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# Scorpion speciation in the Holy Land: Multilocus phylogeography corroborates diagnostic differences in morphology and burrowing behavior among *Scorpio* subspecies and justifies recognition as phylogenetic, ecological and biological species \*



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#### ABSTRACT

Scorpio Linnaeus, 1758 (family Scorpionidae Latreille, 1802) was considered monotypic for over a century, and comprised a single species, Scorpio maurus Linnaeus, 1758, with 19 subspecies, distributed from West Africa, throughout the Maghreb and the Middle East, to Iran. Two parapatric subspecies, Scorpio maurus fuscus (Ehrenberg, 1829) and Scorpio maurus palmatus (Ehrenberg, 1828), have long been recognized in the eastern Mediterranean region. We examined morphological variation, burrow architecture and genetic divergence among 39 populations across the distribution of the two subspecies to assess whether they are conspecific and, if not, how many species might be involved. Cuticle coloration, pedipalp chela digital carina condition, and selected measurements were recorded. Sixty burrows were excavated and examined for burrow structure and depth. A multilocus dataset comprising concatenated fragments of one nuclear (28S rDNA) and three mitochondrial (12S rDNA, 16S rDNA, Cytochrome c Oxidase Subunit I) loci, totaling ca. 2400 base-pairs, was produced for 41 individuals, and a single-locus dataset comprising 658 base-pairs of the COI locus for 156 individuals. Despite overlapping ranges in morphometric characters of pedipalp chela shape, the putative subspecies were easily distinguished by cuticle coloration and condition of the pedipalp chela digital carina, and were also found to differ significantly in burrow architecture and depth. Phylogeographical analyses of the COI and multilocus datasets recovered seven distinct clades. Separate analyses of mitochondrial sequences, and combined analyses of mitochondrial and nuclear sequences support most clades. The two major clades corresponded with the geographical distributions of S. m. fuscus and S. m. palmatus in the region. Specimens from these clades were genetically distinct, and exhibited different burrow structure in geographically-proximate localities, suggesting reproductive isolation. The palmatus clade included two distinct subclades of specimens from localities adjacent to the Dead Sea. Three other clades, comprising specimens from the most northeastern localities, were tentatively assigned to subspecies previously recorded in neighboring Jordan and Syria. The morphological, behavioral and genetic evidence supports previous suggestions that Scorpio maurus is a species complex and justifies the following taxonomic emendations: Scorpio fuscus (Ehrenberg, 1829), stat. nov.; Scorpio kruglovi Birula, 1910, stat. nov.; Scorpio palmatus (Ehrenberg, 1828), stat. nov.; Scorpio propinquus (Simon, 1872), stat. nov.

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## 1. Introduction

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*Scorpio* Linnaeus, 1758 is one of four genera in the Old World scorpion family Scorpionidae Latreille, 1802 (Prendini et al., 2003; Prendini and Wheeler, 2005). The genus originally

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accommodated a number of species (e.g., Simon, 1872; Pocock, 1900), some of which were subsequently transferred to other genera (e.g., *Heterometrus* Ehrenberg, 1828 and *Pandinus* Thorell, 1876). By 1910, however, only a single species, *Scorpio maurus* Linnaeus, 1758, containing 11 subspecies, was recognized (Birula, 1910). Additional subspecies were subsequently added (Pallary, 1928; Werner, 1929, 1932, 1936; Schenkel, 1949; Bouisset and Larrouy, 1962) but the genus remained monotypic for almost a century, by which time *S. maurus* comprised 19 recognized subspecies, distributed from West Africa, throughout the Maghreb and the Middle East, to Iran (Fet, 2000).

Levy and Amitai (1980) stated that there are no reliable characters to distinguish the subspecies of S. maurus, and agreed with Vachon (1950) that the absence of such characters precludes their recognition as species. Nevertheless, the mostly allo- or parapatric subspecies of S. maurus could be distinguished on the basis of somatic characters presented in several identification keys (Birula, 1910; Vachon, 1950, 1952; Levy and Amitai, 1980) leading Prendini et al. (2003) to suggest that they are in fact diagnosable (i.e., phylogenetic) species. The limited ecological data available also suggested that multiple ecological species, with different climatic requirements and ecological niches, might be involved across the broad distribution of S. maurus. For example, Scorpio maurus palmatus Ehrenberg, 1828 occurs near the Dead Sea, 400 m below sea level, whereas Scorpio maurus fuliginosus (Pallary, 1928) occurs at 3000 m in the Atlas Mountains, thousands of kilometers away (Levy and Amitai, 1980). Although all subspecies construct and live in burrows with characteristic oval or crescent-shaped entrances (Levy and Amitai, 1980; Prendini et al., 2003), burrows vary in length, depth and configuration (straight, or with twists and turns).

More recently, considerable divergence in DNA sequences from the mitochondrial Cytochrome c Oxidase Subunit I (COI) gene, used for DNA barcoding (Hebert et al., 2003), was reported among subspecies of *S. maurus* in Morocco (Froufe et al., 2008). The following year, seven Moroccan subspecies of *S. maurus* were elevated to the rank of species (Lourenço, 2009), while four new species of *Scorpio* were described from Cameroon, Chad, Niger and Sudan, extending the known distribution of the genus within sub-Saharan Africa (Lourenço, 2009; Lourenço and Cloudsley-Thompson, 2009, 2012; Lourenço et al., 2012).

