). ***Euscorpius concinnus*** (C.L. Koch, 1837): FRANCE, Alpes-Maritimes, Grasse, 43°40'N, 06°55'E, September 2004, leg. E. Ythier, VF-0782, AMSCO049-10 (HM418287). ***Euscorpius hadzii***

Caporiacco, 1950: BULGARIA, Blagoevgrad District, Gorna Breznitsa, 41°45'N, 23°07'E, 27 May 2005, leg. V. Fet & D. Dobrev, VF-0798, AMSCO059-10 (HM880289); MONTENEGRO, Budva District, Visnjevo, 42°17'52"N, 18°46'37"E, sea level, 29 October 2005, leg. F. Franeta, VF-0807, AMSCO066-10 (HM418296). ***Euscorpius flavicaudis*** (DeGeer, 1787): FRANCE, Vaucluse, Pernes-les-Fontaines, 43°59'55"N, 05°03'35"E, 230 June 2007, leg. V. Fet, VF-0700, AMSCO001-10 (HM418267).

NOTE. Additional specimens of *E. sicanus* (not included in the DNA study) were collected from the same area by us (N.A. and P.S.): 2 juv., NE Tunisia, Zaghouan Governorate, Jebel Zaghouan Mts., surroundings of a small limestone cave 'Gouffre du courant d'air', 36°21.980'N, 10°05.513'E, 561 m a.s.l., *Quercus ilex, Pistacia lentiscus, Jasminum fruticans*, under stones and leaf litter, 17 March 2008, N. Akkari & P. Stoev leg.

Fig. 2. Dorsal view of the habitus of *Euscorpius sicanus* (C.L. Koch) female collected from Tunisia for which a DNA barcode was sequenced and analyzed in this study. Note a weak darker reticulation pattern on carapace, typical of *E. sicanus*. Photo: P. Stoev and R. Bekchiev.

Molecular Techniques: The V.F. lab used a DNeasy Blood & Tissue Kit (Qiagen) to isolate genomic DNA from leg or muscle tissue. A portion of the mitochondrial protein-coding gene cytochrome oxidase subunit I (*cox1*) was then amplified and sequenced using primers Nancy (Simon *et al.*, 1994) and LCO (5' – GGT CAA CAA ATC ATA AAG ATA TTG G – 3') following protocols outlined by Simon *et al.* (1994).

Barcodes (*cox1* sequences) generated at the Canadian Centre for DNA Barcoding, University of Guelph, were obtained using standard protocols for DNA extraction, polymerase chain reaction (PCR) and sequencing (Ivanova *et al.*, 2006, DeWaard *et al.*, 2008). In brief, tissue from a single scorpion leg was used for extraction of genomic DNA using a 96 AcroPrep™ 1 ml filter plate (PALL) with 3.0 µm Glass fiber. DNA was eluted in 40 µl of dH2O. Full-length *cox1* barcodes (649 bp) were amplified using two newly designed primer sets (Ivanova, unpublished): ScorpF1_t1 (5' – TGTAAAACGACGG CCAGTTTTCTACTAATCAYAAAGAYATTGG – 3') and ScorpR1_t1 (5' – CAGG AAACAGCTATGACGGRTGTCCAAAAAAYCAAAAYAAATG – 3'). All PCR products were sequenced bi-directionally on an ABI3730XL using the primer pair of

M13F and M13R (Messing, 1983). The forward and reverse sequences were used to generate a single consensus sequence using CodonCode Aligner v. 3.0.2 (CodonCode Corporation). *Cox1* was chosen because it is commonly used in barcoding and has been demonstrated as highly effective in discriminating among insect (Zhang & Hewitt, 1997; Foottit *et al.*, 2009; Zhou *et al.*, 2009) and arachnid species (e.g. Barrett & Hebert, 2005; Thomas & Hedin, 2008; Wang *et al.*, 2008; Robinson *et al.*, 2009; Graham *et al.*, 2012; Sousa *et al.*, 2012).

