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PRIMARY RESEARCH PAPER

Molecular evidence for the presence of cryptic evolutionary lineages in the freshwater copepod genus *Hemidiaptomus* G.O. Sars, 1903 (Calanoida, Diaptomidae)

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Abstract The pattern of morphological and mtDNA cytochrome *b* diversity of three calanoid copepod species belonging to the diaptomid genus *Hemidiaptomus* has been investigated with the aim of checking the reliability of the morphological characters currently used for species identification, and the possible presence of cryptic taxa. A sharply different molecular structuring has been observed in the studied species: while *Hemidiaptomus amblyodon* exhibits a remarkable constancy throughout the European range of its distribution area (maximum inter-populations cytochrome *b* divergence of 3%), observed distances between presumed conspecific lineages of *Hemidiaptomus gurneyi* (maximum divergence of 21.5%) and *Hemidiaptomus ingens* (maximum 19.1%) suggest that under these binomens are in fact included complexes of cryptic, or currently just unrecognized, independent evolutionary lineages. The application of the "4x rule" shows that the two lineages singled out within *H. ingens* are in fact independent evolutionary units, while the complex molecular structure observed in *H. gurneyi* s.l. could not be resolved based on the currently available data. Applying standard crustacean mtDNA evolutionary rates to the observed divergence values, the separation of the main lineages within both *H. ingens* and *H. gurneyi* might dates back to the Miocene; however, it has also to be considered that the rate of mtDNA evolution might be accelerated in copepods, as already observed in other arthropod taxa. Present results gives further evidences of the high potential for copepod speciation with no or little morphological changes, and stress the need of a revision of the most controversial Palaearctic diaptomid genera.

Keywords Calanoid copepods · *Hemidiaptomus amblyodon* · *H. gurneyi* · *H. gurneyi canaanita* · *H. ingens* · Cryptic evolutionary lineages · mtDNA · Cyt *b* · Molecular systematics

Introduction

The copepod genus *Hemidiaptomus* Sars is composed of 18 large-bodied species (up to 7 mm in length) spread in the Palaearctic region, from Mongolia to Spain and Morocco (Dussart & Defaye, 2002; Stepanova, 2005). The genus is exclusive of temporary water bodies, where it establishes large monovoltine populations usually in association with other

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diaptomid copepods (e.g.,: Gauthier, 1928; Alonso, 1998; Marrone & Naselli-Flores, 2004). Like several other diaptomids, *Hemidiaptomus* species survive the dry phase of the inhabited water bodies through the production of resting eggs (De Stasio, 1989); these are also used for the passive dispersal of the species through zoochory and anemochory (Figuerola & Green, 2002; Cáceres & Soluk, 2002; Graham & Wirth, 2008).

The genus *Hemidiaptomus* currently comprises three subgenera, i.e. *Hemidiaptomus* s.s. Sars, *Gigantodiaptomus* Kiefer, and *Occidodiaptomus* Borutzky. Recently, Stepanova (2005) proposed a revision of the whole genus based on morphology, suggesting to rise *Occidodiaptomus* to generic rank and to include some species formerly belonging to the subgenus *Hemidiaptomus* s.s. in the new subgenus *Balcanodiaptomus.* However, in this article, we follow the classification proposed by Borutzky et al. (1991), which is currently the more widespread and accepted one among the scientific community.

The three subgenera of the genus *Hemidiaptomus* are mostly parapatric and present only limited co-occurrence areas: the subgenus *Hemidiaptomus* s.s. occurs from north-eastern Algeria and south-eastern Europe to Mongolia, *Gigantodiaptomus* is known from Central Europe to Siberia, and *Occidodiaptomus* is exclusive of the European and Maghrebian countries of the western Mediterranean area. However, the subgenera *Occidodiaptomus* and *Gigantodiaptomus* share a limited sympatric area in France, while the subgenus *Hemidiaptomus* s.s. co-occurs with *Occidodiaptomus* in southern Italy and north-eastern Maghreb, and with *Gigantodiaptomus* in the Caucasus (Dussart & Defaye, 2002).