Two subspecies of S. maurus, distinguished by the coloration of the cuticle and subtle differences in the shape and carination of the pedipalp chelae, have long been recognized from the eastern Mediterranean region, including Israel, Sinai, the Golan Heights and the Palestinian territories (Levy and Amitai, 1980). Scorpio maurus fuscus (Ehrenberg, 1829) is dark brown to greenish black in color (Fig. 1A) whereas S. m. palmatus is light olive-brown to yellow (Fig. 1B). The two subspecies are parapatric, occurring in different habitats, and appear to have distinct ecological requirements (Fig. 2A). Scorpio m. fuscus is restricted to areas with a Mediterranean climate, where the average rainfall may exceed 800 mm, in the northern coastal plain, northern Israel and Lebanon, and occurs mostly on terra rossa, basalt, rendzina and brown-red sandy soils. Scorpio m. palmatus is restricted to arid and semi-arid habitats of the southern coastal plain, Negev and Judaean deserts, extending through Sinai to Egypt, where the annual rainfall may average less than 50 mm, and occurs on brown-red sandy soils, loess, alluvial soils and in stony desert. Burrow architecture and depth also appear to differ between the two subspecies (Levy and Amitai, 1980). Scorpio m. fuscus usually constructs a simple, straight tunnel to a depth of about 40 cm and the burrow entrance is usually situated at the base of a stone, whereas S. m. palmatus usually constructs a spiral burrow up to 70 cm deep, with the opening always situated in open ground. Physiological studies revealed that S. m. fuscus and S. m. palmatus differ in several traits concerning water relations, conferring better desiccation resistance to *S. m. palmatus*, which occurs in more arid areas (Gefen and Ar, 2004; Gefen, 2011; Kalra and Gefen, 2012).

Levy and Amitai (1980) reported that both subspecies co-exist in the northern Negev and Judaean foothills (Fig. 2A), where intermediate color morphs are found, and suggested this was evidence for hybridization (and, hence, a single biological species). Morphological and ecological differences between the two subspecies, mixed color populations (Fig. 1C and D), and suggestions that *S. maurus* is a species complex (Prendini et al., 2003; Froufe et al., 2008) motivated us to examine morphological variation, burrow architecture, and genetic divergence among populations of *S. m. fuscus* and *S. m. palmatus* in the eastern Mediterranean region to assess whether they are conspecific and, if not, how many species might be involved. We analyzed DNA sequences from four gene loci, both mitochondrial and nuclear, separately and in combination.

#### 2. Material and methods

#### 2.1. Fieldwork and material

Scorpions were collected from 39 sites in Israel, the Golan Heights and the Palestinian territories, hereafter referred to as the 'study area' (Fig. 2B; Supplementary material S1), by turning stones and excavating burrows during the day, and by detection with ultraviolet flashlights on warm, moonless nights. After collection, scorpions were preserved individually in 50 ml vials of 99% ethanol and frozen at -20 °C until DNA extraction.

Tissue samples were deposited in the Ambrose Monell Collection for Molecular and Microbial Research at the American Museum of Natural History (AMNH), New York (Supplementary material S2). Adult voucher specimens, collected from the same populations, were deposited in the AMNH Arachnida and Myriapoda Collections.

#### 2.2. Morphological characters

Cuticle base coloration and condition of the digital carina of the pedipalp chela (partially costate vs. entirely granular) were noted for individuals collected (Supplementary material S1, S2). Coloration was assessed by eye in freshly-collected specimens. Pectinal tooth counts and seven measurements, i.e., carapace length and six measurements capturing dimensions of the pedipalp chela (Supplementary material S3), were recorded for 12 male and 35 female specimens from 14 sampling sites. The six pedipalp chela measurements of female specimens were normalized against the first measurement, carapace length, a standard proxy for total body size, and plotted to assess their utility as diagnostic characters.

#### 2.3. Burrow architecture and depth

Sixty burrows of *S. m. fuscus* and *S. m. palmatus* (thirty per subspecies at two sites each) were excavated and examined for burrow structure and depth at four locations across a 33 km north-south transect in southwestern Israel: *S. m. fuscus* at Gevim (site 18) and Nahal Bohu (site 20), and *S. m. palmatus* at Sharsheret (site 25) and Tze'elim (site 29) (Supplementary material S1, Fig. 2B). Each burrow was carefully excavated to expose the burrow structure, from the entrance to the basal chamber. Burrow depth was measured along a perpendicular line, from the surface of the entrance to the surface of the terminal chamber, using a spirit level and a ruler. The configuration of each burrow was captured by a count of the number of curves with angles greater than 30°.



**Fig. 1.** Typical coloration of the species of *Scorpio* Linnaeus, 1758 occurring in the eastern Mediterranean region, as recognized in the present contribution: *Scorpio fuscus* (Ehrenberg, 1829) (A) from the Nofit (site 7) and *Scorpio palmatus* (Ehrenberg, 1828) (B) from Sde Boqer (site 37), both in Israel. Individuals with variable cuticle coloration from Susita (site 6; C, D) and Buq'ata (site 3; E, F) in the Golan Heights, assigned to *Scorpio kruglovi Birula*, 1910 and *Scorpio propinquus* (Simon, 1872), respectively. Site numbers follow Supplementary material S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

#### 2.4. Taxonomic and geographical sampling

The outgroup comprised a specimen of *S. fuliginosus* from Morocco. The ingroup comprised 85 brown individuals corresponding to *S. m. fuscus* and 70 yellow individuals corresponding to *S. m. palmatus*, collected throughout the distribution of the two putative subspecies in the study area (Fig. 2B; Supplementary material S1). Fifteen sampling sites were located in the northern part of the study area (Upper and Lower Galilee, northern Jordan Valley and Golan Heights) and 24 in the southern part (Negev and Judaean deserts; Supplementary material S1).

