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EUSCORPIUS CARPATHICUS (LINNAEUS, 1767) (SCORPIONES: EUSCORPIIDAE) FROM ROMANIA: MITOCHONDRIAL DNA DATA

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ABSTRACT.- The new mitochondrial 16S rRNA DNA data support the phylogenetic position of *Euscorpius carpathicus* (Linnaeus, 1767) (recently restricted to Romania) as compared to several other species of "*E. carpathicus*" complex. Phylogenetic and biogeographic implications are discussed.

KEY-WORDS.- *Euscorpius carpathicus*, Mitochondrial 16S rRNA, Phylogeny, Romania

RESUME.- Des nouvelles données mitochondriales 16S rARN ADN appuient la position phylogénétique d'*Euscorpius carpathicus* (Linnaeus, 1767) (population limité à la Roumanie) quand comparée à d'autres espèces du complexe « *E. carpathicus* ». Des implications phylogénétiques et biogéographiques sont discutées.

MOTS-CLES.- *Euscorpius carpathicus*, 16S rARN mitochondrial, Phylogénie, Roumanie

INTRODUCTION

Scorpions of the genus *Euscorpius* Thorell, 1876 (Scorpiones: Euscorpiidae) are very common in southern Europe (FET & SISSOM, 2000). Ecologically diverse, they occupy a variety of habitats from xeric to mesic, from the Mediterranean shoreline to the high altitudes of the Alps and Balkans. LINNAEUS (1767: 1038) described *Scorpio carpathicus* from Transylvanian Alps (Romania) (now *Euscorpius carpathicus*, the type species of the genus).

As a result of an extensive recent revision by FET and SOLEGLAD (2002), the European scorpion species *E. carpathicus* was restricted only to Romania, its type locality. The name *E. tergestinus* (C. L. Koch, 1837) was applied to most of the "western" populations of former *E. carpathicus*. Across the Balkans, several more forms of the "*E. carpathicus*" complex are present. During the revisions of this complex by GANTENBEIN *et al.* (2001) and FET and SOLEGLAD (2002) using morphological and molecular information, the following species have been established: *E. balearicus* Caporiacco, 1950 (Baleares, Spain), *E. tergestinus* (C.L. Koch, 1837) (France, Italy, western Balkans), *E. carpathicus* (Linnaeus, 1767) (Romania), *E. hadzii* Caporiacco, 1950 (Balkans), and *E. koschewnikowi* Birula, 1900 (Greece). Several additional forms of this species complex are currently under detailed investigation. The present paper reports the first molecular (mitochondrial DNA) data on the Romanian population of *E. carpathicus*, and also the first DNA information on *E. hadzii* from Croatia.

MATERIAL AND METHODS

Material. Two adult females and one adult male of E. carpathicus were collected by Valentin D. Popa on June 21, 2001 at Baile Heruclane, Romania. Scorpions were preserved in 96% ethanol and sent for DNA analysis to Marshall University, West Virginia, USA.

DNA analysis. Comparative analyses of the mitochondrial 16S ribosomal RNA gene has been recently used for resolving species-level phylogeny of *Euscorpius* (GANTENBEIN *et al.*, 1999, 2000, 2001; SCHERABON *et al.* 2000); for the detailed DNA analysis procedures and phylogenetic tree-building algorithms, see GANTENBEIN *et al.* (1999, 2000). Total DNA was extracted from fresh or preserved (95 % ethanol) muscle tissue (a leg) using a Qiagen™ DNeasy extraction kit. An approximately 400 bp fragment of the mitochondrial (mt) 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using the primers 16Sbr, or LR-J-12887 (CGATTTGAACTCAGATCA; forward, 18-mer) and a scorpion-specific reverse primer (GTGCAAAGGTAGCATAATCA, 20-mer). These primers corresponded to the positions 11,173-11,190 and 11,625-11,606 in the *Limulus polyphemus* mitochondrial genome (LAVROV *et al.*, 2000). The resulting PCR product was verified on 1% agarose electrophoretic gel and purified by Ultrafree MC 30000 cellulose filters (Millipore, Inc.). Automated Sanger dideoxy sequencing of the double-stranded PCR product was performed at the Molecular Genetics Instrumentation Facility, University of Georgia (Athens, GA) on the ABI 9600 Sequencer.