The molecular systematics and phylogeography of inland waters calanoid copepods have till recently received little attention when compared with their marine counterparts (e.g.,: Lee, 2000; Papadopoulos et al., 2005; Bucklin & Frost, 2009, and references therein). Among the inland water calanoid taxa, the phylogeny and molecular systematics of Nearctic diaptomids and Neotropical centropagids have been investigated using both nuclear (Thum, 2004; Marszalek et al., 2009) and mitochondrial markers (Adamowicz et al., 2007; Thum & Derry, 2008; Thum & Harrison, 2009), while the only molecular data currently available on Palaearctic diaptomid copepods deal with population genetics (Bohonak et al., 2006; Zeller et al., 2006), with no direct taxonomical or phylogenetic implications.

Interestingly, contrasting data regarding the relative rates of morphological and molecular evolution have been registered in different taxonomical groups: Adamowicz et al. (2007) observed in South American freshwater Centropagidae shallow intra-specific and large inter-specific cytochrome *c* oxidase subunit I (cox I) distances, in good accordance with the morphological identification of the studied specimens and the general mitochondrial molecular pattern observed in other crustacean groups (Costa et al., 2007); conversely, Boileau (1991), Thum & Derry (2008), and Thum & Harrison (2009) observed unexpectedly large molecular divergences within some alleged North American diaptomid species, which were then considered by the authors rather as complexes of cryptic species, which were hard or impossible to distinguish on morphological bases.

In light of the apparent high potential for copepod speciation to occur with little or no morphological changes (Thum & Harrison, 2009, and references therein), we have investigated the pattern of molecular diversity of three species of the genus *Hemidiaptomus* with the aim of checking the reliability of the morphological characters currently used for species identification, and the possible presence of evolutionary independent lineages within the currently accepted species of the genus. One species from each *Hemidiaptomus* subgenus was included in the analyses: (i) *H.* (*Gigantodiaptomus*) *amblyodon* (Marenzeller), occurring throughout Central Europe north of the Alps and Pyrenees to western Asia; (ii) *H.* (*Hemidiaptomus*) *gurneyi* (Roy), a temperate species currently known to occur from the Balkans to north-eastern Algeria passing through Peninsular Italy and the larger Tyrrhenian islands (i.e., Sicily and, likely, Sardinia); and (iii) *H.* (*Occidodiaptomus*) *ingens* (Gurney), a steno-Mediterranean species currently known from the Western Mediterranean countries only (i.e., the Maghreb, Sicily, Corsica, and southern Italy) (Fig. 1). In the light of the studies of de Queiroz (2005, 2007) and Hey (2006), in the frame of present study, we consider the "lineages evolving separately from others" as good species following De Queiroz's "unified species concept" (De Queiroz, 2007).

Thum and Harrison (2009) showed that in the diaptomids the cytochrome *b* has a similar evolutionary rate to that of the 16S-rDNA and cox I, which