#### 2.5. DNA sequencing

DNA isolation, PCR amplification and sequencing were conducted at the Department of Biology and Environment, University of Haifa – Oranim and the AMNH Sackler Institute for Comparative Genomics, using standard protocols (Prendini et al., 2002, 2003, 2005) and primers (Supplementary material S4). A multilocus dataset was produced for 41 individuals (21 brown individuals corresponding to *S. m. fuscus*, 19 yellow individuals corresponding to *S. m. palmatus*, and the outgroup, *S. fuliginosus*) from four gene markers selected based on previous studies of scorpions and other arachnids (Prendini et al., 2003, 2005): a 329–331 base-pair (bp) fragment of the 12S rDNA (12S), a 482–483 bp fragment of the 16S rDNA (16S) and a 1078 bp fragment of the Cytochrome c Oxidase Subunit I (COI), from the mitochondrial genome, and a 514 bp fragment of the D3 region of the 28S rDNA (28S), from the nuclear genome. The combined length of the four concatenated fragments was 2403–2406 bp.

A shorter, 658 bp fragment (the anterior 60%) of the COI was sequenced for a further 115 individuals, yielding a more detailed single-locus dataset for 156 individuals (85 brown individuals corresponding to *S. m. fuscus*, 70 yellow individuals corresponding to *S. m. palmatus*, and the outgroup, *S. fuliginosus*). In total, 279 sequences were generated (Supplementary material S2): 41 sequences of 28S, 12S and 16S, and 156 sequences of COI.



**Fig. 2.** Records of *Scorpio maurus fuscus* (Ehrenberg, 1829) and *Scorpio maurus palmatus* (Ehrenberg, 1828) in the eastern Mediterranean region, from Levy and Amitai (1980), superimposed on a climatic map of the region (after Dan and Raz, 1970) (A). Study area showing sampling sites for phylogeographical analysis of the species of *Scorpio* Linnaeus, 1758, recognized in the present contribution, numbered according to Supplementary material S1. Black, yellow and red circles represent clades A (*S. fuscus*), B, F, G (*S. palmatus*) and C, D, E (Golan Heights *Scorpio* spe), respectively (see Section 3) (B). Two pairs of geographically-proximate sampling sites in the northwestern Negev, Nahal Bohu (site 20) and Nahal Sharsheret (site 25) (C), and near Lahav (sites 23, 24) in the northern Negev (D), Israel. Codes in C and D denote Site No.-Color-Sample ID (Supplementary material S2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Genomic DNA was extracted from leg or pedipalp muscle or from the hepatopancreas using the Qiagen DNeasy Blood and Tissue Kit. DNA was amplified using PureTaq-Ready-To-Go PCR Beads (GE Healthcare) or RANGER DNA Polymerase (BIOLINE), 1  $\mu$ l (10  $\mu$ M) of each primer and 1–4  $\mu$ l of DNA template. The PCR program consisted of an initial denaturing step at 94 °C for 5 min, 30–35 amplification cycles (94 °C for 15 s, 49 °C for 10 s, 72 °C for 15 s), and a final step of 72 °C for 7 min, in a GeneAmp PCR System 9700 thermocycler. Specific conditions were optimized for primer pairs (e.g., a lower annealing temperature was used for COI). PCR products were examined on 1–1.5% agarose-TBE gels, and purified with QIAquick columns (Qiagen, GmbH), illustra ExoProstar, or Ampure Magnetic Beads Purification System (Agentcourt), followed by direct sequencing using an ABI automatic sequencer.

The accuracy of sequences was verified by independently amplifying and sequencing the complementary strands of all fragments. Primer sequences were removed and complementary strands of DNA assembled into consensus sequences, edited, and checked for quality using Sequencher 5.0 (Gene Codes Corporation). If complementary strands disagreed (besides minor mismatches), the sample was reamplified and sequenced to resolve discrepancies.

#### 2.6. Phylogeographical analysis

DNA sequence alignment was trivial. There was no difference in length among the 28S and COI sequences, a single bp difference among the 16S sequences and a 2 bp difference among the 12S sequences. Static alignments of the 12S and 16S gene fragments were generated with MEGA6 (Tamura et al., 2013) or MAFFT (Katoh and Standley, 2013) using default settings. The aligned 12S and 16S fragments were then concatenated with the COI and 28S fragments to create the multilocus dataset.

Phylogenetic analyses were performed on the multilocus dataset (combined analysis) (41 sequences with an aligned length of 2405 bp), as well as on the individual loci (separate analysis) of the mitochondrial 12S (41 sequences with an aligned length of 331 bp), 16S (41 sequences with an aligned length of 484 bp) and COI datasets (156 sequences of 658 bp), and the nuclear 28S (41 sequences of 514 bp) (de Queiroz et al., 1995). The separate analyses were performed to determine the phylogenetic signal in the individual loci. Phylogenetic reconstruction was conducted with both maximum likelihood (ML) and parsimony using MEGA6 (Tamura et al., 2013) and PAUP version 4 (Swofford, 1998).

A general time reversible (Lanave et al., 1984) model with gamma distributed rate variation among sites (G), and proportion of invariable sites (I) was selected for the ML analyses using ModelTest (Posada and Crandall, 1998) as it provided the highest Bayesian Information Criterion (Schwarz, 1978) and Akaike Information Criterion (Akaike, 1974) scores among all gene fragments. The parsimony analyses applied 1000 rounds of Subtree-Pruning-Regrafting on ten initial trees (generated with random addition), retaining 100 trees at each, from which a 50% majority rule consensus was generated. Nodal support was assessed with 500 bootstrap replicates for both the ML and parsimony trees, and augmented with jackknife analysis (Farris et al., 1996) for the parsimony tree, using PAUP version 4 (Swofford, 1998).

