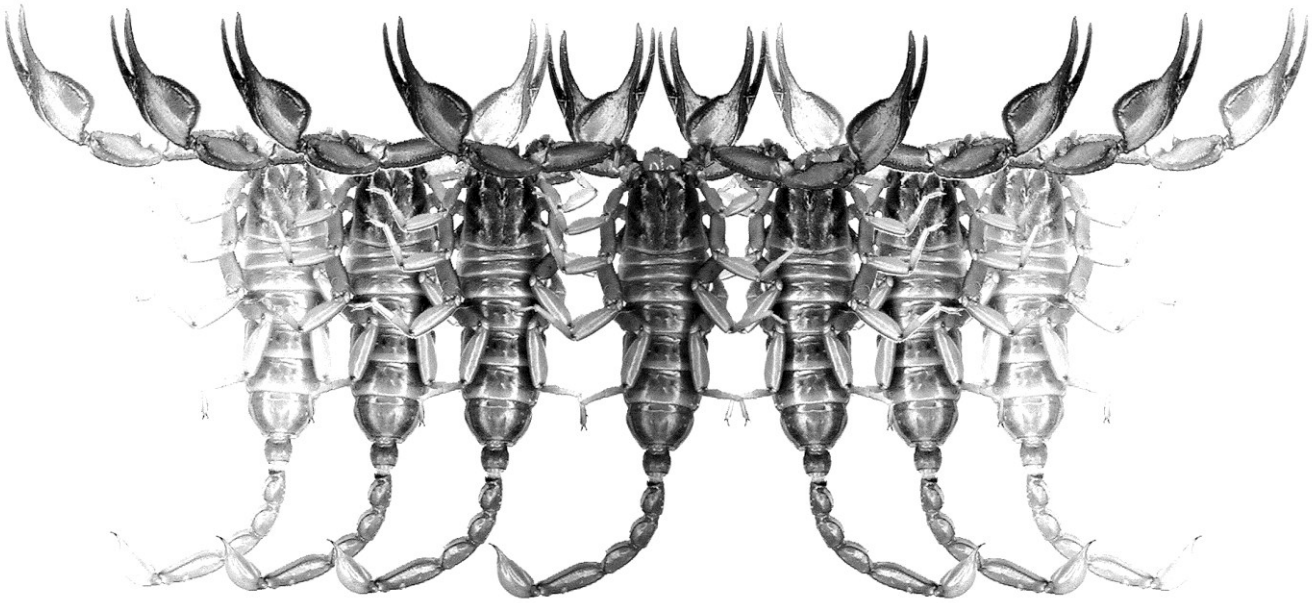
Euscorpius

Occasional Publications in Scorpiology



DNA Barcoding Indicates Hidden Diversity of *Euscorpius* (Scorpiones: Euscorpiidae) in Turkey

Victor Fet, Matthew R. Graham, Gergin Blagoev, Ayşegül Karataş & Ahmet Karataş

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Euscorpius — Occasional Publications in Scorpiology. 2016, No. 216

DNA barcoding indicates hidden diversity of *Euscorpius* (Scorpiones: Euscorpiidae) in Turkey

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Summary

The Anatolian fauna of the genus *Euscorpius* (Scorpiones: Euscorpiidae) is in the process of reassessment. Twelve species of this genus are currently recognized for Anatolia, of which seven have been recently described on the basis of morphology. We demonstrate additional cryptic diversity in Anatolian *Euscorpius* by applying molecular markers (mitochondrial *COI* and *16S* rDNA genes) from 14 populations, of which 13 were morphologically characterized by "*em*=3," a phenotypic marker on the pedipalp patella. All studied Anatolian forms are strongly supported as a single clade compared to the European (from the Alps to the Balkans) taxa of the subgenus *Alpiscorpius*. Of these, six are assigned to known species (*E. ciliciensis*, *E. mingrelicus, E. eskisehirensis, E. phrygius,* and *E. uludagensis*); and three (Aksaray, Ankara, Sakarya) are closely related to a clade containing *E. phrygius* and *E. uludagensis*. Four clades represent undescribed taxonomic forms, possibly of species level: Balikesir/Canakkale (Kazdağları National Park), Konya, Denizli, and Trabzon, all with *em*=3. Another putative species from Kayseri Province, Aladağlar (=Antitaurus) Mts., is related to (*E. ciliciensis* + *E. eskisehirensis*) clade; however, it exhibits *em*=4, which appears to be the first case of reversal in this important trait for the genus *Euscorpius*.

Introduction

The taxonomy of Anatolian scorpions of the genus *Euscorpius* is being reassessed. At present, 12 valid species are recognized from the territory of Turkey (Tropea et al., 2014, 2015; Tropea & Yağmur, 2015). Historically, only two species, *Euscorpius* (*Polytrichobothrius*) *italicus* (Herbst, 1800) (Black Sea coast) and *E. mingrelicus* (Kessler, 1876) were long considered valid for Turkish fauna (Kinzelbach, 1975, 1982). However, various pilot studies indicated that the species diversity is much higher than just two taxa, and modern molecular techniques are beginning to delimit cryptic *Euscorpius* species (Parmakelis et al., 2013).

Forms of "*Euscorpius mingrelicus* complex" were traditionally diagnosed by having three trichobothria in the external patellar *em* series, a well-known phenotypic marker that applies currently to subgenus *Alpiscorpius* Gantenbein et al., 1999 (although it is also independently derived in some other clades of genus *Euscorpius*). Fet (1985) studied the types of the old species *E. ciliciensis* Birula, 1898 from Bolkar Mts.; and Fet et al. (2003a) published the first pilot DNA phylogeny, based on *16S rRNA* markers, for two populations from Anatolia, then tentatively assigned to *E. ciliciensis*.