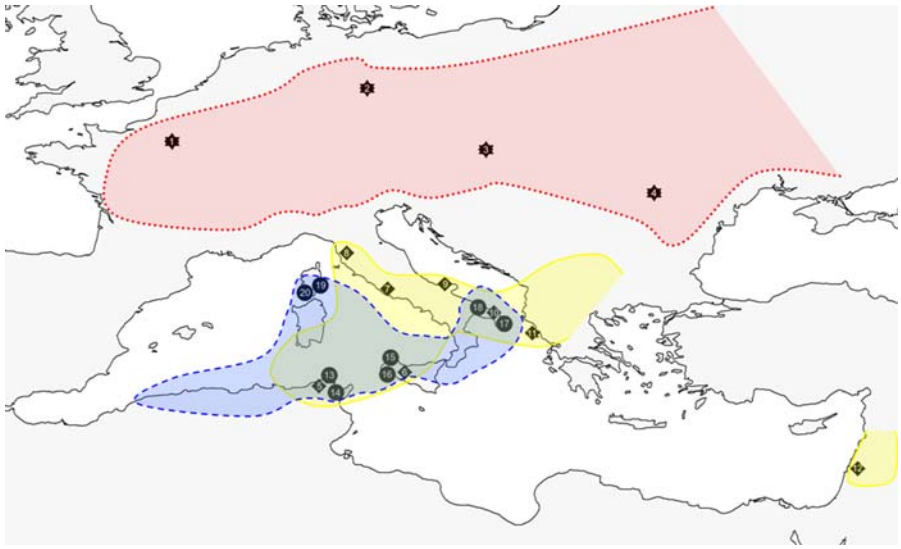

Fig. 1 Location of the collection sites for *H. amblyodon* (*stars*, sites 1–4), *H. gurneyi* (*rhombuses*, sites 5–12), and *H. ingens* (*circles*, sites 13–20). See Table 1 for "Location ID" legend. Currently known distribution area of *H. amblyodon* is represented with a dashed line, that of *H. gurneyi* with a solid line, and that of *H. ingens* with a dotted line

are considered good markers for inter-specific discrimination in the Crustacea (e.g.,: Costa et al., 2007; Elias-Gutierrez et al., 2008). The cytochrome *b* (cyt *b*) is thus a suitable marker for testing the possible presence of cryptic species within the genus, and we used it in the frame of our study.

Materials and methods

The *Hemidiaptomus* specimens were collected from 20 sites (four sites for *H. amblyodon*, eight for *H. gurneyi*, and eight for *H. ingens*) scattered throughout the known geographical distribution ranges of the studied species, and fixed in situ in 80% ethanol (Table 1; Fig. 1). When available, ten males and ten females from each studied population were prepared according to Huys & Boxshall (1991) and Dussart & Defaye (2001), dissected under the stereomicroscope, and identified according to Kiefer (1978), Stella (1984), and Einsle (1993).

Given that a *Hemidiaptomus gurneyi* subspecies (i.e., *H. gurneyi canaanita* Dimentman & Por) was described based on the morphology of the female right fifth pair of legs, particular attention was paid to this character on all the studied specimens, and it was photographed in a single individual *per H. gurneyi* population with a LEICA D-LUX 3 "LMS" camera mounted on a LEICA DM4000 microscope. The same procedure has been applied to male and female fifth pair of legs in *H. ingens*, which is known to show a certain variability in the species (Marrone & Naselli-Flores, 2004, and references therein).

Two further adult specimens *per* population were identified under the stereomicroscope and included in the molecular analysis; the French *H. amblyodon* population from Batignolles is the only case in which the molecular analyses were performed from a single specimen. Single specimens of *Diaptomus serbicus* (Gjorgjewic) and *Metadiaptomus chevreuxi* (de Guerne & Richard) have been included in the analyses as outgroups (Table 1).

Prior to DNA extraction, specimens were carefully cleaned and soaked in double-distilled water for 3 h. DNA was then extracted using whole specimens and the "DNEasy® Animal Tissue Kit" (QIAGEN). A partial sequence of the mitochondrial gene cyt *b* was then amplified using the degenerate primers "L10319" (5′-CCTTGGGGKCAGATGTCTTTTTGGG- 3′) and "H10648" (5′-GATAAAATTTTCWGGGTC-3′), described by Machida et al. 2004, for *H. amblyodon, H. ingens*, *D. serbicus*, and *M. chevreuxi*; the degenerate primers "UCYTB151-F" (5′-TGTGGRGCNAC YGTWATYACTAA-3′) and "UCYTB270-R" (5′-A ANAGGAARTAYCAYTCNGGYTG-3′), described by Merrit et al., 1998, were used for *H. gurneyi*.

PCR mix consisted, with both primer pairs, of 2.5 µl of Buffer 10× (THERMOSCIENTIFIC), 2 µl of 25 mM $MgCl_2$, 0.5 µl of each dNTP 10 mM, 0.3 µl of each 50 µM primer, 0.3 µl of Red Hot Taq Polymerase 5U/µl (THERMOSCIENTIFIC), 0.6 µl of DNA template, and 17 µl of double-distilled water, for a