The topological distinctiveness of the tree obtained by separate analysis of the COI sequences of the 41 samples included in the multilocus dataset was evaluated using the Kishino-Hasegawa Test (KHT; Kishino and Hasegawa, 1989), the Shimodaira-Hasegawa Test (SHT; Shimodaira and Hasegawa, 1999), and the Likelihood Ratio Test (LRT; Stuart and Ord, 2009). Although KHT is the most widely used, the stricter SHT was recommended for ML analyses (Goldman et al., 2000). As the consensus tree obtained from the parsimony bootstrap was used for comparison, rather than the ML topology, the analysis was augmented with an alternative to the conventional LRT (after Anisimova and Gascuel, 2006). The LRT approach deals with nested models where the nested model is a restricted version of the other model, meaning that several variables are confined to pre-assigned values. By definition, the nested model is inferior to the more general model in terms of likelihood score. Hence twice the difference in the (log) likelihood scores is approximately distributed as a two random variable with degrees of freedom as the difference in free variables. In the present context, a contracted branch is therefore equivalent to a zero-length branch. Therefore, when allowing the branch length to take any value, the model was generalized with one more variable, such that a greater likelihood score is expected.

#### 2.7. Genetic divergence

Genetic Divergence was calculated on the COI sequences for 41 individuals collected from 39 localities in the study area, and the outgroup, *S. fuliginosus*, using the Maximum Composite Likelihood model (Tamura et al., 2004) in MEGA6 (Tamura et al., 2013). First, second and third codon positions were included for the COI dataset. Positions containing missing data were omitted. The final dataset comprised 1050 bp. Mantel tests (999 random permutations), comparing between- and within-group dissimilarities, were applied as a non-parametric substitute for Analyses of Variance (Sokal and Rohlf, 2012) and conducted in R (R Core Team, 2013), using the 'vegan' package (Oksanen et al., 2013).

#### 3. Results

#### 3.1. Morphological characters

Cuticle base coloration was very consistent within populations. Most northern sample sites were inhabited by brown individuals corresponding to *S. m. fuscus* (Fig. 1A; Supplementary material S1), but a population of yellow individuals (resembling *S. m. palmatus*) together with one brown individual (resembling *S. m. fuscus*) was discovered at Susita (site 6) in the southern Golan Heights (Fig. 1C and D). This was the only case in which a marked difference in coloration was observed among individuals from the same population. Six of the 24 southern sites (Negev and Judaean desert) were inhabited exclusively by brown individuals corresponding to *S. m. fuscus* and the rest by yellow individuals corresponding to *S. m. palmatus* (Fig. 1B; Supplementary material S1).

The condition of the digital carina of the pedipalp chela was also found to be very consistent within populations. The digital carina was partially costate in all populations with brown coloration (*S. m. fuscus*) and entirely granular in all populations with yellow coloration (*S. m. palmatus*), including all individuals from Susita, the population of yellow individuals including one brown individual (Supplementary material S1).

Meristic data were less informative. Pectinal tooth counts revealed little difference between the sexes either within or between the putative subspecies (Supplementary material S3). The pedipalp chela measurements, normalized against carapace length, revealed a tendency toward longer fingers (resulting in an overall longer chela) and a broader, deeper manus in *S. m. fuscus* than *S. m. palmatus* (Fig. 3). However, the lower ends of the ranges in *S. m. fuscus* overlapped with the upper ends of the ranges in *S. m. palmatus*, limiting their utility as diagnostic characters.

#### 3.2. Burrow architecture and depth

In both putative subspecies, the crescent-shaped burrow entrance leads to a straight section which extends into the soil at an angle of  $20-40^{\circ}$ . In *S. m. fuscus*, the burrow usually continues downwards as a simple, straight or gently curving tunnel whereas, in *S. m. palmatus*, the straight section of the burrow ends with an abrupt curve after 5–10 cm, and is usually followed by another straight segment that ends in one or more curves, creating a spiral structure.

Significant variation in burrow depth across a relatively short transect in the northwestern Negev was observed between two populations of *S. m. fuscus* at Gevim (site 18) and Bohu (site 20) and two populations of *S. m. palmatus* at Sharsheret (site 25) and Tze'elim (site 29; Table 1) These two pairs of localities represent populations situated, respectively, in clades A and B (see below). The burrows of *S. m. palmatus*, constructed in open ground, were,



**Fig. 3.** Pedipalp chela measurements plotted against carapace length (mm), a standard proxy for total body size, for the species of *Scorpio* Linnaeus, 1758 occurring in the eastern Mediterranean region, as recognized in the present contribution. Dataset includes 35 female specimens from 12 localities (Supplementary material S3). Symbols with error bars represent species mean ± standard deviation.

on average, 6–10 cm deeper than those of *S. m. fuscus*, constructed under stones. A distinct difference in structure was also evident between the burrows of *S. m. fuscus* at Gevim (site 18) and Bohu (site 20) and those of *S. m. palmatus* at Sharsheret (site 25) and Tze'elim (site 29) (Table 1). The burrows of *S. m. fuscus* were considerably less spiral than those of *S. m. palmatus*, a difference that could not be explained by variation in soil type (Table 1). Despite the similar soil type, burrows of *S. m. palmatus* at Tze'elim were significantly deeper than burrows of *S. m. fuscus* at Bohu ( $t_{28}$  = 7.4, *P* < 0.001). Whereas all fifteen burrows at Tze'elim were markedly spiral, the number of curves ranging from 2 to 5, at

nearby Bohu, six burrows exhibited a single curve and five others (N = 15) exhibited none at all.

