Genetic cohesion of Eresus walckenaeri (Araneae, Eresidae) in the eastern Mediterranean

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The eresid spider genus Eresus is morphologically and ecologically conservative. At least three species occur in Europe. However, deep genetic divergence among geographical samples within two species, E. cinnaberinus and E. sandaliatus, may suggest more cryptic species. In the present study we investigate the genetic cohesion of the third species, Eresus walckenaeri, throughout its eastern Mediterranean distribution range, relative to the E. cinnaberinus–E. sandaliatus species complex. Eresus walckenaeri specimens were monophyletic. Genetic discreteness of E. walckenaeri in a region of sympatry with its sister species in Greece provides evidence for species integrity of E. walckenaeri within the European Eresus species complex. Eresus walckenaeri exhibited high concordance between geographical location and mtDNA genealogy. Two major phylogeographical clades were found in the Greek–Turkish and Syrian–Israel parts of the investigated area, respectively (~6.5% sequence divergence). Concordance between geography and genetic divergence was further observed between Aegean island samples and their corresponding Greek and Turkish mainland samples, suggesting regional subdivision with gradual but potentially high dispersal propensity. Monophyly and limited regional distribution indicate Mediterranean endemic origin. © 2005 The Linnean Society of London, Biological Journal of the Linnean Society, 2005, 86, 1–9.


INTRODUCTION

Cryptic species diversity arises when reproductive isolation evolves faster than morphological diversification. Several processes may rapidly enhance reproductive isolation, including disruption selection for differential resource utilization and sexual selection (e.g. Rice, 1987; Panhuis et al., 2001). Secondary contact between vicariant lineages may further shape diversification. This may be a common feature of regions where species ranges are continuously redistributed due to, for example, climatic oscillations (Hewitt, 1996; Comes & Kadereit, 1998). Upon secondary contact, reinforcement may strengthen isolation (e.g. Coyne & Orr, 1997; Bordenstein, Drapeau & Werren, 2000); otherwise, renewed gene flow can either break down specificity (Rhymer & Simberloff, 1996; Clarke et al., 2002) or, alternatively, facilitate new specific trajectories via introgressive hybridization (Arnold, 1997; Harani & Ramachandra, 2003). Quantifying historical distribution (dispersal) as well as delimiting genetic variance among species variants therefore provides insights into processes that shape species-level complexes (Templeton, 2001).

The spider genus Eresus Walckenaer, 1805 (Araneae: Eresidae) is morphologically conservative. At least three species occur in Europe, Eresus walckenaeri Brullé, 1832, E. cinnaberinus Olivier, 1789, and E. sandaliatus Martini & Goeze, 1780. The latter has only recently been elevated to species rank (Ratschker & Bellmann, 1994, 1995) and there may be more cryptic species (Bellmann, 1997). All three species live underground in silk-lined burrows. Morphological identification is based mostly on male leg coloration and female head colour and size. The pedipalp conductors differ slightly between E. cinnaberinus and
E. sandaliatus but species determination is complicated by intraspecific variation. Common for the two species presently recognized in northern and eastern European, E. cinnaberinus and E. sandaliatus, are highly structured populations (Johannesen et al., 1998; Johannesen & Veith, 2001). This type of population structure, coupled with repeated range shifts during interglacial periods, may encourage rapid genetic divergence. Indeed, the variant E. sandaliatus has divergence genetically for allozymes and is morphologically distinct, despite being part of an E. cinnaberinus mtDNA sublineage (Johannesen & Veith, 2001). Thus, E. cinnaberinus is paraphyletic; it includes three ancient lineages that are geographically distributed with origins corresponding to Western Europe, the Balkans and Italy. Eresus sandaliatus is included in the Balkan sublineage, and is therefore possibly an ‘island’ offshoot. However, evidence for introgression between E. sandaliatus and E. cinnaberinus in an area of post-glacial colonization in Germany (Johannesen & Veith, 2001) questions the species integrity of the European E. cinnaberinus and E. sandaliatus as defined by the biological species concept.