Recently, Tropea et al. (2015c) revised and outlined four species of the genus *Euscorpius* with *em*=3 from Anatolia, which formerly were included under *E. mingrelicus*, limiting and clarifying the status of all subspecies described from Turkey, especially the unclear taxa of Lacroix (1995). They confirmed species status of four taxa: *Euscorpius mingrelicus* (Kessler, 1876); *E. ciliciensis* Birula, 1898; *E. phrygius* Bonacina, 1980 (=*E. mingrelicus ollivieri* Lacroix, 1995); and *E. uludagensis* Lacroix, 1995 (=*E. mingrelicus legrandi* Lacroix, 1995). In addition, most recently two new "*em*=3" species were described: *Euscorpius arikani* Yağmur et Tropea, 2015 from Antalya Province (Yağmur & Tropea, 2015), and *E. eskisehirensis* Tropea et Yağmur, 2015 from Eskişehir Province (Tropea & Yağmur, 2015.

Therefore, material published so far outlines in Anatolia six species with *em*=3 (Tropea et al., 2015c; Tropea & Yağmur, 2015a, 2015b). These studies have not yet included a number of natural *Euscorpius* populations found elsewhere in Anatolia, especially in the

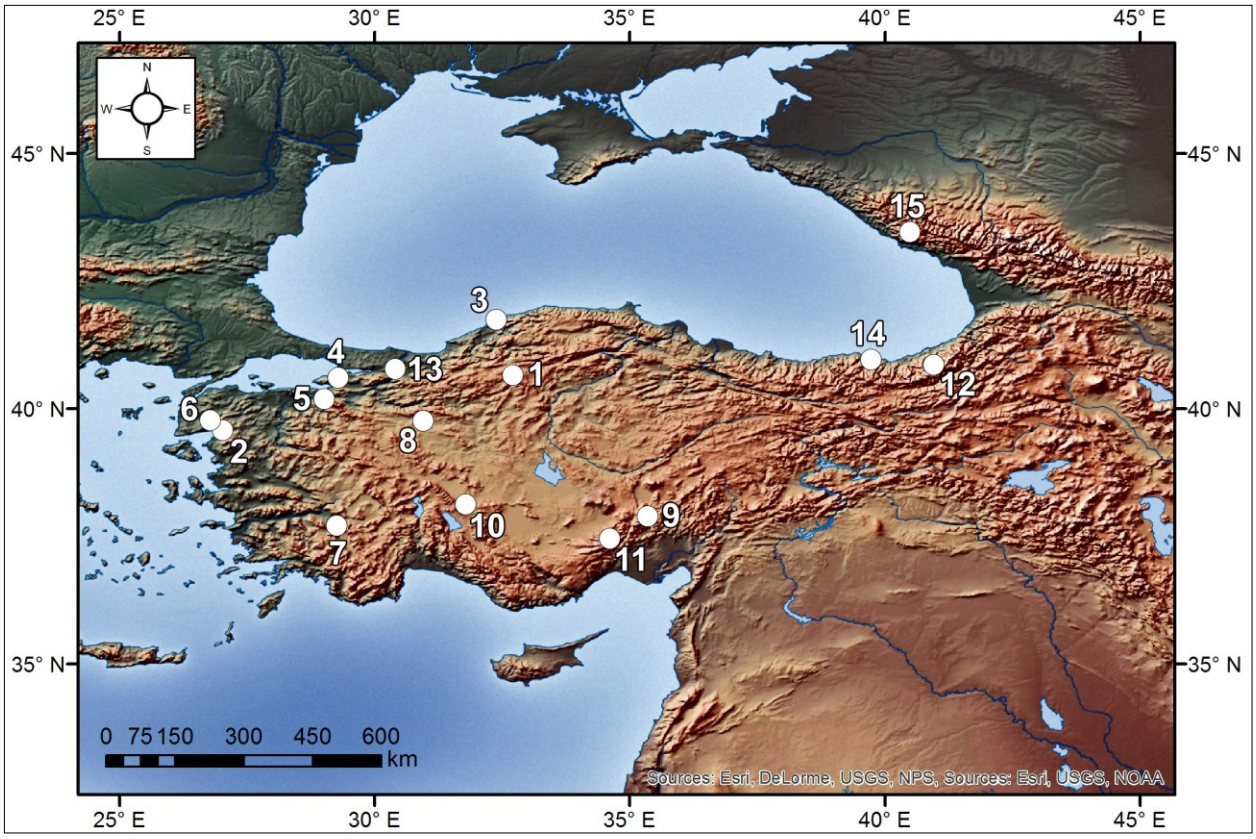

Figure 1: Distribution of 15 populations of Anatolian-Caucasian *Euscorpius* used in the present study.

western part of the peninsula. Specimens of three of these localities from Western Anatolia were listed already by Vachon (1951) (as "*E. germanus ciliciensis*") from Eğridir (Cire pinari, 1000 m; Akpunar pinari, 1000 m), "Honaz Dagi, near Denizli, 1000-1500 m and 2000 m", and "Acipayam [SSE Denizli]" (Vachon, 1951: 342; Kinzelbach, 1975, 32: Abb. 16). Specimens from these locations were collected by Dr. Curt Kosswig in 1945-46. Vachon (1951) commented that these populations could belong to a variety of "*E. germanus ciliciensis*" or to another subspecies.