# 3.3. Phylogeographical analysis

Parsimony and ML analyses of the multilocus dataset for 41 individuals and the more detailed single-locus (COI) dataset for 156 individuals all recovered seven major clades (Fig. 4), corresponding closely with the geography of the study area (Supplementary material S1; Fig. 2B). Clade A includes typical populations of *S. m. fuscus* (type locality: Beirut, Lebanon) with dark

#### Table 1

Dimensions of burrows (N = 15 per species), situation of entrances (under stone vs. in open ground) and soil characteristics of two species of *Scorpio* Linnaeus, 1758, recognized in the present contribution, collected at four nearby localities in northern and northwestern regions of the Negev Desert, Israel. Site numbers in parentheses follow Fig. 2B and Table 1.

	S. fuscus		S. palmatus	
	Gevim (18)	Nahal Bohu (20)	Sharsheret (25)	Tze'elim (29)
Burrow depth (mm)	$160.9 \pm 7.3$	150 ± 8.7	265.6 ± 13	208.3 ± 12.3
Number of curves	$1.0 \pm 0.3$	$0.9 \pm 0.2$	$3.3 \pm 0.2$	$2.1 \pm 0.3$
Under stone/in open	15/0	15/0	0/15	0/15
Soil type	Hamra	Loess	Loess	Loess

blackish-brown base coloration of the cuticle and a longer pedipalp chela with a partially costate digital carina (Fig. 1A), from 16 sites. into This clade was further subdivided several geographically-delimited subgroups: western and Lower Galilee, northern Jordan Valley. Mt. Carmel and western and northern Negev (Fig. 4). Typical populations of *S. m. palmatus* (type locality: Egypt), characterized by yellow base coloration and a shorter pedipalp chela with an entirely granular digital carina (Fig. 1B), from 18 sites, grouped into Clades B, F and G, consisting of three geographically-delimited subgroups from the Negev, northern Judaean Desert and southern Judaean Desert, respectively (Fig. 4).

The analyses recovered three additional clades representing five sites in the northernmost part of the study area (Upper Galilee and Golan Heights; Fig. 2B). Clade C comprised individuals from Susita (site 6) in the southern Golan Heights. Most of these specimens resembled *S. m. palmatus* in exhibiting yellow base coloration and a shorter pedipalp chela with an entirely granular digital carina, but many were more infuscate on the carapace and tergites than typical populations of *S. m. palmatus* (Fig. 1D). One dark brown individual, also with a shorter pedipalp chela and an entirely granular digital carina (Fig. 1C) grouped with the other yellow specimens collected at the same site (Fig. 4; Tables 3 and 4). The yellow specimens from Susita, conspecific with material examined from Jordan and Turkey, are tentatively assigned to *Scorpio maurus kruglovi* Birula, 1910 (type locality: Deir-Zor, Syria).

The two remaining clades each comprised individuals from two sites, in the Upper Galilee and northern Golan Heights (clade D, sites 1 and 2; Fig. 2B), and the eastern Golan Heights (clade E, sites 3 and 4; Fig. 2B). The specimens from these sites exhibited a shorter pedipalp chela with a partially costate digital carina and mixed cuticular coloration (Fig. 1E and F). For example, several individuals exhibited a dark prosoma and mesosoma with yellow-ish pedipalps, legs and metasoma (Fig. 1E) whereas others were more uniformly dark reddish-brown (Fig. 1F). These specimens are tentatively assigned to *Scorpio maurus propinquus* (Simon, 1872) (type locality: Damascus, Syria) although clades D and E were monophyletic only in the ML analysis of the multilocus data-set (Fig. 4).

Parsimony and ML analyses of the multilocus dataset differed from one another and from the analyses of the single-locus (COI, 16S and 12S) datasets concerning the arrangements among the recovered clades. In the parsimony analysis of the multilocus dataset (Fig. 4), clades A (*S. m. fuscus*) and C (*S. m. kruglovi*) formed a monophyletic group, sister to clade D, and to the exclusion of clade E, rendering *S. m. propinquus* paraphyletic. Clades B, F and G (*S. m. palmatus*) grouped sister to the monophyletic group comprising the other clades. In the ML analysis of the multilocus dataset (Fig. 4), clades B, F, G (*S. m. palmatus*) and C (*S. m. kruglovi*) formed a monophyletic group, sister to the monophyletic group comprising

comprising clades D and E (*S. m. propinquus*), to the exclusion of clade A (*S. m. fuscus*).

Separate analyses of the single-locus datasets revealed varying levels of support for the clades recovered by the multilocus datasets, consistent with their phylogenetic information content (Tables 2 and 3). The nuclear 28S contained the fewest informative sites among the four fragments and, when analyzed separately, did not support the clades recovered by analyses of the multilocus dataset. In contrast, separate analyses of the mitochondrial COI, and to a lesser extent, the 16S and 12S, supported most clades recovered by analyses of the multilocus dataset (Table 3). All three loci supported clades B, C and F when analyzed separately using parsimony, and clades A, B, C and F when analyzed separately using ML.