Although E. sandaliatus belongs to the paraphyletic mtDNA E. cinnaberinus-Balkan clade, it has diverged genetically for allozymes and morphologically away from other clade members. If one considers that E. cinnaberinus holds ancient lineages, this may imply several cryptic species within the morphotype. In this case, morphological and genetic differentiation have not reacted jointly. The third European Eresus species, Eresus walckenaeri, seems to constitute an eastern Mediterranean isolate, although distribution ranges of E. cinnaberinus and E. walckenaeri overlap west of the Bosphorus (Bellmann, 1997). Eresus walckenaeri is common in Greece, the Aegean and Turkey (Bellmann, 1997) and is reported from Sicily (University Catania spider checklist). Morphological identification of E. walckenaeri is based on male leg coloration, which is longitudinally striped with yellowish hairs, and female size, which is twice that of E. cinnaberinus. Typically, E. walckenaeri builds webs under stones in xerothermic habitats but can build nests on and under bark at heights of up to 1.5 m (our pers. observ.). It is found from sea level and up to 1200 m elevation. It is presumed that dispersal can take place by ballooning (Ratschker, 1995; Bellmann, 1997).

The aim of the current paper is to investigate the genetic cohesion of the species variant E. walckenaeri throughout its eastern Mediterranean distribution. First we study the phylogenetic position of E. walckenaeri relative to the E. cinnaberinus species complex for delimitation of E. walckenaeri as a cohesive genetic entity (which is not the case for E. cinnaberinus). Hereafter, we study the phylogeography and historical demography of E. walckenaeri based on the geographical distribution of mitochondrial DNA lineages on islands and surrounding mainlands. In this respect we address endemism of E. walckenaeri.

MATERIAL AND METHODS

Some confusion pertains to the nomenclature of Eresus walckenaeri. In this paper we follow the nomenclature used by the World Spider Catalog (Platnick, 2004).

Spiders were collected in the eastern Mediterranean between 1997 and 2002 (Fig. 1, Table 1). Spiders were brought live to the laboratory and conserved in an ultra-low-temperature freezer at −80 °C until analysis.

For analyses we sequenced partial 16S and ND1 genes of the mitochondrial DNA. The sequencing protocol and primers are identical to those given in Johannesen & Veith (2001). Sequences were aligned using the program Sequence Navigator (ABI). Subsequently, all aligned sequences were checked manually. A partition-homogeneity test implemented in PAUP was employed to test whether the partial 16S and ND1 genes could be analysed combined.

We used two approaches to evaluate molecular evolution of E. walckenaeri haplotypes. First, we tested for monophyly of E. walckenaeri haplotypes relative to European E. cinnaberinus [GenBank accession nos. AF374171 (Western Germany, Rotenhels), AF374175 (Italy, Lago di Gada), AF374176 (Greece, Milotades)] and E. sandaliatus [AF374177 (Denmark, Gammel Rye)] (Johannesen & Veith, 2001) and an Eresus from Morocco (species uncertain; this study). To view in more detail the ambivalent mtDNA phylogenetic position of E. sandaliatus within E. cinnaberinus please refer to Johannesen & Veith, 2001.) Outgroup was the eresid Stegodyphus lineatus (AF374183). Phylogenetic analyses were performed with PAUP version 4.08b for the Macintosh (Swofford, 1999) applying maximum parsimony (MP), neighbour joining analysis (NJ) and maximum likelihood (ML). For MP all characters were weighted equally. Indels were treated as a fifth base. Haplotype relationships were analysed with a heuristic search with random addition of sequences. For NJ analysis we chose the distance model with the highest likelihood ratio found by MODELTEST 3.06 (Posada & Crandall, 1998). The likelihood ratio was estimated among in-group haplotypes (i.e. Eresus species). The gamma distribution was estimated from the data. For all tree algorithms a strict consensus tree was computed based on bootstrap search. For MP and NJ we performed 2000 replicates. Owing to computational limits we performed only 100 resamplings for ML. We
Figure 1. Sample locations of *Eresus walckenaeri* in the eastern Mediterranean.