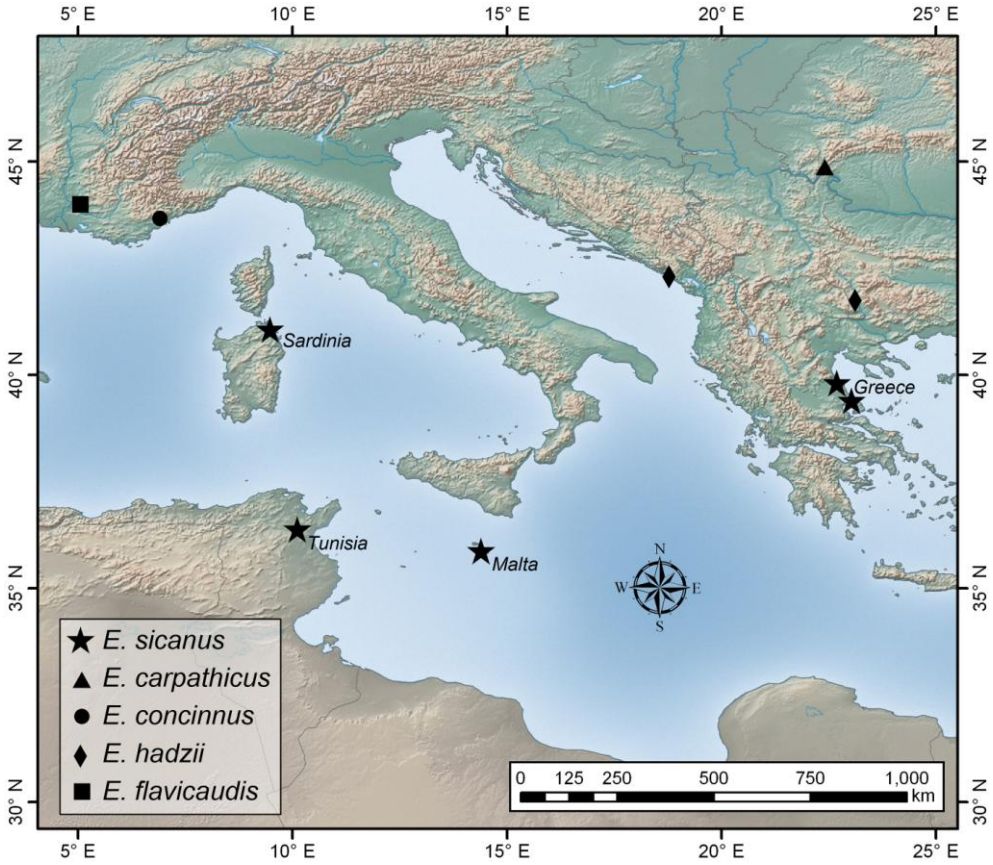

Fig. 3. Map depicting locations for *Euscorpius* Thorell specimens used in this study.

Phylogenetic analysis and divergence time estimation: Sequences were aligned using SEQUENCHER v. 4.9 (Gene Codes Corp., Inc., Ann Arbor, MI, USA) and verified by eye. The alignment was then imported into the program MEGA 5 (Tamura *et al.*, 2011) which was used to find a suitable model for nucleotide substitution through the Akaike Information Criterion (Posada, 2008). The program chose the GTR+I+G model, so phylogeny was then estimated via this model and the criterion of Maximum Likelihood (ML) with 1,000 bootstrap replicates, again using MEGA 5.

We also estimated tree topology and divergence dates for the *Euscorpius* samples in BEAST v. 1.5.3 (Drummond & Rambaut, 2007) using the same substitution model. We applied the Yule tree prior and a mutation rate of 0.007 substitutions/site/million years for *cox1* (Gantenbein *et al.*, 2005), and set the mean standard deviation to 0.003 to accommodate a similar rate estimated for 16S (Gantenbein & Largiadèr, 2002). Analyses were conducted for 40 million Markov Chain Monte Carlo generations, sampling every 1,000 generations, and with the first 20% of the generations discarded as burn-in. We used LOGCOMBINER v. 1.6.1 (Drummond & Rambaut, 2007) to combine trees and parameter estimates, and TRACER to examine the estimated sample sizes (ESS) to avoid poor estimates of the parameters (ESS < 200).

