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# *Euscorpius carpathicus* (L., 1767) in Austria: phylogenetic position clarified by mitochondrial DNA analysis (Scorpiones: Euscorpiidae)

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## Summary

*Euscorpius carpathicus* (L., 1767) was recorded for the Austrian fauna in three disjunct localities (Federaun and Burg Hochosterwitz in Carinthia, and Krems in Lower Austria) (Scherabon, 1987). We studied samples from all three populations using mitochondrial DNA analysis. Identical molecular data suggest the same source for three populations, most likely human introduction from the south (Slovenia).

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## Introduction

The boundary of the continuous range of *Euscorpius carpathicus* (L., 1767) (Euscorpiidae) in southern Europe goes from west to east through northern Italy and Slovenia. In Austria, this scorpion species has been reported from three disjunct localities (Scherabon, 1987; Komposch & Scherabon, 1999; Komposch & Komposch, 2000): two from Carinthia (Federaun, or Unterfederaun, near Warmbad Villach, and Burg Hochosterwitz), and one from Lower Austria (Krems). The Krems population has been described by Ferrari (1872) as a separate subspecies, *E. tergestinus* var. *austriacus*; this taxon has been for a long time since synonymized with *E. carpathicus* (Fet & Sissom, 2000).

Morphological variation in *E. carpathicus* is extremely complex; it has a large number of valid subspecies (Caporiacco, 1950; Fet & Sissom, 2000). Scherabon (1987), in a detailed review of Austrian, and especially Carinthian scorpions, confirmed that three Austrian populations belong to *E. carpathicus*, but did not assign it to any subspecific form. Analysis of morphology based on 72 Austrian specimens showed that they usually have from 10 to 12 ventral and 25 to 26 external trichobothria on pedipalp patella. Such range of meristic characters is common for many populations of *E. carpathicus* in southern Europe, especially in Italy (Caporiacco, 1950). Thus, relationship of Austrian populations to those within the continuous range was not clear. In order to verify the possible origin of the three populations of *E. carpathicus* in Austria, we applied recently established mitochondrial gene markers (Gantenbein *et al.*, 1999, 2000). We were particularly interested if these populations are of autochthonous origin, and therefore can be regarded as glacial refuge populations or, alternatively, if these populations were recently introduced by man—which is the more likely hypothesis.

## Material and methods

Live specimens used for molecular analysis were collected in Austria by D.H., V.F. and B.S. in Federaun, near Warmbad Villach; by D.H. in

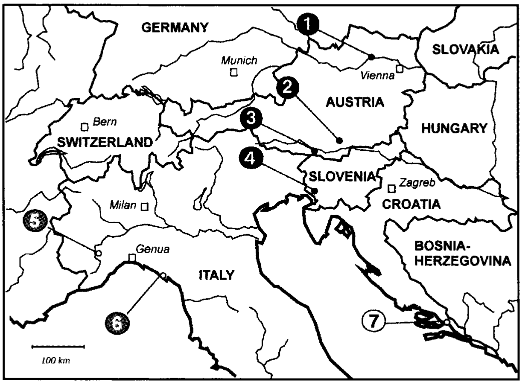

Fig. 1: Sampling sites of *E. carpathicus* populations used in this study: Black circles = "Slovenian clade": 1 Krems; 2 Burg Hochosterwitz; 3 Federaun, all Austria; 4 Črniče, near Nova Gorica, Slovenia. Grey circles = "North Italian clade": 5 La Morra, Piemonte, Italy; 6 Vernazza, Liguria, Italy. White circle = "Croatian clade": 7 Mala Duba, Croatia.

Krems; and by W. Rothmann in the historical castle Burg Hochosterwitz. For comparisons, we used specimens of *E. carpathicus* from Slovenia (Črniče near Nova Gorica, coll. M. Kuntner), Italy (Vernazza in Liguria, coll. C. R. Largiadèr; and La Morra in Piemonte, coll. I. & B. Gantenbein), and Croatia (Mala Duba, 20 km south of Makarska, coll. A. Winkler). The sampling sites are shown in Figure 1.

For the DNA analysis procedures, see Gantenbein *et al.* (1999). Total DNA was extracted from fresh or preserved (95% ethanol) muscle tissue using a standard extraction method. An approximately 450 bp fragment of the mitochondrial 16S rRNA gene was amplified using the universal forward primer LR-J-12887 and a scorpion-specific reverse primer (Gantenbein *et al.*, 1999). The sequence of the double-stranded PCR product was obtained by cycle sequencing using the forward primer LR-J-12887 (fluorescent dye end-labelled, IRD800; LI-COR™). The single stranded fragments were then resolved on an automated sequencer (LI-COR™ model 4200). Detailed PCR and sequencing protocols are given in Gantenbein *et al.* (1999). Sequencing reactions were produced with the thermosequenase kit by Amersham™ according to the recommendations of the distributors.