Table 1. Sampling locations and haplotype distributions of *Eresus walckenaeri*

<table>
<thead>
<tr>
<th>Area</th>
<th>Locality</th>
<th>Coordinates N/E</th>
<th>Coordinates N/E</th>
<th>Haplotype</th>
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</thead>
<tbody>
<tr>
<td>1. Greek Mainland</td>
<td>Pindos Mts</td>
<td>39 34/21 04 3 1</td>
<td></td>
<td>1 1</td>
</tr>
<tr>
<td>2. Peloponnesus</td>
<td>Zachlourou</td>
<td>38 07/28 10 2</td>
<td></td>
<td>1 1</td>
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<tr>
<td></td>
<td>Kalávrita</td>
<td>38 02/28 06 1</td>
<td></td>
<td>1 1</td>
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<tr>
<td></td>
<td>Petra</td>
<td>37 25/21 56 6</td>
<td></td>
<td>1 1 1 1 1</td>
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<tr>
<td></td>
<td>Dirrachi</td>
<td>37 11/22 13 2</td>
<td></td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>Evrotas-Valley</td>
<td>36 59/22 36 2</td>
<td></td>
<td>1 1 1 1 1</td>
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<tr>
<td></td>
<td>Itilio</td>
<td>36 42/22 23 3</td>
<td></td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>3. Crete</td>
<td>Zenia</td>
<td>35 13/25 36 4</td>
<td></td>
<td>1 1</td>
</tr>
<tr>
<td>4. Rhodes</td>
<td></td>
<td>36 25/28 09 1</td>
<td></td>
<td>1 1</td>
</tr>
<tr>
<td>5. Turkey</td>
<td>Hurma</td>
<td>36 51/30 35 4</td>
<td></td>
<td>1 1</td>
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<tr>
<td></td>
<td>Arif</td>
<td>36 30/30 03 2</td>
<td></td>
<td>1 1</td>
</tr>
<tr>
<td></td>
<td>Gögceovaçik</td>
<td>36 47/28 59 1</td>
<td></td>
<td>1 1</td>
</tr>
<tr>
<td>6. Bulgaria</td>
<td>Harmanli</td>
<td>41 57/25 53 1</td>
<td></td>
<td>1 1</td>
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<tr>
<td>7. Israel</td>
<td>Mt. Hermon</td>
<td>33 19/35 48 1</td>
<td></td>
<td>1 1</td>
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<tr>
<td>8. Syria</td>
<td>Slunfeh, northern</td>
<td>35 40/36 13 1</td>
<td></td>
<td>1 1</td>
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<tr>
<td></td>
<td>Jabal Ansarija Mts</td>
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<td>13 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</td>
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tested the goodness by running 100 000 quartet puzzles and compared the topology of the original ML consensus tree with the quartet puzzle tree. They did not differ. Branch-swapping was computed with the tree bisection–reconnection algorithm. Second, to analyse the geographical distribution of genetic divergence within *E. walckenaeri*, we constructed a minimum spanning network using the program TCS 1.13 (Clement, Posada & Crandall, 2000). The network differs from traditional cladistic methodologies by acknowledging the presence of tree-internal haplotypes.

Historical dispersal can be tested if mtDNA haplotypes evolution is neutral, which is expected when edging the presence of tree-internal haplotypes. From traditional cladistic methodologies by acknowledging the presence of tree-internal haplotypes.

The network differs from traditional cladistic methodologies by acknowledging the presence of tree-internal haplotypes. Historical dispersal can be tested if mtDNA haplotypes evolution is neutral, which is expected when edging the presence of tree-internal haplotypes.

RESULTS

The 557 characters assayed from 34 *E. walckenaeri* individuals revealed 17 haplotypes (Table 1) containing 59 variable sites [accession nos. AF374181, AF374182 (Johannesen & Veith, 2001); AY739876–739893 (this study)]. Haplotype 16 had a unique 5-bp deletion at position 101–105. This deletion was weighted as a single mutation (gap) in parsimony and TCS analyses; in all other analyses gaps were deleted from analysis.