The topologies recovered by analyses of the COI dataset of 156 samples closely resembled those recovered by analyses of the multilocus dataset (Fig. 4): clades A (*S. m. fuscus*) and B, F, G (*S. m. palmatus*) formed a monophyletic group, sister to clade C (*S. m. kruglovi*), and to the exclusion of clades D and E, again rendering *S. m. propinquus* paraphyletic. The Likelihood Ratio, Kishino–Hasegawa and Shimodaira–Hasegawa tests on the tree obtained by separate analysis of the COI sequences of the 41 samples included in the multilocus dataset (Table 4) confirmed significant support for Clades A–E and two additional clades (F and G) comprising samples from the vicinity of the Dead Sea, on the northeastern margin of the distribution of *S. m. palmatus* (Clade B; Fig. 2B).

## 3.4. Genetic divergence

Analysis of genetic divergence among COI sequences of specimens collected from 39 localities (Supplementary material S5) recovered significant between-group dissimilarities (Mantel statistic: r = 0.834, P < 0.001). Pairwise comparisons resulted in significant divergence (r = 0.866, P < 0.001) between clades A (*S. m. fuscus*) and B (*S. m. palmatus*), and between these clades and the pooled Golan Heights specimens in clades C–E (r = 0.851, P < 0.001 and r = 0.969, P < 0.001, respectively).

# 4. Discussion

Although *Scorpio* originally accommodated a number of species (e.g., Simon, 1872; Pocock, 1900), only a single widespread, polymorphic species was recognized by 1910, a status quo that persisted for almost a century, on the grounds that no reliable characters could distinguish its 19 subspecies and circumstantial evidence of hybridization between the two subspecies occurring in the eastern Mediterranean region (Vachon, 1950; Levy and Amitai, 1980). The fact that the subspecies could be diagnosed on the basis of somatic characters presented in several keys (Birula, 1910; Vachon, 1950, 1952; Levy and Amitai, 1980), however, prompted the suggestion that most if not all should be recognized as phylogenetic species (Prendini et al., 2003), a move that has recently begun to be implemented (Lourenço, 2009), albeit without rigorous reanalysis of the variation.

We present the most comprehensive study to date, based on molecular phylogenetic analysis, of species limits in *Scorpio*. Our results support earlier suggestions that *Scorpio maurus* is a species complex (Prendini et al., 2003; Froufe et al., 2008), rather than a single widespread polymorphic species, with many subspecies, as portrayed by earlier literature (e.g., Birula, 1910; Vachon, 1950, 1952; Levy and Amitai, 1980). Seven geographically-delimited clades of *Scorpio maurus*, corresponding to at least four currently recognized subspecies, appear to occur in our study area in the eastern Mediterranean region, representing a small fraction of



**Fig. 4.** Phylogenetic trees for the species of *Scorpio* Linnaeus, 1758 occurring in the eastern Mediterranean region, as recognized in the present contribution, inferred from parsimony (A) and maximum likelihood (B) analyses of concatenated mitochondrial Cytochrome c Oxidase Subunit I (COI), 12S rDNA and 16S rDNA sequences, and nuclear 28S rDNA sequences for 41 samples (top), and COI sequences for 156 samples (bottom). Codes for terminals denote Site No.-Color-Sample ID (Supplementary material S2). Bootstrap support values are indicated at nodes.

the geographical distribution of the former *S. maurus*, which extended from West Africa to Iran (Fet, 2000).

Clades A and B encompassed the widest geographical distributions within the study area. Clade A included specimens collected at sites ranging from the western Galilee in the north to the northwestern Negev in the south (Fig. 2B, black circles). Specimens comprising clades B, F and G were collected in southern and southeastern regions of the study area, in the Negev and Judaean Desert (Fig. 2B, yellow circles). Unsurprisingly, the two clades correspond roughly with the geographical distributions of *S. m. fuscus* and *S. m. palmatus*, the two subspecies traditionally recognized in the north and south of the study area (Fig. 2A), respectively. This pattern of



Fig. 4 (continued)

distribution, associated with a gradient of increasing aridity southwards and eastwards (Fig. 2A), has been shown to correlate with the land snail fauna (Kadmon and Heller, 1998), anatomical variation in the buthid scorpion, *Leiurus quinquestriatus* (Ehrenberg, 1828) (Warburg and Elias, 1998), genetic differentiation in aphids (Avrani et al., 2012) and speciation in vertebrates (Nevo, 2013).

Despite an overlap in the ranges of morphometric characters of pedipalp chela shape between the putative subspecies, a difference is evident in the average, with *S. m. fuscus* displaying longer fingers (resulting in an overall longer chela) and a broader, deeper manus than *S. m. palmatus* (Fig. 3). *Scorpio m. fuscus* and *S. m. palmatus* are

consistently distinguished by the coloration of the cuticle as well the condition of the digital carina of the pedipalp chela: dark blackish-brown coloration with a partially costate digital carina in *S. m. fuscus* and pale yellow coloration (sometimes with infuscation) and an entirely granular digital carina in *S. m. palmatus* (Supplementary material S1). These consistent diagnostic differences imply that the two putative subspecies are phylogenetic species.

The putative subspecies differ further in burrow architecture and depth, implying that they are also distinct ecological species. The burrows of *S. m. fuscus* are significantly shallower and simpler,

Table 2

Phylogenetic information content (number of invariant and parsimony-informative sites) of sequences, and number and length of most parsimonious trees (MPTs) inferred from separate analyses of mitochondrial Cytochrome c Oxidase Subunit I (COI), 16S rDNA (16S), and 12S rDNA (12S) and nuclear 28S rDNA (28S) sequences for 41 samples of *Scorpio* Linnaeus, 1758 from the eastern Mediterranean region.