All new *Euscorpius* sequences were deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk) with the following accession numbers: *E. carpathicus*: 1 specimen from Vernazza, Liguria, Italy (= *Ec*VE1, AJ298062); 2 specimens from Mala Duba, Croatia (= *Ec*MD1, AJ298063; =*Ec*MD2, AJ298064); 2 specimens from Burg Hochosterwitz, Austria; 2 specimens from Krems, Austria; 1 specimen from Federaun near Warmbad Villach, Karinthia, Austria (all Austrian haplotypes were identical = *Ec*HO1, AJ298065); 1 specimen from Črniče, Slovenia, (= *Ec*CE2, AJ298066); *E. italicus*: 2 specimens from Tortoreto, Italy (identical haplotypes = *Ei*TO1, AJ298067). The sequence of the specimen from La Morra, Piemonte, Italy (*Ec*LM1) was recently used in Gantenbein *et al.* (1999) and was taken from EMBL (accession number AJ389377).

We aligned 14 sequences by eye, of which seven sequences were excluded for phylogenetic analysis because of identical haplotypes. We included three outgroup taxa: *Euscorpius italicus* (Herbst, 1800) collected by Marco Bellini at Tortoreto (Abruzzi, Adriatic coast of Italy; *Euscorpius flavicaudis* from Lauris (France), EMBL accession number AJ389381; and *Hadrurus concolor* Stahnke, 1969 (Iuridae), EMBL accession number AJ389382. *E. italicus*, a Mediterranean species, is probably the closest relative (= sister taxon) of *E. carpathicus*, whereas *E. flavicaudis* can be considered a more distant, plesiomorphic outgroup (Gantenbein *et al.*, 1999).

For phylogenetic analyses we stripped all ambiguities out as described elsewhere (Gantenbein *et al.*, 2000) and tested for the most appropriate DNA substitution model using hierarchical likelihood ratio tests; for details, see Huelsenbeck & Rannala (1997) and Huelsenbeck & Crandall (1997). Used computer programs and detailed description of phylogenetic analyses are given in Gantenbein *et al.* (2000). Recently developed methods allow evaluation of the limits of parsimony (Templeton *et al.*, 1992, 1995). Therefore, we calculated a cladogram which only connects haplotypes with a probability of a 95% confidence limit using the program TCSalpha v1.01 by Clement *et al.* (2000). From the length of the DNA sequences we estimated the maximum number of steps haplotypes can differ from each other for a 95% confidence limit.

| | *EcLM1* | *EcVE1* | *EcHO1* | *EcMD1* | *EcMD2* | *EcCE2* | *EiTO1* | *EfLA* | *Hcon* |
|---|---|---|---|---|---|---|---|---|---|
| *EcLM1* | - | 0.03 | 0.03 | 0.03 | 0.04 | 0.03 | 0.05 | 0.11 | 0.22 |
| *EcVE1* | 0.03 | - | 0.03 | 0.02 | 0.03 | 0.03 | 0.05 | 0.11 | 0.22 |
| *EcHO1* | 0.04 | 0.04 | - | 0.02 | 0.02 | 0.00 | 0.05 | 0.10 | 0.22 |
| *EcMD1* | 0.03 | 0.03 | 0.02 | - | 0.01 | 0.02 | 0.05 | 0.10 | 0.22 |
| *EcMD2* | 0.04 | 0.04 | 0.03 | 0.01 | - | 0.03 | 0.06 | 0.11 | 0.22 |
| *EcCE2* | 0.04 | 0.04 | 0.00 | 0.02 | 0.03 | - | 0.05 | 0.10 | 0.22 |
| *EiTO1* | 0.08 | 0.07 | 0.07 | 0.07 | 0.08 | 0.06 | - | 0.09 | 0.21 |
| *EfLA* | 0.22 | 0.21 | 0.17 | 0.20 | 0.22 | 0.18 | 0.17 | - | 0.21 |
| *Hcon* | 1.03 | 1.03 | 0.97 | 0.99 | 1.00 | 0.98 | 0.95 | 0.86 | - |

Table 1: Pairwise distances between taxa: mtDNA 16S mtDNA sequence data (below diagonal: HKY85 + Γ distances; above diagonal: "uncorrected *p*" sequence divergence).