A partition-homogeneity test including outgroups indicated similar evolution of the 16S and ND1 genes included in analysis (P > 0.90).

An MP heuristic search for delimiting *E. walckenaeri* found a single most parsimonious tree (tree length 304, CI = 0.678, RI = 0.754): 381 characters were constant, 69 variable characters were parsimony-uninformative and 107 characters were parsimony-informative. The Tamura–Nei distance model (TrN+G) was the model with the highest likelihood ratio, –LnL = 1729.3550, gamma distribution of rate heterogeneity α = 0.2743. A heuristic search retained two trees with a minimum evolution score = 1.34976. The two trees differed in the relative positions of haplotypes 13 and 14. ML analysis found one most likely tree, –Ln = 1994.73956. All three heuristic searches found monophyly of *E. walckenaeri* but differed by how *E. cinnaberinus* haplotypes were positioned within the clade. Bootstrap analysis for all algorithms (MP, NJ, ML) supported monophyly of *E. walckenaeri* with high bootstrap scores (Fig. 2).

*Eresus walckenaeri* haplotypes grouped into two major clades (Fig. 2). An ‘Aegean’ group consisted of Turkish, Greek and Bulgarian spiders: nucleotide diversity, Π = 0.0137; average number of pairwise differences, k = 7.456. The second, ‘Levant’, group consisted of the two spiders from Syria and Israel, which differed by 4 bp.

The mean sequence divergence (uncorrected *p* distances) between *E. walckenaeri*, *E. cinnaberinus*–*E. sandalitus* and the Moroccan *Eresus* for the total 16S/ND1 sequence was c = 0.115–0.125. This was twice that between *E. walckenaeri* Aegean and Levant clades, c = 0.065. Incidentally, this latter value is equivalent to the mean divergence among the three major European *E. cinnaberinus*–*sandalitus* lineages, 0.068. Relative divergence of the 16S and ND1 subsequences behaved as the total sequence. 16S divergence ranges were as follows: between species, 0.061–0.093; between Aegean and Levant *E. walckenaeri*, 0.040–0.056; between *E. cinnaberinus* lineages, 0.031–0.061. ND1 divergence ranges were: between species, 0.140–0.168; between Aegean and Levant *E. walckenaeri*, 0.072–0.081; between *E. cinnaberinus* lineages, 0.071–0.085.

TCS analysis of the Aegean group revealed a haplotype network with four sublineages (Fig. 3). Two sublineages (A and B) were found syntopically in one population in the middle of Peloponesus (Table 1). Otherwise lineage A was found only in north Peloponesus whereas lineage B, although found in all Greece, dominated on south Peloponesus and Crete. The third sublineage, C, was found on the Greek mainland. It had a phylogenetically and geographically intermediate position to lineage D, which was found in southeastern Turkey and on the nearby island of Rhodes (Figs 3, 4). The mean divergence (uncorrected *p* distances) among the Aegean clades ranged between 0.7 and 3% (Table 2).

Tests for molecular clock evolution of haplotypes (branch lengths) were equivocal. The total sequence
**Figure 2.** Neighbour-joining tree showing monophyly of *Eresus walckenaeri*. Bootstrap scores for MP, NJ (TrN+G) and ML analyses, respectively. Bootstrap scores lower than 50 are denoted with a dash.

**Figure 3.** Minimum spanning networks of Aegean and Levant *Eresus walckenaeri* haplotype clusters; the joining position of the networks is indicated with an asterisk.

was not in accordance with a molecular clock (likelihood ratio test statistic delta: 32.60, d.f. 19, \( P < 0.05 \)). Sequential exclusion of haplotypes identified haplotype 15 as the cause of the deviation. Haplotype 15 occupies a genealogical tip position (Fig. 3). However, tested separately and including haplotype 15, neither the 16S nor the ND1 subsequence deviated from a molecular clock (\( P > 0.75 \) and \( P > 0.30 \), respectively). We therefore assume that the tip position, which involves five substitutions, caused rejection of the molecular clock for the total sequence. As all other sequences conformed to clock expectations and haplotype 15 was observed only once, we proceeded with the demographic analysis assuming neutrality. Neither Fu and Li’s \( D^* = 0.35, P > 0.10 \), nor \( F^* = 0.089, P > 0.10 \), deviated significantly from expectation.