The "*em*=3" forms are not the only *Euscorpius* inhabiting Anatolia. Karataş (2006) reviewed all Anatolian forms with *em*=4, addressed at the time as "*Euscorpius carpathicus*". These, as discovered later, mostly belonged to undescribed taxa. Some of these populations have been mentioned earlier in literature (Vachon, 1951; Kinzelbach, 1975). Recently, five Anatolian forms, all with *em*=4, have been described as separate species: *Euscorpius avcii* Tropea et al., 2012 (Aydın Province); *E. gocmeni* Tropea et al., 2014 (Antalya Province); *E. koci* Tropea et Yağmur, 2015 (Mersin Province); *E. lycius* Yağmur et al., 2013 (Muğla Province); and *E. rahsenae* Yağmur et Tropea, 2013 (Bursa Province) (Tropea et al., 2012, 2014; Yağmur et Tropea, 2013; Yağmur et al., 2013; Tropea & Yağmur, 2015). Of these, DNA markers are available only for *Euscorpius avcii*, and an examination of this species showed that it closely matched a population from a nearby Samos Island (Greece) (Parmakelis et al., 2013a, 2013b). *E. avcii*, along with *E. tauricus* (C. L. Koch, 1837) from Crimea (Ukraine), *E. drenskii* Tropea et al., 2015 from Bulgaria (an independently derived "*em*=3" form unrelated to subgenus *Alpiscorpius*), and *E. popovi* Tropea et al., 2015 from Greece and Bulgaria, all group outside of the traditional subgenus *Euscorpius* (Parmakelis et al., 2013b; Tropea et al., 2015a, 2015b). For additional Anatolian species with *em*=4, DNA marker sequences are not yet available, and their phylogenetic placement is unclear.

The goal of this study was to study the phylogenetic relationships of additional Anatolian *Euscorpius* populations with "*em*=3". In addition, we used divergence dating to place the timing of diversification in a temporal context, thus allowing us to explore the biogeographic history of the group.

Methods & Material

Specimens used for DNA barcoding. Table 1 and map on Fig. 1 lists all localities and specimen labels used for DNA marker sequencing. Anatolian specimens (n=14)

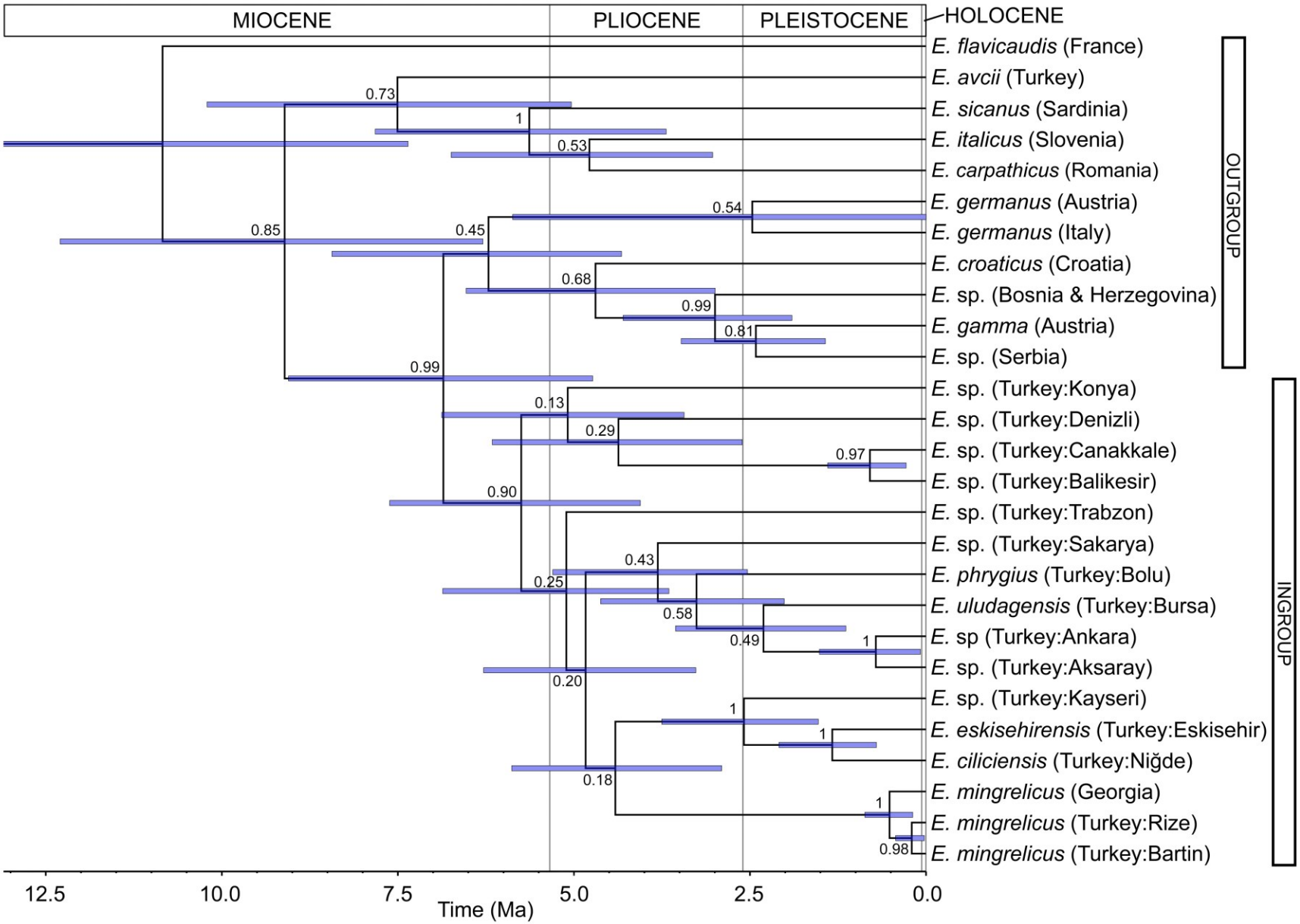

Figure 2: Maximum clade credibility tree from a BEAST analysis of DNA barcode data from 15 populations of Anatolian-Caucasian *Euscorpius*. Numbers at nodes are posterior probabilities. Outgroups: six populations of European "*em*=3" *Euscorpius* as well as five other species of *Euscorpius* including the Anatolian "*em*=4" species *E. avcii*.