Locus	Sites	Invariant sites	Parsimony- informative sites (%)	MPTs	Parsimony score (length)
COI	658	512	112 (17)	4340	381
16S	484	400	53 (11)	2	140
12S	331	261	39 (12)	156	118
28S	514	504	4 (1)	13	1

the entrances always situated under or at the base of stones, than those of S. m. palmatus, which are deep and spiral, the entrances always situated in open ground. Variation in burrow structure among populations of S. maurus was previously reported, and attributed to abiotic factors prevailing in their respective habitats (Abdel-Nabi et al., 2004). However, significant differences in burrow structure at two geographically-proximate sites sharing similar soil characteristics (Fig. 2C; Table 1) suggest that behavioral constraints, e.g., burrow construction behavior, may also contribute to determining the geographical distribution of S. m. fuscus. The shallower burrow of S. m. fuscus (Table 1) and its requirement for rocky microhabitats may restrict its ability to colonize semi-arid habitats like the barren loess plains inhabited by S. m. palmatus (Levy and Amitai, 1980; Fig. 2A). The mesic distribution of S. m. fuscus is evidently also associated with its considerably higher susceptibility to dehydration (Gefen, 2011; Kalra and Gefen, 2012). These morphological, behavioral, ecophysiological and distributional differences are supported by the phylogeographical analyses of specimens from 30 sampling sites included in clades A (S. m. fuscus) and B (S. m. palmatus) (Fig. 4).

According to Levy and Amitai (1980), brown and yellow color forms co-occur in peripheral populations of *S. m. fuscus* and *S. m. palmatus* (Fig. 2A), with individuals of intermediate cuticle coloration providing evidence for hybridization (and, hence, a single biological species). Our data suggest otherwise. For example, in the northwestern Negev, a clear distinction is evident between the dark brown populations assigned to *S. m. fuscus* and the yellow populations assigned to *S. m. palmatus*. All specimens from Nahal Bohu (site 20, N = 8) formed a monophyletic group within clade A (*S. m. fuscus*), whereas those from Sharsheret (site 25, N = 7), only 6 km away (Fig. 2C), formed a monophyletic group within clade B (*S. m. palmatus*) (Fig. 4). The significant genetic divergence between the clade from Nahal Bohu, which grouped with other dark brown populations from much further away (Fig. 2B), and the clade from geographically-proximate Sharsheret, demonstrated that the two

Table 3

Nodal support (parsimony bootstrap/parsimony jackknife/maximum likelihood bootstrap) values for clades of *Scorpio* Linnaeus, 1758 occurring in the eastern Mediterranean region inferred from separate analyses of mitochondrial Cytochrome c Oxidase Subunit I (COI), 16S rDNA (16S), and 12S rDNA (12S) sequences for 41 samples. – denotes no clade support.

Clade	COI	16S	12S
А	100/100/99	-/-/42	80/78/88 <sup>a</sup>
В	100/92/99	86/82/93	94/88/96 <sup>b</sup>
С	100/100/98	98/96/99	100/98/100
D	60/62/84	-/-/-	-/-/-
E	100/100/100	-/-/-	91/76/99
F	100/100/100	100/99/100	91/82/90
G	100/70/95	77/69/85	-/-/39

<sup>a</sup> Including sites 25 and 28, and sites 2 and 29, respectively grouped into clades A and D in the COI tree.

<sup>b</sup> Excluding sites 25, 28, 29 and 35 that form part of clade A in the COI tree.

color forms are reproductively isolated and represent different biological species despite the absence of any geographical barrier between the two localities (Fig. 2C). Additional evidence is available from Lahav (sites 23, 24), where both color forms were found almost in sympatry (Fig. 2D) yet the dark brown and yellow specimens were strongly divergent genetically, and placed into clades A (*S. m. fuscus*) and B (*S. m. palmatus*), respectively (Fig. 4).

The genetic, morphological, behavioral and physiological evidence all justify elevating the two forms, currently recognized as S. m. fuscus and S. m. palmatus, to the rank of species, as implemented below. Moreover, we provide evidence for further genetic divergence within the study area. Specimens collected in five Upper Galilee and Golan Heights localities (sites 1–4 and 6) were not only genetically divergent from clades A (S. m. fuscus) and B (S. m. palmatus), but exhibited considerable divergence among themselves (Fig. 4: Tables 3 and 4), consistent with subtle morphological differences, implying that additional species are involved. Whereas cuticular coloration was a consistent morphological indicator separating clades A and B, dark brown and yellow specimens collected at Susita in the southern Golan Heights (site 6; Figs. 1C, D, and 2A) were closely related and placed within clade C (Fig. 4; Tables 3 and 4). The nearest records of previously described S. m. palmatus specimens west of the Jordan River are more than 100 km south of Susita, just north of the Dead Sea (Levy and Amitai, 1980), suggesting that the Susita Scorpio specimens represent the westernmost tip of the distribution of a third species which inhabits neighboring Jordan and/or Syria. Scorpio m. kruglovi is reported from these countries, although the type locality is in Syria (Fet, 2000). We tentatively assign clade C to S. m. kruglovi based on conspecific material examined from Jordan and Turkey and elevate it accordingly (below).