The mismatch distribution of Aegean animals was bimodal. The first peak included zero values while the second peak arose from distances between sublineages. The mismatch was marginally but not significantly different from a sudden expansion model (Fig. 5) (\( N = 32 \); mean mismatch \( = 8.28, SSD = 0.043, P = 0.09 \); raggedness index \( r = 0.047, P = 0.10 \)). Lack of significance was true also for Greek spiders (excluding Rhodes) (\( N = 23 \); mean mismatch \( = 7.95, SSD = 0.067, P = 0.30; r = 0.057, P = 0.65 \)) and for Peloponnesus spiders (\( N = 16 \); mean mismatch \( = 6.58, SSD = 0.058, P = 0.42; r = 0.097, P = 0.77 \)).

**Figure 4.** Distribution of haplotype clades as observed from the current data set showing a discrete geographical divergence between Greece (areas A, B and C), Turkey (D) and the Levant (E).

**Table 2.** Mean sequence divergence (uncorrected \( p \) distances) among the Aegean clades A–D. Above diagonal, ND1; below diagonal, 16S

<table>
<thead>
<tr>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td>A</td>
<td>–</td>
<td>0.030</td>
<td>0.012</td>
<td>0.021</td>
</tr>
<tr>
<td>B</td>
<td>0.019</td>
<td>–</td>
<td>0.024</td>
<td>0.025</td>
</tr>
<tr>
<td>C</td>
<td>0.018</td>
<td>0.012</td>
<td>–</td>
<td>0.019</td>
</tr>
<tr>
<td>D</td>
<td>0.014</td>
<td>0.008</td>
<td>0.007</td>
<td>–</td>
</tr>
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</table>

**Figure 5.** Mismatch distribution for Aegean *Eresus walckenaeri*. Full line, observed mismatch; dashed line, mismatch expected under population expansion.
DISCUSSION

Phylogenetic analysis of Mediterranean Eresus walckenaeri revealed a cohesive genetic group relative to the E. cinnaberinus–sandaliatus species complex. The genetic divergence of E. walckenaeri was dominated by gradual change on a regional level, climaxing in the Aegean and Levant groups. The strict division in the Aegean and Levant groups may be caused by small sample size. In other organisms, by contrast, this distribution corresponds to genetic–biogeographical divisions (e.g. Suzuki et al., 1996; Orth, Auffray & Bonhomme, 2002). Population subdivision could even be found on a local scale on Peloponnesus where two lineages (A and B), although found in all Greece, predominantly were found in northern and southern individuals, respectively, and meet at the centre of the peninsular. These findings suggest restricted gene flow in E. walckenaeri. However, dispersal ability may be higher than the regional division implies because island samples from Crete and Rhodes had similar or identical haplotypes to the those on the nearest mainland. However, anthropogenic dispersion, e.g. in logs, in historical time may also explain wide dispersal and the successful crossing of saltwater barriers.