			GenBank accession number		Taxonomy
	Ingroups	**Label & Habitat Data**	***COI***	***16S* rDNA**	
1	*E.* sp. (Turkey: Ankara)	TURKEY. *Ankara Province*: Kizilcahamam District, Soğuksu National Park, 40.6611°N, 32.7078°E, 1000 m, 08 August 2002, leg. A. & A. Karataş, VF-0752, AMSCO031-10. *Habitat*: dense pine forest; border of dry Central Anatolian steppe and humid Black Sea coast.	KT764039	KT764032	*Euscorpius* sp.
2	*E.* sp. (Turkey: Balıkesir)	TURKEY. *Balikesir Province*: Edremit District, Levent Bogazi, Kazdağları National Park, Ceyiz Deresi, 39.5833°N, 27.0166°E, 28 May 2004, leg. A. Karataş, VF-0754, AMSCO032-10. *Habitat*: a dense pine forest, at lower altitudes mixed with deciduous forest.	KT764040	N/A	*Euscorpius* sp.
3	*E. mingrelicus* (Turkey: Bartin)	TURKEY. *Bartin Province*: Amasra District, TTK Lojmanlar, 41.75°N, 32.3833°E, 13 August 2004, leg. A. Karataş, VF-0755, AMSCO033-10. *Habitat*: a town with dense vegetation.	KT764041	N/A	*Euscorpius mingrelicus* (Kessler, 1876)
4	*E. phrygius* (Turkey: Bolu)	TURKEY. *Bolu Province*: Mudurnu District, Abant Lake Nature Park (type locality), 40.6166°N, 29.2833°E, 1600 m, 11 August 2002, leg. A. & A. Karataş & H. Karakaya, VF-0756, AMSCO034-10. *Habitat*: a dense pine forest; specimens are found in the forest and under rocks along forest trails.	KT764042	KT764033	*Euscorpius phrygius* Bonacina, 1980 [type locality]
5	*E. uludagensis* (Turkey: Bursa)	TURKEY. *Bursa Province*: Osmangazi District, Uludağ Mt., near Inkaya Village, 15 August 2002, 40.1953°N, 29.0115°E, ca. 1000 m, leg. A. & A. Karataş & H. Karakaya, VF-0757. *Habitat:* mixed forest (mostly coniferous). Uludağ Mt. is the highest point south of the Marmara Sea.	N/A	KT764034	*Euscorpius uludagensis.*Lacroix, 1995 [type locality]
6	*E.* sp. (Turkey: Canakkale)	TURKEY. *Canakkale Province*: Bayramic District, Evciler, Ayazma, 39.7755°N, 26.7717°E, 08 September 2004, leg. A. Karataş, VF-0758, AMSCO036-10. *Habitat*: the locality is close to Edremit (Balıkesir; No 3 above), located on the northern slope of the same Kazdağları Mts. This part of the Kazdağları National Park has dense forested and mixed forests in lower altitude, but coniferous (*Pinus* and endemic *Abies*	KT764043	N/A	*Euscorpius* sp.

		nordmanniana ssp. *equi-trojani*) forests at higher elevations.			
7	*E.* sp. (Turkey: Denizli)	TURKEY. *Denizli Province*: Honaz District, Honaz Dağ National Park, 37.7184 °N, 29.2511°E, 1500 m, 25 July 2001, leg. E. Şıvkın, VF-0759, AMSCO037-10. *Habitat*: a pine forest.	HM418280	KT764035	*Euscorpius* sp.
8	*E. eskisehirensis* (Turkey: Eskişehir)	TURKEY. *Eskişehir Province*: Mihalıççık District, Otluk Village, 39.7667 N, 30.95 E, 1200 m, 06 April 2001, leg. F. Çalıskan (ZDUN/S-2001/41). *Habitat*: xeric vegetation with some pine forest; located in transitional zone between Central Anatolian steppe and Boreal zoogeographic provinces.	KT764046	AY152395 (Fet et al. 2003a)	*Euscorpius eskisehirensis* Tropea et Yağmur, 2015 [type locality]
9	*E.* sp. (Turkey: Kayseri)	TURKEY. *Kayseri Province*: Yahyali District, Kapuzbaşi Waterfall, 37.90°N, 35.35°E, 11 May 2003, leg. A. Karataş, VF-0761, AMSCO039-10. *Habitat*: a valley in Alağdalar Mts (=Antitaurus) Mts; tree vegetation sparse; xeric climate; transition zone between Mediterranean and Irano-Turanian zoogeographic provinces.	HM418281	N/A	*Euscorpius* sp.
10	*E.* sp. (Turkey: Konya)	TURKEY. *Konya Province*: Doğanhisar District, Başköy Village, Kirazlidere, 38.1333°N, 31.7833°E, 1300 m, 21 August 2001, leg. A. Ersöz, VF-0762, AMSCO040-10. *Habitat*: Mountain pine forest.	HM418282	KT764036	*Euscorpius* sp.
12	*E. ciliciensis* (Turkey: Niğde)	TURKEY. *Niğde Province*: Ulukisla District, Çiftehan, Gümüş-Maden road, 5th km, 1750 m, 37.4667°N, 34.6167°E, 23 May 2001, leg. A. Karataş, VF-0461. *Habitat*: Alpine zone, at timberline level, just above *Pinus brutia* forest patches.	KT764047	AY152394 (Fet et al., 2003a)	*Euscorpius ciliciensis* Birula, 1898 [close to type locality]
12	*E. mingrelicus* (Turkey: Rize)	TURKEY. *Rize Province*: Çamlıhemşin District, near Şenköy Village, 40.8644° N, 40.9647°E, 720 m, 24-25 August 2001, leg. A. & A. Karataş, VF-0765, AMSCO041-10. *Habitat*: dense mixed forest with spruce (*Picea orientalis*); specimens were found along the road, among rocks in crevices.	KT764044	KT764037	*Euscorpius mingrelicus* (Kessler, 1876)
13	*E.* sp. (Turkey: Sakarya)	TURKEY. *Sakarya Province*: Adapazari District, Kocaeli-Adapazarı road, 40.7833°N, 30.4°E, 30 July 2004, leg. A. Karataş, VF-0767, AMSCO043-10. *Habitat*: deciduous forest.	HM418283	N/A	*Euscorpius* sp.