Results

ML and Bayesian analyses produced identical topologies. We chose to present the Bayesian tree with both posterior probabilities and bootstrap support values for each node (Fig. 4, Table 1). A total of 6 out of 9 nodes were supported under BI (PP > 0.9), and 5 nodes were supported by the ML (bootstrap values > 0.75).

Table 1. Molecular clock estimates and support values for nodes presented in Fig. (4).

Node	Age	95% HPD	Posterior Probability	ML Bootstrap (%)
a	25.57	14.32 - 39.56	1	100
b	15.28	9.74 - 22.7	1	100
c	12.65	8.23 - 18.54	0.74	59
d	9.73	5.38 - 15.4	0.98	64
e	8.59	5.52 - 12.25	1	81
f	7.18	4.44 - 10.13	0.66	37
g	5.56	3.29 - 8.14	0.81	38
h	4.67	2.7 - 6.99	1	98
i	3.57	1.72 - 5.67	1	79

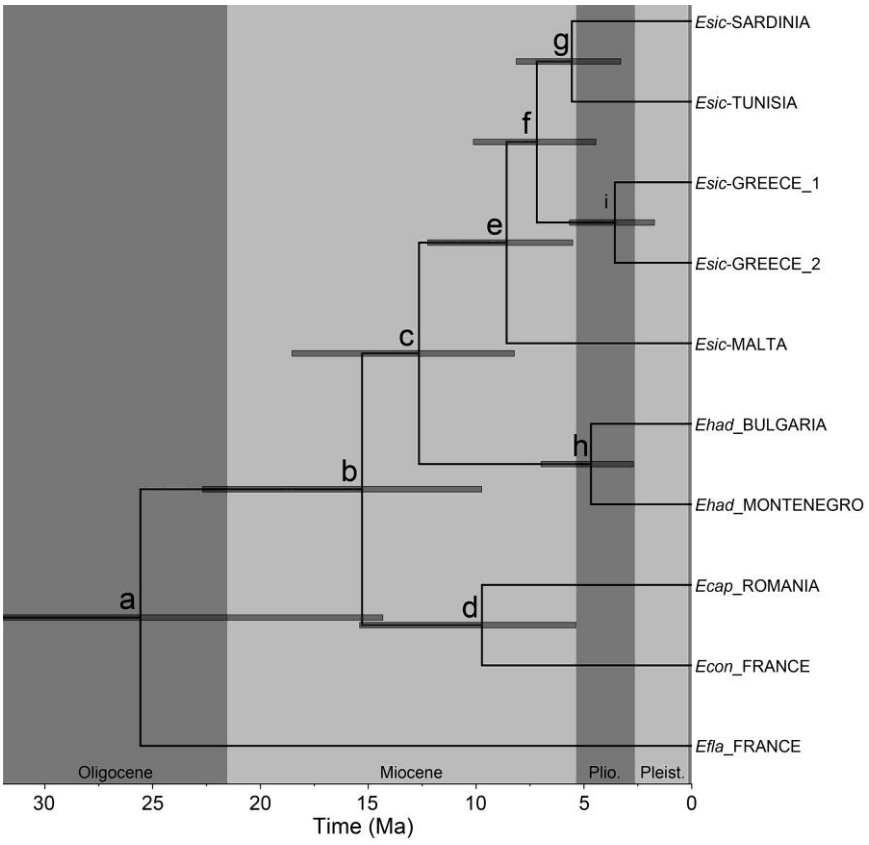

Fig. 4. Ultrametric tree estimated in BEAST. Mean divergence times, 95% highest posterior densities (HPD), and support values for nodes (a - i) are presented in Table 1. Dark bars represent variation (95% HPD) for the age estimate of each node.