## Results and discussion

All specimens from three Austrian populations of *E. carpathicus* produced 100% identical mtDNA sequences. When this Austrian haplotype, represented by Burg Hochosterwitz (= *Ec*HO1) population, was compared to those from Slovenia and Italy, it grouped extremely closely to a Slovenian sequence from Črniče, near the Italian border with Slovenia, *Ec*CE2 (0.2%) (Table 1 difference corresponds to a minimal possible mismatch of one base pair). On the other hand, samples from northwestern Italy (Liguria and Piemonte) were 2 to 3% distant from Austrian and Slovenian samples (Table 1; Fig. 2A, B).

The hierarchical likelihood ratio tests suggested the substitution model of Hasegawa *et al.* (1985) (HKY85 + Γ) with a gamma approximated correction for multiple hits (Yang, 1994). The hypothesis for a molecular clock was rejected at the 0.01 level (details of this test are given in Huelsenbeck & Crandall, 1997)), therefore, tree search was carried out without clock enforcement. The Maximum Likelihood analysis revealed a single tree with a -Likelihood of 1058.05. The base pair frequencies and other estimated parameters are given in Figure 1A. The weighted Maximum Parsimony analysis revealed in general a tree topology identical to the ML analysis. Transversions (tv) were weighted three times over transitions (ti) according to the ML-estimated ti/tv ratio. In this tree topology the outgroup *E. italicus* is confirmed by a rather high bootstrap value (91 and 98%, respectively). The ingroup of *E. carpathicus* was split into three (ML analysis, Fig. 2A) or four (MP analysis, Fig. 2B) lineages. The two haplotypes from Burg Hochosterwitz (*Ec*HO1) and from Črniče (Slovenia) (*Ec*CE2) were found to form a well-supported clade in both analyses (83 and 88%, respectively) (= "Slovenian clade"). The haplotypes from Mala Duba, Croatia (*Ec*MD1 and *Ec*MD2), however, clustered as a second independent clade (= "Croatian clade"). Finally, the haplotypes from Vernazza and La Morra clustered as a third clade (= "North Italian clade").

The analysis using the confidence approach on parsimony cladogram estimation by Templeton *et al.* (1992) revealed that only the shortest connection between the haplotypes from Črniče (*Ec*CE2) and from Burg Hochosterwitz (*Ec*HO1) is supported by a confidence interval of 95%. All other haplotypes were connected by more than 8 steps with each other and, therefore, their connections are not supported with this confidence limit. We interpret this result as a strong evidence for recent artificial introduction of *E. carpathicus* into Austria rather than the presence of glacial relict populations. If these populations were to be considered relicts of glacial era, we would expect higher level of divergences between isolated mtDNA sequences. Assuming the scorpion mtDNA evolves as fast as the oft-cited mtDNA clock by Brown *et al.* (1979) with 2% sequence divergence per Ma, and considering that these Austrian populations should have been isolated at least since the onset of the last ice age (Würm period, about 70,000 yr ago) from lineages in Slovenia, we would expect a sequence divergence of 0.14% (Hewitt, 1996).

As a much more likely hypothesis, we suggest that Austrian populations are a local introduction by humans from more southern areas (Slovenia) along the trade routes, and were established recently. Human introduction of scorpions is