Except for Misgav Am (site 1; Fig. 2B), the populations comprising the three northern clades C, D and E are geographically separated from the populations of clade A (*S. m. fuscus*) by the Hula Valley and the Sea of Galilee. Together with the Anti-Lebanon Mountains, these depressions, comprising a section of the Syrian–African Great Rift Valley in northern Israel, probably provided a geographical barrier to gene flow resulting in the isolation and divergence of the populations on either side. Although a possible barrier between the Susita population (clade C) and the remaining four populations from the Golan Heights (clades D and E) is less obvious, the genetic and morphological evidence suggests that two species may be present (Fig. 4). A similar divergence occurs in the blind mole rat, which comprises different chromosomal species in the northern and southern Golan Heights (Ben-Shlomo et al., 1988).

Clades D and E were monophyletic only in the topology obtained by the ML analysis of the multilocus dataset. However, based on morphological similarity we consider the four populations comprising these clades to be conspecific, and tentatively assign them to *S. m. propinquus*, which we elevate accordingly (below).

#### Table 4

Statistical support for clades of *Scorpio* Linnaeus, 1758 occurring in the eastern Mediterranean region based on Likelihood Ratio Test (LRT), Kishino–Hasegawa Test (KHT) and Shimodaira–Hasegawa Test (SHT) conducted on mitochondrial Cytochrome c Oxidase Subunit I sequences for 41 specimens.

Clade	LRT	KHT	SHT
А	$\chi^2 = 58.4; P < 0.001$	<i>P</i> < 0.001	<i>P</i> = 0.003
В	$\chi^2 = 26.4; P < 0.001$	P < 0.001	P = 0.025
С	$\chi^2 = 10.0; P = 0.002$	<i>P</i> < 0.001	P = 0.133
D	$\chi^2 = 3.9; P = 0.048$	<i>P</i> < 0.001	P = 0.195
Е	$\chi^2 = 35.4; P < 0.001$	<i>P</i> < 0.001	P = 0.011
F	$\chi^2 = 41.4; P < 0.001$		
G	$\chi^2 = 19.2; P < 0.001$		

Geographical isolation resulting from the Great Rift could also explain the four genetically-distinct populations comprising clades F and G (Fig. 4). These populations occupy extremely dry habitats around the Dead Sea (Fig. 2), isolated from the Judaean Desert to their west by a steep ridge and a considerable distance from populations in the Negev Desert. However, additional data (e.g., morphology and ecology) are required to justify their separation from *S. palmatus*.

The evidence of cryptic species among the populations of Scorpio occurring in the eastern Mediterranean region, presented here, is probably a function of their sedentary lifestyle and stenotopic ecological requirements (Prendini, 2001), as well as the topographical and geological complexity of the region (but see Hausdorf and Hennig, 2006). Scorpions in general exhibit extremely low metabolic rates compared with arthropods of similar body size (Lighton et al., 2001). The obligatorily fossorial lifestyle of Scorpio and its "sit-and-wait" foraging strategy suggest that the energetic cost of burrow construction (as well as exposure to desiccation and predation risk while on the surface) will select for homing behavior (e.g., Polis et al., 1986) and thus limit dispersal. Low dispersal rates and philopatry, in turn leading to high burrow densities and reduced gene flow between demes and larger populations, probably exacerbate isolation imposed by stenotopic habitat preferences (Prendini, 2001), ultimately resulting in substantial genetic divergence over short geographical distances, e.g., between Mt. Carmel and the Lower Galilee populations within clade A or the Negev and Judaean Desert populations in clade B (Fig. 4; Supplementary material S5).

In conclusion, we provide genetic, morphological, and behavioral support for previous suggestions that *Scorpio maurus* is a species complex, comprising multiple distinct phylogenetic, ecological and biological species, rather than a single widespread polymorphic species. Our data justify elevating *S. m. fuscus*, *S. m. palmatus*, and two additional subspecies in the northeastern region of the study area, to the rank of species. Additional taxon sampling beyond the study area may help to resolve disagreement among the various analyses concerning the relative positions of the clades, and confirm the monophyly of populations tentatively identified as *S. propinquus*. Considering the extensive geographical distribution of *Scorpio*, the considerable genetic divergence discovered in the small study area reported here highlights the need to undertake a thorough systematic revision of the genus using modern phylogenetic methods.

#### 5. Taxonomic emendations

Based on the conclusions above, we propose the following taxonomic emendations: *Scorpio fuscus* (Ehrenberg, 1829), stat. nov.; *Scorpio kruglovi* (Birula, 1910), stat. nov.; *Scorpio palmatus* (Ehrenberg, 1828), stat. nov.; *Scorpio propinquus* (Simon, 1872), stat. nov.

As discussed above, we are confident that *S. fuscus* (clade A) and *S. palmatus* (clades B, F and G) are phylogenetic, ecological and biological species, that clades C–E are not conspecific with the latter, and that clade C is not conspecific with clades D and E. Separation of clades F and G from *S. palmatus* merits further assessment.

*Scorpio kruglovi* is reported from Jordan and Syria, although the type locality is in Syria. We tentatively assign clade C to *S. kruglovi* based on conspecific material examined from Jordan and Turkey and elevate it accordingly.

Fet (2000) suggested that *S. m. propinquus* is dubious, apparently because Birula (1910) noticed that the type locality (Damascus, Syria) falls within the range of *S. m. fuscus*. However, the type locality of *S. m. fuscus* (Beirut, Lebanon) is on the coast, separated from the type locality of *S. m. propinquus* by the

Anti-Lebanon Mountains, a major southwest–northeast mountain range, which presents a potentially formidable barrier to scorpion dispersal, suggesting that the populations on either side are unlikely to be conspecific. Given the relative proximity of Damascus to the Golan Heights, we tentatively assign the material from this area (clades D and E) to *S. propinquus* and elevate it accordingly.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.04. 028.

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