Table 1 Populations of diaptomid copepods sampled in this study

ID	Taxon	Site	Country	Coordinates	Collector
1	*H. amblyodon*	Batignolles, Savigny en Veron	France	n.d.	NR
2	*H. amblyodon*	Breitenhagen, Saxony-Anhalt	Germany	51.92 N; 11.95 E	MK
3	*H. amblyodon*	Lanzhot, Moravia	Czech Republic	48.66 N; 16.96 E	VK
4	*H. amblyodon*	Simbod, Harghita	Romania	46.25 N; 25.87 E	LD
5	*H. gurneyi*	Oued El Amor, Tabarka	Tunisia	36.92 N; 8.75 E	FM
6	*H. gurneyi*	Monte Carcaci, Sicily	Italy	37.72 N; 13.50 E	FM
7	*H. gurneyi*	Castel Romano, Latium	Italy	41.72 N; 12.45 E	AH
8	*H. gurneyi*	San Rossore, Tuscany	Italy	43.71 N; 10.32 E	GR & VP
9	*H. gurneyi*	Lesina, Gargano, Apulia	Italy	41.89 N; 15.35 E	GA
10	*H. gurneyi*	Le Cesine, Salento, Apulia	Italy	40.35 N; 18.33 E	GA
11	*H. gurneyi*	Giannades, Corfu	Greece	39.61 N; 19.82 E	FM
12	*H. gurneyi*	Moshav Ishrash	Israel	31.90 N; 34.83 E	HH
13	*H. ingens*	Sejenane, Bizerte	Tunisia	37,13 N; 9,37 E	FM
14	*H. ingens*	Raoued, Ariana	Tunisia	36,95 N; 10,22 E	FM & ST
15	*H. ingens*	Monte Cofano, Sicily	Italy	38.10 N; 12.67 E	FM
16	*H. ingens*	Gallitello, Sicily	Italy	37.85 N; 12.92 E	FM
17	*H. ingens*	Patula Mancina, Apulia	Italy	39,98 N; 18,31 E	GA
18	*H. ingens*	Sandonaci, Apulia	Italy	40,43 N; 17,91 E	GA
19	*H. ingens*	Padule de Suartone, Corsica	France	41.46 N; 9.23 E	FM & FS
20	*H. ingens*	Santa Manza, Corsica	France	41.40 N; 9.19 E	FM & FS
21	*Diaptomus serbicus*	Piano Bùffali, Sicily	Italy	37.87 N; 14.67 E	FM
22	*Metadiaptomus chevreuxi*	Oued El Mekta, Tunisia	Tunisia	35.61 N; 10.17 E	FM

NR Nicolas Rabet; *MK* Michael Korn; *VK* Vladimir Korinek; *LD* Laszlo Demeter; *FM* Federico Marrone; *AH* Anna Hundsdoerfer; *GR* Giampaolo Rossetti; *VP* Valentina Pieri; *GA* Giuseppe Alfonso; *HH* Hervé Huet; *ST* Souad Turki; *FS* Fabio Stoch

total reaction volume of 25 µl. The amplification consisted of an initial denaturation step of 94°C for 3 min followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 9 min.

The PCR products were separated by electrophoresis on a 2% agarose gel at 70 V for 1 h and visualized with a UV Transilluminator. When amplified bands were sharp and clean, they were cut, purified from agarose gel using the "Qiaquick® Gel Extraction Kit" (Qiagen) and sequenced in both forward and reverse directions with a Roche Genome Sequencer FLX. Chromatograms were imported and edited with Chromas Lite 2.01 (Technelysium Pty Ltd) and exported to be aligned with ClustalX (Thompson et al., 1997).

A reconstruction of the phylogenetic relationships among the studied species and of the molecular structure of each alleged species was carried out using Neighbour Joining and Maximum Parsimony methods as implemented in Paup 4.0b10 (Swofford, 1998). The best evolutionary model for the dataset was established by Akaike Information Criterion, performed with the software jModelTest (Guindon & Gascuel, 2003; Posada, 2008).