Phylogenetic analysis. Nine mtDNA sequences representing different haplotypes were aligned using Clustal X 1.81 (THOMPSON *et al.*, 1997). Three new DNA sequences where deposited to GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers AY172337 (EcaRO1), AY172338 (EcRO2), and AY172339 (EhDU2). Six DNA sequences publshed earlier by our research group and its collaborators (GANTENBEIN *et al.*, 1999, 2000, 2001; HUBER *et al.*, 2001) were extracted from the GenBank online database. The corresponding taxa, their geographic origin, abbreviations and accession numbers were: *E. flavicaudis* (DeGeer, 1778): Lauris, Vaucluse, France, EfLA (AJ389381); *E. germanus* (C.L. Koch, 1837), Austria, Carinthia, Oberdrauenburg, EgOB (AJ249553); *balearicus* Caporiacco, 1950: Mallorca, Baleares, Spain (AJ309208), EbBA1; *E. tergestinus* (C.L. Koch, 1837): Mathis, Alpes-Martitimes, France, EtMA1 (AJ389376); Mala Duba, Croatia, EtMD2 (AJ298063); and *E. "carpathicus candiota"* Birula, 1903: Kallikratis, Crete, Greece, EcKA2 (AJ309214). As an outgroup, we used *E. flavicaudis.* The software package PAUP* Version 4.0b10 (Swofford, 1998) was used for sequence analysis to perform genetic distance calculation, Maximum Parsimony (MP), and Neighbor Joining (NJ) algorithms. The statistical support of inner clades of the phylogenetic tree was determined by bootstrapping (1000 pseudoreplicates).

RESULTS

Exhaustive Search under PAUP* found six shortest MP trees, 96 steps long, under various weightings. A highly supported (80%) monophyletic lineage among the studied material included "*E. carpathicus* complex" taxa: Romanian specimens, *E. hadzii* from Croatia, *E. tergestinus* from France and Croatia, and *E. "carpathicus candiota"* from Crete. The consensus tree yielded a polytomy of the branches within this lineage; *E. balearicus* was supported as a sister group to this polytomy. The clades with a high statistical support within the polytomy were two Romanian specimens (bootstrap 93 %) and two *E. tergestinus* populations (bootstrap 59 %). The branching order under most Neighbor Joining distance models studied (Kimura, Felsenstein, Jukes-Cantor, HKY85,

Tamura-Nei), placed *E. tergestinus* as the sister group to the Romanian population of *E. carpathicus*. Only under absolute distance model the Romanian population formed a sister group to a clade including *E. tergestinus* and *E. "carpathicus candiota"*. The bootstrap under distance criterion yielded polytomy of Romanian specimens, *E. tergestinus* and *E. "carpathicus candiota* supported by 56% bootstrap (Fig. 1). High bootstrap value (85%) supported this group's association with *E. hadzii*, as opposed to *E. balearicus*. In all analyses, *E. balearicus* formed a sister group to other "*E. carpathicus* complex" taxa, and *E. germanus* grouped outside of *E. balearicus*. Distance data (Table I) are presented for absolute and Kimura distances.