Given the regional division one might expect the demographic imprint from mismatch distributions to show old stationary populations. However, despite pronounced regional division and bimodal mismatch distribution of the Aegean samples, the mismatch did not deviate significantly from a sudden expansion model. The first peak of the bimodal mismatch distribution was characterized by zero values caused by two haplotypes common on Peloponnesus and in Turkey, respectively, while the second peak was dominated by divergence between regional clades. A unimodal peak, characteristic of an expanding population, may show up in subdivided populations if these were structured in recent time but had random mating before that (Rogers, 1995). Limited gene flow leads to relatively rapid coalescence events (zero values) and to bimodal mismatch distributions with many zero values, while the phylogenetic tree shows short and long branches (Ray, Currat & Excoffier, 2003). Such a tree was observed for E. walckenaeri. Thus, the concordance between geography and genealogy, and the phylogenetic tree of E. walckenaeri may support contemporary separation in regional populations but range expansion throughout the Aegean in the recent past. The branching pattern of the Aegean phylogenetic tree suggests a historical split between a ‘southern lineage’ (clade B) and an early expanding ‘northern lineage’, consisting of clades A, C and D, that settled through northern Greece to Turkey. The two now meet on Peloponnesus. The presence of the divergent Aegean sublineage B on Peloponnesus is in agreement with a continuous presence in Greece throughout periods of Pleistocene climate fluctuations and corroborates that the Balkans show the highest biodiversity of spiders in Europe (Delschev, 1999). Although we do not know the exact rate of divergence for the ND1 gene in Eresus, the standard rate divergence of 2% per million years for ND1 (Desalle et al., 1987) suggests that the Aegean clades started diverging about 750 000 years ago. This date correlates exactly with the onset of the last intense glaciations due to Milankovitch oscillations 700 000 years ago. The distribution of genetic variability therefore suggests a two-level process of local dispersal but restricted gene flow between regions. It furthermore suggests gradual dispersal rather than repeated admixture of discrete lineages, implying range expansion during warm (interstadial) periods and range contraction during cold (stadial) periods. This pattern corresponds broadly to that of the Aegean water frogs Rana ridibunda and R. bedriagae, in which gradual differentiation from Turkish to Greek populations started around 1 Mya (Beerli, Hotz & Uzzell, 1996).

Certainly E. walckenaeri has diverged both genetically and morphologically relative to its two European sister species. Assuming a 2% rate of divergence per Myr for ND1, the species split occurred about 3.5–4 Mya during an extended cold period in the early Pliocene (Müller, 1985). This extended cold period probably triggered speciation in several organisms in the Mediterranean (Bianco, 1990; Veith, Kosuch & Vences, 2003). Given that E. walckenaeri is monophyletic and restricted in occurrence, sequence linearity supports that it originated within the region presently occupied rather than it being a relic from past invasions.

The question remains as to whether Aegean and Levant lineages of E. walckenaeri and the ancient E. cinnaberinus lineages have diverged enough to be considered separate (sub)species? Deep sequence divergence may not be uncommon in morphologically and ecologically conservative species of spider but may also be due to cryptic species (Bond et al., 2001; Hedin & Wood, 2002). The splits between the E. walckenaeri Aegean and Levant clades probably occurred during the same geological period as that that led to the three ancient E. cinnaberinus clades in Europe (Johannesen & Veith, 2001). This period corresponds to the fluctuating cold period in the late Pliocene about 1.8–2 Mya. Studies of the Turkish herpetofauna have shown that Pleistocene climatic oscillations caused parapatric distributions that led to incipient species by ‘trapping’ lineages in refugial areas (Tarkhnishvili, Hille & Böhme, 2001; Veith et al., 2003a, b). The deep intraspecific divergence in Eresus is indeed based on regional separation. The three ancient E. cinnaberin-
Eresus lineages are found, as far as we know, west of the Rhine, east of the Rhine (possibly as far as Iran, our unpubl. data) and Italy south of the Alps. However, the one exception to regional isolation, Eresus sandaliatus, is included within the east Rhine (Balkan) lineage, thus making E. cinnaberinus paraphyletic. The incongruence between sequence divergence and morphological divergence in the morphotype E. sandaliatus suggests that it has undergone a major (behavioural) shift, faster than the level of sequence divergence. Morphological divergence of E. sandaliatus relative to E. cinnaberinus is associated with areas where the species have probably experienced contact during post-glacial range expansion. Perhaps morphological diversity is triggered only in contact zones? Secondary contact during interglacial periods may have reinforced the discreteness of E. sandaliatus but kept stasis of the remaining non-mixing E. cinnaberinus. Future studies must be conducted to reveal whether more Eresus species exist. Although the status of the ancient intraspecific lineages remains uncertain, the picture that emerges for E. walckerneari compared with E. cinnaberinus–E. sandaliatus is one of species distinction and range conservation in the eastern Mediterranean.

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