14	*E.* sp. (Turkey: Trabzon)	TURKEY. *Trabzon Province*, Çağlayan District, Çakılca, 40.9667°N, 39.7333° E, 500 m, 22 August 2001, leg. A. & A. Karataş, VF-0766, AMSCO042-10. *Habitat*: karstic rocky area in hazelnut plantation in a mixed chestnut-beech deciduous forest.	KT764045	KT764038	*Euscorpius* sp.
15	*E. mingrelicus* (Georgia)	GEORGIA. *Abkhazia*: Ritsa Lake, 43.4731°N, 40.50°E, 14 October 2004, leg. Yu. Marusik, VF-0747, AMSCO027-10.	HM418279 (Graham et al., 2012b).	N/A	*Euscorpius mingrelicus* (Kessler, 1876) [close to type locality]
	Outgroups				
16	*E. avcii* (Turkey)	TURKEY. *Aydin Province*: Kuşadasi District, Güzelçamli, 37.71667°N, 27.2333°E.	KF030934 (Parmakelis et al., 2013a).	KF030936 (Parmakelis et al., 2013a).	*Euscorpius avcii* Tropea et al., 2012 [type locality]
17	*E. carpathicus* (Romania)	ROMANIA. *Caraş-Severin County*: Băile Herculane, 44.8786°N, 22.4141°E, 4 June 2008, leg. F. Šťáhlavský, VF-0768, AMSCO044-10.	HM418284 (Graham et al., 2012a).	N/A	*Euscorpius (Euscorpius) carpathicus* (L., 1767) [type locality]
18	*E. carpathicus* (Romania)	ROMANIA. *Caraş-Severin County*: Băile Herculane, 44.8786°N, 22.4141°E, 21 June 2001, leg. V. Popa, VF-0465.	N/A	AY172337 (Fet et al., 2002)	*Euscorpius (Euscorpius) carpathicus* (L., 1767) [type locality]
19	*E. croaticus* (Croatia)	CROATIA. *Krk Island*: Dobrinj District, Rudine, Biserujka Cave, 45.1831°N, 14.6131°E, 1997, VF-0824, AMSCO082-10.	HM418306 (Graham et al., 2012a)	N/A	*Euscorpius croaticus* Di Caporiacco, 1950
20	*E. gamma* (Austria)	AUSTRIA. *Carinthia*: Trögerner-Klamm, 46.4577°N, 14.5005°E, 13 June 1999, leg. V. Fet & B. Scherabon, VF-0732, AMSCO014-10.	HM418273) (Graham et al., 2012b).	AJ249553 (Scherabon et al., 2000)	*Euscorpius (Alpiscorpius) gamma* Di Caporiacco, 1950
21	*E. germanus* (Italy)	ITALY. *Trentino-Alto Adige*: Egna (Neumarkt), 46.3170°N, 11.26702°E, 214 m, leg. F. Štáhľavský, VF-0726, AMSCO011-10.	HM418272) (Graham et al., 2012b).	N/A	*Euscorpius* (*Alpiscorpius*) *germanus* (C. L. Koch, 1837)
22	*E. germanus* (Austria)	AUSTRIA. *Carinthia*: Oberdrauburg, EgOB.	N/A	AJ249553 (Scherabon et al., 2000)	*Euscorpius* (*Alpiscorpius*) *germanus* (C. L. Koch, 1837)
23	*E. flavicaudis* (France)	FRANCE. *Vaucluse*: Pernes-les-Fontaines, 43.9990°N, 05.0600°E, 230 June 2007, leg. V. Fet, VF-0700, AMSCO001-10.	HM418267 (Graham et al., 2012a).	N/A	*Euscorpius* (*Tetratrichobothrius*) *flavicaudis* (DeGeer, 1787)
24	*E. flavicaudis* (France)	FRANCE. *Vaucluse*: Lauris, EflaLA.	N/A	AJ389381) (Gantenbein et al., 1999)	*Euscorpius* (*Tetratrichobothrius*) *flavicaudis* (DeGeer, 1787)
25	*E. italicus* (Slovenia)	SLOVENIA. *Koper District*: Marezige, 45.5097°N, 13.8030°E, 11 August 2002, leg. T. Brstilo, VF-0706, AMSCO005-10.	HM418270 (Graham et al., 2012b).		*Euscorpius* (*Polytrichobothrius*) *italicus* (Herbst, 1800).
26	*E. italicus* (Slovenia)	SLOVENIA. *Gorizia*: Aidovščina, Brje, Dobravlje, 45.8840°N, 13.8470°E , 7 August 2000, leg. B. Sket (VFPC), *Ei*SL1.	N/A	AJ512752 (Fet et al., 2006)	*Euscorpius* (*Polytrichobothrius*) *italicus* (Herbst, 1800)
27	*E. sicanus* (Italy:	ITALY. *Sardinia*, S. Niccolo	JX133089	N/A	*Euscorpius*