The tree is rooted with *Euscorpius flavicaudis*, which was estimated to have diverged from the remaining samples sometime between the mid-Oligocene and mid-

Miocene. The next oldest node diversified between the Late Oligocene and Middle Miocene, resulting in two clades: one that was strongly supported by BI but weakly supported by ML consisting of *E. concinnus* and *E. carpathicus*, and another showing sister relationships between *E. hadzii* and *E. sicanus*, which is supported by morphological data (Fet & Soleglad, 2002, 2007; Fet *et al.*, 2003). The *E. concinnus* and *E. carpathicus* were estimated to have diverged in the Middle to Late Miocene. Within the other clade, *E. hadzii* and *E. sicanus* were estimated to have split sometime in the Middle Miocene. Of the *E. sicanus*, the specimen from Malta was most basal and estimated to have diverged from the rest between the Middle and Late Miocene. Of the remaining *E. sicanus*, two specimens from Greece (eastern Thessaly) formed a strongly supported group that was predicted to have diverged from the Late Miocene to early Pleistocene. Although poorly supported, the specimens from Sardinia and Tunisia grouped together in both analyses and were predicted to have diverged from the other *E. sicanus* in the Late Miocene to early Pliocene. The Tunisia specimen was estimated to have diverged from the specimen from Sardinia sometime between the Late Miocene and late Pliocene, with a mean divergence date estimate of 5.56 Ma (Table 1).

Discussion

In a review of *E. sicanus*, Fet *et al.* (2003) wrote that "No DNA is available from the northern African enclaves yet; it remains to be seen if these are true relict populations or if they have been introduced via human activity." The analyses presented herein support the former hypothesis, that North African *E. sicanus* from Tunisia are genetically distinct and represent a relict population. Our sample of *E. sicanus* from Tunisia grouped most closely with a sample from Sardinia. Both Sardinia and Tunisia samples were more distantly related to samples from Greece and Malta. Molecular dating estimated samples from Sardinia and Tunisia to have diverged between the Late Miocene (3.29 Mya) and early Pliocene (8.14 Mya), with a mean estimate near the Mio-Pliocene transition (5.56 Mya), suggesting that the disjunction across the Mediterranean Sea is quite ancient (Fig. 4, Table 1). Intriguingly, this timeframe very closely matches that of a widespread drying and refilling of the Mediterranean Basin in the late Miocene (more precisely the Messinian).

Approximately 5.96 Mya, marine gateways between the Atlantic Ocean and Mediterranean Sea closed due to uplift along the African and Iberian continental margins (Duggen *et al.*, 2003). This resulted in a pervasive desiccation of the Mediterranean Basin known as the 'Messinian salinity crisis', which was one of the most dramatic earth history events during the Cenozoic era (Krijgsman, 2002). Evaporation of the Mediterranean Sea is thought to have allowed many terrestrial organisms that were previously isolated by marine waters (e.g. Martín-Piera & Sanmartín, 1999; Sanmartín, 2003; Wilke, 2003), to more easily disperse throughout the region. Tectonic subsidence then allowed Atlantic water to make its way through the Gibralter Strait at 5.33 Mya. This refilling of the Mediterranean Basin, known as the 'Zanclean' or 'post-Messinian' flood, then appears to have caused vicariance between terrestrial organisms in North Africa and Europe (Sanmartín, 2003). Such a scenario could account for the genetic divergence between *E. sicanus* from Tunisia and Sardinia. Although we have not studied samples from the Italian mainland (Apennine Peninsula), paleogeographic reconstructions suggest that terrestrial connections occurred between Italy, North Africa, Sicily, Sardinia, and Corsica until the Late Miocene or Pliocene (Rosenbaum & Lister, 2002). Therefore, *E. sicanus* may have dispersed between these regions, which may have been made even easier during the Messinian salinity crisis. Increased longitudinal crustal extension could have then worked synergistically with the refilling of the Mediterranean

basin to effectively sever land connections between our samples from Sardinia and Tunisia, which is concordant with our estimated divergence dates (Fig. 4, Table 1).