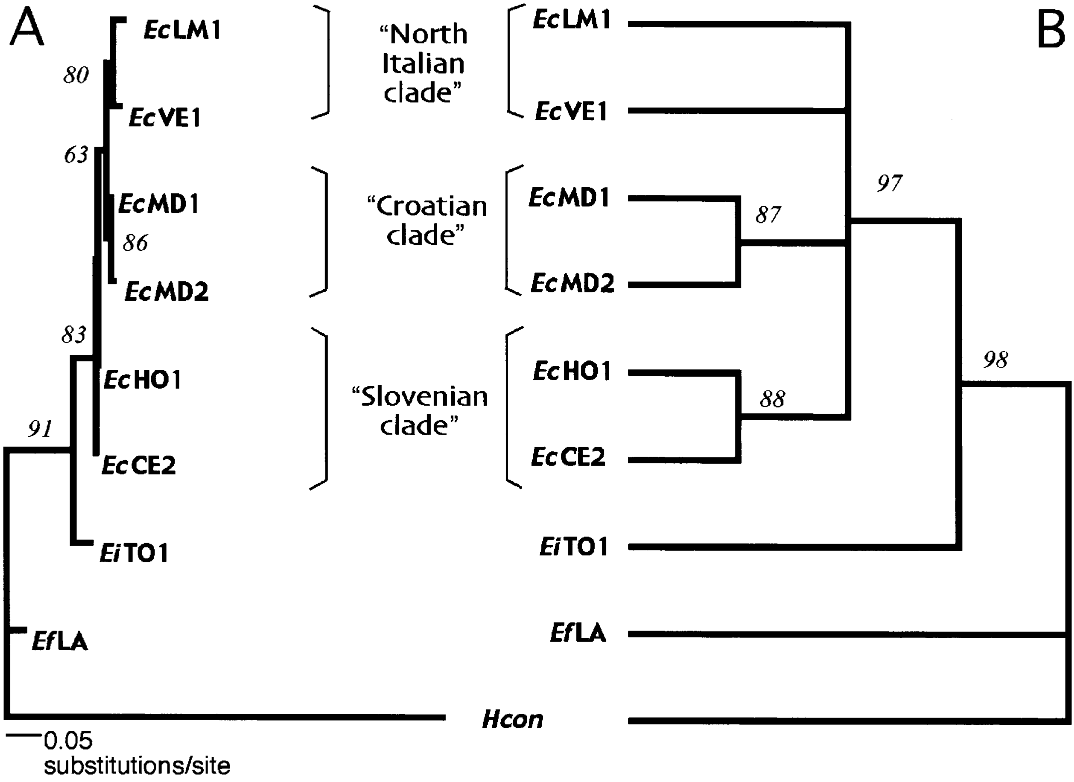

Fig. 2: Phylogeny of *Euscorpius carpathicus* 16S mtDNA sequences (373 bp) including three outgroup taxa, *E. italicus* (*Ei*TO1), *E. flavicaudis* (*Ef*LA) and *Hadrurus concolor* (Iuridae) (*Hcon*). A Maximum Likelihood (ML) tree (-ln L = 1056.90) of 16S mtDNA sequences of *E. carpathicus* from Austria using the HKY85 + Γ model. The paramters were ML-estimated to $\pi_A$=0.39, $\pi_C$=0.13, $\pi_G$=0.11, and $\pi_T$=0.37, $\alpha$=0.21, transition (ti)/transversion (tv) ratio = 3.13627 (κ = 8.50132); B Strict consensus tree of weighted Maximum Parsimony analysis (weighting ti three times over tv). The exhaustive tree search revealed three equally parsimonious trees with 134 steps (CI=0.93, RI=0.79). Numbers at nodes correspond to bootstrap values > 70%. Bootstrap values in (A) are from Neighbour-Joining (NJ) analysis. Abbreviations for haplotypes are given in **Material & methods.**

common (Goyffon *et al.*, 1992); certain species of the European–Mediterranean genus *Euscorpius* established viable, reproducing populations in such places as Great Britain and Uruguay (Benton, 1992; Toscano-Gadea, 1998; Fet & Sissom, 2000).

The origin of the Austrian *Euscorpius* species was also discussed in Thaler & Knoflach (1995). These authors doubt the autochthonous origin of the Austrian populations of *E. carpathicus*.

A similar artificial transplantation was suspected for the isolated Austrian finding places of *Euscorpius germanus* (C. L. Koch, 1837) in the Inn Valley between Landeck and Kufstein (Thaler & Knoflach, 1995). This species is usually found south of the Reschenpass (1510 m). A recent phylogeographic study (Gantenbein *et al.*, 2000) that applied the same mitochondrial markers as the present study, showed that the populations of the Inn valley must have passed very recently via the Reschenpass. Because *E. germanus* was found up to 2200 m (Braunwalder & Tschudin, 1997), we cannot fully exclude natural colonization. However, given the low genetic distances calculated from allozyme data between these Inn populations and the populations from the region of Trentino-Alto Adige, and that these populations share an identical haplotype this hypothesis seems very unlikely.

It would be interesting to see if the only Central European population of *E. carpathicus* to the north of Austria (near Prague in Czech Republic, recorded since the 1950s) is related to the Austrian populations; however, Kovařík (1998) indicates that this population might be already extinct.

Regardless of their possible introduced origin, populations of *E. carpathicus* in Austria should be protected as a rare natural object (Komposch & Scherabon, 1999; Komposch & Komposch, 2000). Currently, such protection is secure at Burg Hochosterwitz, which is a historical castle. The Krems and Federaun sites are both private residential properties.

From these phylogenetic analyses it becomes obvious that different phylogenetic lineages are present in *E. carpathicus,* and that there is evidence that this species is truly polytypic as suggested by Caporiacco (1950). We uncovered the presence of a "North Italian lineage", a "Slovenian lineage" and a "Croatian lineage" (Fig. 2A, B). However, in order to recover true phylogeographic patterns and to evaluate the validity of the 22 formally valid subspecies of *E. carpathicus* (Fet & Sissom, 2000), population samples from around the Mediterranean region are urgently needed, covering the entire range of this species (or species complex). The reconstruction of true phylogeography might be especially difficult in this species because of human introductions, such as reported in this paper.

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