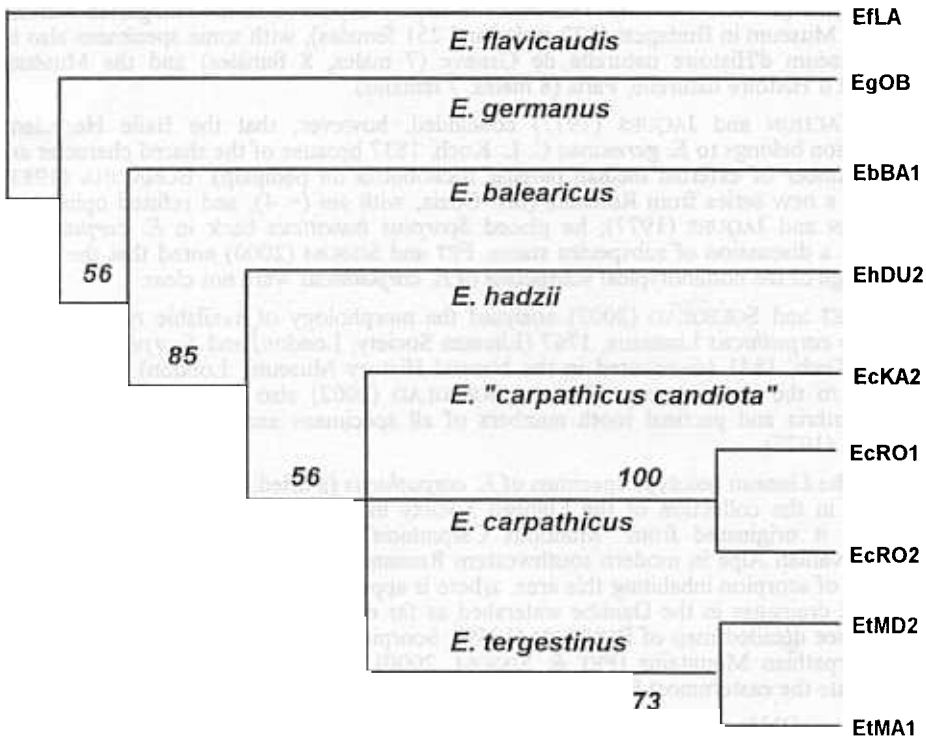

Fig. 1. Neighbor Joining (NJ) phenogram based on the Kimura distance (KIMURA, 1980). Numbers designate bootstrap values.

DISCUSSION

Populations of *E. carpathicus* from Romania (the easternmost boundary of the genus' continuous range in Europe) have been recorded and studied for many years by a number of zoologists since LINNAEUS (1767). However, these disparate data were never collated or compared to the original Linnean holotype specimen. C. L. KOCH (1841, 1842) described two new species of *Euscorpius* from nearly the same place in modern Romania (territory of Hungary in the 1830s), *Scorpius banaticus* and *S. oravitzensis*.

Both of these were synonymized to *E. carpathicus* (see e.g. KRAEPELIN, 1899) and Romanian populations were addressed as such, without any comparative analysis (ZOTTU, 1927; CALINESCU & CALINESCU, 1930; CALINESCU, 1956; BUNESCU, 1959). Some authors, like CHYZER (1900) still used the "old name" as *Euscorpius banaticus*.

Among numerous subspecific forms deemed valid by CAPORIACCO (1950), the nominotypic subspecies, *"E. carpathicus carpathicus"* was poorly defined both in morphology and geographic range. CAPORIACCO (1950) analyzed only a single specimen from "Hungary" (now Romania) and was not conclusive as of the geographic range of the nominotypical subspecies. KINZELBACH (1975) published a short information (based on a letter of Dr. O. Kraus) on the Linnean holotype, deposited in the Linnean Society of London. VACHON and JAQUES (1977) analyzed a very large series (403 specimens) of "*Scorpius banaticus*" collected by C. Chyzer from Herkulesfürdo, now Baile Herculane, in Romania (historical Banat). This series is largely deposited in the Hungarian Natural History Museum in Budapest (122 males and 251 females), with some specimens also in the Muséum d'Histoire naturelle de Genève (7 males, 8 females) and the Muséum national d'Histoire naturelle, Paris (8 males, 7 females).

VACHON and JAQUES (1977) concluded, however, that the Baile Herculane population belongs to *E. germanus* C. L. Koch, 1837 because of the shared character *em* = 3 (number of external median patellar trichobotria on pedipalp). BONACINA (1983) studied a new series from Romania (Mt. Cozia, with *em* (= 4), and refuted opinion of VACHON and JAQUES (1977); he placed *Scorpius banaticus* back in *E. carpathicus*, without a discussion of subspecies status. FET and SISSOM (2000) noted that the scope and range of the nominotypical subspecies of *E. carpathicus* were not clear.