	Sardinia)	Gerrei, near Grotta Saturru, 39.4981°N, 09.3150°E, 395 m, April 2006, leg. A. v. d. Mejden, VF-0789, AMSCO052-10.	(Graham et al., 2012a)		(*Euscorpius*) *sicanus* (C.L. Koch, 1837)
28	*E. sicanus* (Italy: Sardinia)	ITALY. *Sardinia*, Lu Fraili, 16 August 2001, leg. V. Vignoli, EcSDI.	N/A	AY090083 (Fet et al., 2003b)	*Euscorpius* (*Euscorpius*) *sicanus* (C.L. Koch, 1837)
29	*E.* sp. (Bosnia & Herzegovina)	BOSNIA & HERZEGOVINA. *Herzegovina*: Lebršnik, 43.2019°N, 18.6378°E, 1700 m, 9 September 2006, leg. I. Karaman, VF-0737, AMSCO018-10.	HM418276 (Graham et al., 2012b; listed as *E. mingrelicus*).	N/A	*Euscorpius* (*Alpiscorpius*) sp.
30	*E.* sp. (Serbia)	SERBIA. Tara River, Kaluderske Bare, 43.906°N, 19.527°E, 14 July 2007, leg. D. Pavicevic, VF-0744, AMSCO024-10.	HM418278 (Graham et al., 2012b; listed as *E. mingrelicus*).	N/A	*Euscorpius* (*Alpiscorpius*) sp.

Table 1: Specimens of *Euscorpius* used for DNA phylogeny.

were tentatively identified according to Tropea et al. (2015c) and Tropea & Yağmur (2015). Of 23 ingroup sequences used for our analyses, 20 are reported here for the first time and deposited at Genbank under the accession numbers HM418280-HM418283 and KT764032-KT764047; three others were published by Fet et al. (2003a) and Graham et al. (2012b). Graham et al. (2012b) used two of these Anatolian populations (Konya and Denizli) for morphological comparisons in their study of *E. croaticus*. All barcode sequences used as outgroups were previously published in our studies (Gantenbein et al., 1999; Graham et al., 2012a, 2012b; Fet et al., 2002, 2003b, 2006; Parmakelis et al., 2013a; Scherabon et al., 2000).

Molecular Techniques and Divergence Dating. To assess the phylogenetic position of these specimens, we sequenced standard DNA markers or "barcodes" (mitochondrial *COI* and *16S* rDNA sequences). Mitochondrial barcodes (*COI* sequences) were generated at the Canadian Centre for DNA Barcoding, University of Guelph using standard protocols as outlined in Graham et al. (2012a, 2012b). All new barcodes were submitted to GenBank and can be accessed through BOLD (http://www.boldsystems.org, Ratnasingham & Hebert, 2007) under Dataset DS-AMSCO3 "Scorpions of the Ancient Mediterranean 3" (DOI: dx.doi.org/10.5883/DS-AMSCO3). Voucher specimens are in a private collection of V. Fet. Two additional barcodes (*COI* sequences) used were obtained in V.F. in 2003 using universal "Ron and Nancy" *CO1* primers. The sequences of 16S rDNA gene were obtained in V.F. in 2003 using 16S rDNA-specific primers, as described in Gantenbein et al. (1999).

We simultaneously assessed phylogenetic relationships and estimated timing of diversification among Anatolian *Euscorpius* using BEAST v. 1.8.0 (Drummond & Rambaut, 2007). First, we visually checked chromatograms and assembled contigs in Geneious 6.1.6 (Biomatters, http://www.geneious.com). We aligned consensus sequences for both loci in Geneious using MUSCLE (Edgar, 2004), checked the alignments by eye for accuracy, and trimmed ends to minimize missing characters. The final alignments contained 363 bp for 16S and 649 bp for *COI*, for a total of 1012 bp in the concatenated alignment. The best-fit model of nucleotide substitution was determined for each gene partition with MEGA v. 5.2 (Tamura et al., 2011) using the Bayesian Information Criterion. We ran BEAST using the appropriate models (T92+G for *16S* and TN93+G for *COI*) using an uncorrelated lognormal clock for both genes with a Yule tree prior. We set the mean of the ucld.mean to 1.0% per Ma for *16S* and 1.4% per Ma for *COI*. Since *16S* and *COI* have roughly equal mutation rates, as estimated by Gantenbein et al. (2005), we adjusted the stdev of the ucld.mean to 0.25% for each gene partition so that the 95% HPD bounds included both rates. Initial BEAST runs produced low ucld.stdev estimates (<1.0) for both gene partitions, indicating clock-like evolution, so we conducted final analyses using a strict clock. We conducted two MCMC runs with 20^6 generations sampled every 1000 generations. Log files were opened in Tracer v. 1.6 (Drummond & Rambaut, 2007) to assess convergence and adequate (>200) ESS values for each parameter. Output trees were summarized with TreeAnnotator v. 1.8.0 (Drummond & Rambaut, 2007) with the first 20% discarded as burn-in. We visualized the resulting maximum clade credibility tree in FigTree v 1.4.0 (Rambaut, 2009).