If the Zanclean flood was responsible for vicariance in *Euscorpius*, then our rate-calibrated molecular clock was remarkably accurate. Therefore, for similarly distributed taxa (in North Africa and Sardinia) that lack reliable rates, we propose that the Zanclean flood could potentially be used as an incredibly precise geologic calibration. Paleogeographic events like uplift and marine transgressions have commonly been used to date vicariant events, but these events happen gradually and the actual timing of the reduction in gene flow cannot be pinpointed. However, the Zanclean Flood is thought to have filled the Mediterranean in 2 months to 2 years (Garcie-Castellanos *et al.*, 2009), and as similarly proposed for river capture and reversals with freshwater-limited organisms (Waters *et al.*, 2007), the event could potentially be used as a 'sharp' vicariant event, allowing for more precise calibrations. Other authors have already used this sharp vicariant event to calibrate molecular clocks for organisms in the eastern Mediterranean, as it is thought to have isolated populations on Crete and Cyprus (e.g. Beerli *et al.*, 1996; Gantenbein & Keightley, 2004; Lymberakis *et al.*, 2007; Akin *et al.*, 2010; Kornilios *et al.*, 2012). As far as we are aware, however, this method has not yet been employed for organisms from Tunisia and Sardinia.

The placement of our sample from Malta as the most basal lineage within *E. sicanus* is curious. Based on mtDNA data from 16S (Fet *et al.*, 2003), the same specimen is most closely related to samples from Nebrodi, Sicily, which is the type locality for *E. sicanus*. Although Malta is closer to Sicily than Sardinia and Tunisia, this relationship is somewhat surprising when considering earth history. As mentioned above, land connections occurred between Sardinia, Corsica, Sicily, and Tunisia until the Late Miocene to early Pliocene. Although an underwater ridgeline connects the Maltese Islands with Sicily and Tunisia, paleogeographic reconstructions suggest that the archipelago may have remained insular for at least several million years longer (Rosenbaum & Lister, 2002). Therefore, the Maltese Islands could have been colonized by mainland populations of *E. sicanus* that dispersed to the islands prior to the Messinian salinity crisis. Alternatively, *E. sicanus* could have colonized the island of Malta and dispersed to the mainland, probably Sicily, where it may now occur in sympatry with other lineages (represented by our sample in Sardinia) that diverged during the Zanclean Flood. Additional sampling along the along the Apennine Peninsula, Sicily, and the remaining Maltese Islands would be needed to address this hypothesis.

Whatever the mechanism, DNA barcodes imply that North African populations of *E. sicanus* were probably not recently introduced and instead represent an ancient and isolated natural population. If *E. sicanus* had recently colonized the area via human introduction, then the *cox1* barcode should have been similar to those from *E. sicanus* collected in Malta, Greece, or Sardinia, from which the Tunisian population would have most likely been founded. However, we recognize that our sampling is limited, especially in Italy, and that additional cryptic lineages could occur within the species, so recent colonization of North Africa should not be completely ruled out. Furthermore, the age of the intraspecific lineages recovered in *E. sicanus* (some with estimates in the Miocene) suggest that the species might actually represent a cryptic species complex, calling attention to the need for a rigorous and comprehensive assessment of the genus *Euscorpius*. To date, most systematic studies of *Euscorpius* have focused on western Mediterranean and central European species (Gantenbein *et al.*, 2000, 2001; Fet *et al.*, 2003; Salomone *et al.*, 2007). However, recent work has revealed that *Euscorpius* is most diverse in the poorly studied eastern Mediterranean, especially the Balkans, the Aegean region, and Anatolia (Fet *et al.*, in progress). Finally, our analyses provide yet another

example of how DNA barcodes can be used for more than just identifying and discovering species (Hebert *et al.*, 2003; Stoeckle, 2003), and that 'sharp' vicariant events like the Zanclean Flood may be useful for fine-tuning molecular clocks.

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