FET and SOLEGLAD (2002) analysed the morphology of available types of both *Scorpio carpathicus* Linnaeus, 1767 (Linnean Society, London) and *Scorpius banaticus* C. L. Koch, 1841 (discovered in the Natural History Museum, London). Both forms belong to the same species. FET and SOLEGLAD (2002) also included in their study trichobothria and pectinal tooth numbers of all specimens analyzed by VACHON and JAQUES (1977).

The Linnean holotype specimen of *E. carpathicus* (a dried, pinned subadult female) is kept in the collection of the Linnean Society in London. According to LINNAEUS (1767), it originated from "Montibus Carpathicis". This most likely refers to the Transylvanian Alps in modern southwestern Romania (FET *et al.*, 2002). It is the only species of scorpion inhabiting this area, where it appears to be common in at least three isolated drainages in the Danube watershed as far east as the upper reaches of Buzeu River; see detailed map of BUNESCU (1959). Scorpions are not found anywhere else in the Carpathian Mountains (FET & SISSOM, 2000), therefore the Transylvanian Alps constitute the easternmost limit of continuous *Euscorpius* range in Europe.

Our DNA data analysis results confirm the placement of the nominotypic Romanian population together with other southern European taxa of "*E. carpathicus*" complex, which formerly belonged to the species *E. carpathicus*. The closest (sister) groups to the Romanian population, as revealed by Neighbor Joining analysis (Fig. 1) are *E. tergestinus* C. L. Koch and the Greek taxon (putative species) *E. "carpathicus candiota"*. The distance data (Table I) agree with morphospecies-level divergences found in other *Euscorpius* species (e.g., GANTENBEIN *et al.*, 2000, 2001). Therefore, molecular data agree with the delineation of the Romanian *E. carpathicus* by FET and SOLEGLAD (2002) on the basis of morphology, and also do not confirm its proximity to *E. germanus* as assumed by VACHON and JAQUES (1977). The detailed analysis of DNA phylogeny for two Alpine species formerly included under *E. germanus*, was also recently published by our research group (GANTENBEIN *et al.*, 2000).

The locality from which our samples originate is the same as one that provided the numerous Chyzer collection analyzed by VACHON and JAQUES (1977), Baile Herculane (translated as "Herucles' Bath", by the name of the famous spa, known from the Roman

times) in Banat (Caras-Severin county). The locality lies at the altitude 160 m, is located in the Cerna river valley, in the deep intramountain depression with mild climate; the valley is flanked by the Mehedinti Mountains in the east and Cernei Mountains on the west. Low-altitude refugia like this one are likely to house *Euscorpius* species in the marginal parts of their geographic ranges; it remains to be seen if disjunct range of this species in Romania (BUNESCU, 1959) is due to relict distribution or human introduction.

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Table I. A matrix of genetic distances: uncorrected ("p") distance (below the diagonal); Kimura 2-parameter distance (above the diagonal).

		1	2	3	4	5	6	7	8	9
1	EcRO1	-	0.005	0.041	0.049	0.056	0.032	0.072	0.092	0.095
2	EcRO2	0.005	-	0.041	0.052	0.056	0.032	0.072	0.093	0.096
3	EtMD2	0.039	0.039	-	0.052	0.063	0.047	0.091	0.119	0.119
4	EtMA1	0.050	0.050	0.048	-	0.081	0.056	0.104	0.112	0.129
5	EcKA2	0.053	0.054	0.059	0.076	-	0.062	0.087	0.117	0.121
6	EhDU2	0.031	0.031	0.045	0.054	0.059	-	0.062	0.093	0.097
7	EbBA1	0.068	0.068	0.085	0.097	0.082	0.059	-	0.107	0.103
8	EgOB	0.086	0.087	0.109	0.104	0.107	0.088	0.099	-	0.121
9	EfLA	0.089	0.090	0.109	0.118	0.110	0.091	0.096	0.111	-