Results & Discussion

The phylogeny based on concatenated DNA data from two markers (Fig. 1) reveals a high diversity of Turkish species of the genus *Euscorpius*; it recovers

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	**Eavc_Aydin**															
2	**Ecar_Romania**	0.068														
3	**Ecil_Niğde**	0.079	0.068													
4	**Efla_France**	0.108	0.101	0.112												
5	**Egam_Austria**	0.072	0.065	0.043	0.094											
6	**Eita_Slovenia**	0.076	0.047	0.061	0.094	0.058										
7	**Emin_Rize**	0.076	0.072	0.047	0.108	0.040	0.058									
8	**Esp_Ankara**	0.083	0.072	0.040	0.104	0.036	0.072	0.036								
9	**Ephr_Bolu**	0.126	0.108	0.076	0.129	0.072	0.108	0.079	0.058							
10	**Esic_Sardinia**	0.072	0.050	0.065	0.101	0.072	0.040	0.068	0.076	0.119						
11	**Esp_Denizli**	0.086	0.079	0.054	0.108	0.054	0.079	0.058	0.050	0.094	0.083					
12	**Eesk_Eskişehir**	0.076	0.068	0.043	0.108	0.050	0.065	0.047	0.040	0.068	0.068	0.065				
13	**Esp_Konya**	0.090	0.079	0.050	0.112	0.050	0.079	0.036	0.036	0.079	0.083	0.050	0.054			
14	**Esp_Trabzon**	0.097	0.086	0.068	0.126	0.061	0.090	0.058	0.054	0.083	0.090	0.061	0.076	0.058		
15	**Eger_Austria**	0.097	0.086	0.068	0.104	0.068	0.090	0.079	0.079	0.108	0.094	0.086	0.068	0.083	0.094	
16	**Eulu_Bursa**	0.068	0.072	0.047	0.126	0.050	0.076	0.050	0.036	0.072	0.076	0.047	0.050	0.058	0.050	0.079

Figure 3: Genetic distance matrix of 16 populations based on *16S rRNA* mtDNA marker. Includes 9 populations of Anatolian-Caucasian *Euscorpius (Alpiscorpius)*. Outgroups: two populations of European "*em*=3" *Euscorpius (Alpiscorpius)* as well as five other species of *Euscorpius* including *E. avcii*.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	**Eavc_Aydin**														
2	**Ecar_Romania**	0.091													
3	**Ecil_Niğde**	0.109	0.076												
4	**Ecro_Croatia**	0.126	0.103	0.091											
5	**Efla_France**	0.111	0.101	0.111	0.123										
6	**Egam_Austria**	0.113	0.078	0.083	0.076	0.121									
7	**Eger_Italy**	0.118	0.073	0.091	0.073	0.118	0.078								
8	**Eita_Slovenia**	0.101	0.071	0.101	0.091	0.101	0.091	0.083							
9	**Emin_Georgia**	0.113	0.091	0.083	0.101	0.096	0.101	0.098	0.091						
10	**Emin_Bartin**	0.121	0.093	0.083	0.108	0.098	0.103	0.106	0.098	0.013					
11	**Emin_Rize**	0.121	0.093	0.078	0.108	0.098	0.103	0.106	0.098	0.008	0.005				
12	**Esp_Ankara**	0.111	0.078	0.068	0.081	0.096	0.076	0.078	0.071	0.071	0.073	0.073			
13	**Ephr_Bolu**	0.111	0.088	0.071	0.093	0.091	0.093	0.083	0.093	0.083	0.086	0.086	0.045		
14	**Esic_Sardinia**	0.113	0.090	0.111	0.106	0.111	0.100	0.113	0.098	0.100	0.108	0.108	0.100	0.100	
15	**Esp_BosnHerz**	0.113	0.081	0.098	0.060	0.098	0.063	0.078	0.093	0.088	0.096	0.096	0.078	0.086	0.108
16	**Esp_Serbia**	0.116	0.083	0.091	0.086	0.106	0.063	0.088	0.101	0.076	0.083	0.078	0.091	0.093	0.103
17	**Esp_Balikesir**	0.103	0.083	0.096	0.088	0.093	0.060	0.093	0.096	0.083	0.091	0.091	0.086	0.096	0.106
18	**Esp_Canakkale**	0.103	0.081	0.088	0.101	0.091	0.068	0.098	0.091	0.088	0.086	0.086	0.081	0.091	0.111
19	**Esp_Denizli**	0.098	0.076	0.093	0.091	0.101	0.083	0.096	0.098	0.091	0.088	0.088	0.078	0.083	0.108
20	**Eesk_Eskişehir**	0.100	0.064	0.008	0.087	0.108	0.077	0.082	0.095	0.085	0.087	0.082	0.067	0.069	0.097
21	**Esp_Kayseri**	0.111	0.076	0.040	0.083	0.106	0.086	0.081	0.083	0.071	0.078	0.073	0.048	0.053	0.103
22	**Esp_Konya**	0.118	0.091	0.093	0.103	0.093	0.073	0.086	0.083	0.091	0.093	0.093	0.071	0.073	0.111
23	**Esp_Sakarya**	0.113	0.103	0.091	0.098	0.113	0.108	0.113	0.093	0.086	0.093	0.088	0.078	0.071	0.119
24	**Esp_Trabzon**	0.098	0.091	0.083	0.078	0.108	0.093	0.081	0.088	0.076	0.078	0.078	0.076	0.078	0.111

		15	16	17	18	19	20	21	22	23
16	**Esp_Serbia**	0.058								
17	**Esp_Balikesir**	0.068	0.086							
18	**Esp_Canakkale**	0.078	0.091	0.018						
19	**Esp_Denizli**	0.063	0.071	0.083	0.088					
20	**Eesk_Eskişehir**	0.087	0.082	0.095	0.090	0.082				
21	**Esp_Kayseri**	0.093	0.081	0.096	0.093	0.086	0.044			
22	**Esp_Konya**	0.086	0.093	0.083	0.086	0.083	0.090	0.088		
23	**Esp_Sakarya**	0.101	0.101	0.103	0.096	0.093	0.090	0.086	0.096	
24	**Esp_Trabzon**	0.073	0.083	0.081	0.078	0.078	0.087	0.073	0.093	0.088

Figure 4: Genetic distance matrix of 24 populations based on *COI* mtDNA marker. Includes 14 populations of Anatolian-Caucasian *Euscorpius (Alpiscorpius)*. Outgroups: five populations of European "*em*=3" *Euscorpius (Alpiscorpius)* as well as five other species of *Euscorpius* including *E. avcii*.

most of described "*em*=3" species and, in addition, a number of undescribed clades (putative species).

The major observations derived from these DNA phylogenetic data are:

1. All "*em*=3" species (Europe and Anatolia) are clearly separated from all outgroup "*em*=4" species (including *E. avcii* from Turkey (Aydin Province) and all species of the traditional subgenus *Euscorpius* (see Parmakelis et al., 2013b, for details).

Among "*em*=3" species, the European clade (i.e. the original subgenus *Alpiscorpius*, based on the type species *E. germanus*) is clearly separated from the Anatolian-Caucasian clade. This important fact has never been demonstrated before using genetic markers. Further detailed phylogenetic studies and morphological analyses are needed to investigate the structure of subgenus *Alpiscorpius*. This again emphasizes the statement that the Balkan "*em*=3" forms do not belong to *E. mingrelicus* (see Tropea et al., 2015c). The split between European and Anatolian-Caucasian clades can be tentatively assigned to Miocene, between 7.5 to 4 Mya (see divergence estimates in Fig. 1). These approximations correspond well to the general estimates of cladogenesis within the genus *Euscorpius* by Parmakelis et al. (2013b) as 5–8 Mya.

2. We recovered all five "*em*=3" species outlined by Tropea et al. (2015c) and Tropea & Yağmur (2015) (former "*mingrelicus* complex"), which correspond to six out of our 14 Anatolian populations:

   - *E. ciliciensis* (Niğde Province: close to type locality, map No 11)
   - *E. eskisehirensis* (Eskişehir Province: type locality, map No 8)
   - *E. mingrelicus* (Bartin Province, map No 3; Rize Province, map No 12); and also Georgia [close to type locality], map No 15)
   - *E. phrygius* (Bolu Province: Abant, type locality, map No 4)
   - *E. uludagensis* (Bursa Province: Uludağ, type locality, map No 5)

Our material did not include only the southern "*em*=3" form *E. arikani* (Antalya Province) described by Yağmur & Tropea (2015).

3. Among the identified species, *E. mingrelicus* is widely ranging along most of the Black Sea coast where it occupies humid habitats. It is represented by three populations from Bartin to Rize in Turkey, and further to Georgia (type locality). Genetic distance among these populations is low, varies for *COI* between 0.7-1.8%). This allows to assume a recent (Pleistocene, less than 1 Mya) dispersal along the coast (see divergence estimates in Fig. 1).

4. The northern Anatolian clade that includes populations from type localities of *E. phrygius* and *E. uludagensis* is also loosely associated with two other populations from northern Sakarya Province (map No 13) and Ankara Province (Soğuksu; map No 1).

5. We recover at least ***four more*** unique clades (putative species) with "*em*=3" across Anatolia, originating from:
   - two northwestern localities from the mountain forest habitats of the Kazdağları National Park: Balikeşir Province (Edremit; map No 2) and Canakkale Province (Ayazma; map No 6); these populations are closely related (*COI* distance 1.8 %).
   - an isolated southwestern range (Honaz Dağ National Park) in Denizli Province (map No 7, mountain pine forest at 1500 m a.s.l.)
   - Trabzon Province (close to the range of *E. mingrelicus*) (map No 14)
   - Konya Province, Başköy (map No 10, mountain pine forest at 1300 m a.s.l.).

   Relationships between these clades and others remain unresolved.

6. *E. ciliciensis* (a high altitude species) and *E. eskisehirensis* (which inhabits xeric habitats), although geographically distant, appear to be very closely related judging from genetic distance observed for both markers (0.7% for *16S* and 0.8% for *COI*). See Tropea & Yağmur (2015) for diagnostic morphological characters separating these species. This clade is well-separated from a more closely related humid-habitat group of *E. mingrelicus, E. phrygius* and *E. uludagensis*. However, the (*E. ciliciensis* + *E. eskisehirensis*) clade does not group outside the Anatolian "*em*=3" forms, thus indicating that its distinct morphology (Tropea et al., 2015c) could be derived.

7. The clade of (*E. ciliciensis* + *E. eskisehirensis*) includes also a related outgroup population (a putative species, 4 % distance for *COI*) from another xeric habitat in Kayseri Province in Aladağlar (=Antitaurus) Mts (map No 9). Moreover, we discovered that this population exhibits phenotype *em*=4 (confirmed for 4

specimens, for both pedipalps). This is an exceptional observation for subgenus *Alpiscorpius*, a well-defined *em*=3 clade. We suggest that *em*=4 in Kayseri population is a reversal to the plesiomorphic condition, which is the first case when such a reversal is documented in genus *Euscorpius*. Some other populations, morphologically close to *E. ciliciensis*, also exhibit *em*=4, for instance, in Ihlara Valley (Aksaray Province) and Mt. Demirkazik (Aladağlar Mts., Niğde Province) (our unpublished data).

Further detailed analysis of identified populations across Anatolia, their morphological and genetic identity, and biogeographic history of isolation are needed to interpret taxonomic structure of hidden biodiversity of *Euscorpius* in Anatolia.

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