In order to check whether the observed intra-specific clades could be considered separate evolving lineages (i.e., independent species *sensu* De Queiroz, 2007) or the inter-clades distances were just ascribable to random drifts or other transient effects, the "4x rule" (Birky et al., 2005; Birky & Barraclough, 2009, Birky et al., 2010) was applied to molecular distance matrices within and among the detected intra-specific clades based on uncorrected *p*-distance calculated with PAUP 4.0b10 (Swofford, 1998). The "4x rule" is an operational criterion for identifying species based on the idea that a (monophyletic) lineage achieves species status when the molecular differences with its sister lineage are too great to be attributed to random drift alone, i.e., when the mean

sequence divergence between specimens belonging to these lineages (D) is greater than the depth of clades formed by random drift with a 95% confidence ("4 θ", where "θ" is the nucleotide polymorphism of each clade) (Birky et al., 2005). The "4x rule" has been designed for asexual organisms but it might be applied to the mitochondrial genes of gonochoric species assuming that the studied mitochondrial gene mirrors have the behavior of the nuclear genome, and taking into account that mitochondrial genes of two allopatric lineages will become reciprocally monophyletic earlier than nuclear ones, thus giving the earliest evidence of the occurrence of an allopatric speciation event (Birky et al., 2010).

Standard molecular clock evolutionary rates for crustacean mitochondrial DNA (see Thum & Harrison, 2009, and references therein) were applied to the studied species to single out a possible temporal frame in which the separation of the main intra-specific lineages may have occurred.

Results

Morphological analysis

No differences within or among populations were observed in *H. amblyodon* and in *H. gurneyi s.s.* Moreover, the single morphological character currently used to discriminate the nominal subspecies of *H. gurneyi* from its eastern subspecies *H. gurneyi canaanita* unexpectedly proved to be uniform among all the studied *H. gurneyi* s.l. populations (Fig. 2). Conversely, in the studied *H. ingens* populations a certain variability in the ornamentation of the endopodite of the male left P5 was observed, which can bear a variable number of apical setae or can be completely lacking them (Fig. 3A–C), and in the ornamentation of both the endo- and exopodites of the female fifth pair of legs (Fig. 3D–F).

Genetic analysis

The alignment of the amplified cyt *b* fragment of all the different species and the outgroups did not evidence any gap or insertion, and after having trimmed the tails which were not present in all the individuals, led to a cyt *b* aligned fragment of 330 bp.

The four investigated *H. amblyodon* populations show a relatively shallow pairwise sequence divergences, with uncorrected *p*-distance values ranging from 0 to 3.04%, and a clear geographical pattern has not been observed (Appendix 1—Supplementary material and Fig. 4).

The observed uncorrected distances within each *H. gurneyi* population ranges from 0 to 1.5%, while the average pairwise distance between populations ranges from 6% to as much as 21.5% (Appendix 2—Supplementary material). Interestingly, the single Israeli population included in the analysis accounts for most of the observed diversity, while *H. gurneyi* populations from Peninsular Italy are only slightly different from those occurring in Corfu, Sicily, and northern Tunisia. Accordingly, it is possible to single out an "Eastern Mediterranean group", including the single Israeli haplotype, and a "Central Mediterranean group", the latter presenting some substructuring (Appendix 2—Supplementary material and Fig. 4). The average uncorrected *p*-distance "D" between these two groups is 20.4% (Appendix 4—Supplementary material).

In the eight populations of *H. ingens* included in the analysis, the average overall mean uncorrected distance between populations ranges between 0 and 19.1%, and two sharply defined groups can be singled out; the first includes the Sicilian and Tunisian populations, the second includes the populations from Corsica and Apulia (Appendix 3—Supplementary material and Fig. 4). The average uncorrected *p*-distance "D" between the "Siculo-Tunisian" and the "Corso-Apulian" groups is 18% (Appendix 4—Supplementary material), and the mean *p*-distances between specimens within each clade is 2.5% for the first, and 3.2% for the latter.

The Neighbour Joining and the Maximum Parsimony trees (Fig. 4) present the same topology at the higher nodes, and thus confirming the molecular homogeneity of *H. amblyodon* versus the presence of well-supported clades in *H. gurneyi* and *H. ingens*. Nevertheless, The "4x rule" criterion applied to the alleged intra-specific clades supported the reciprocal monophyly of the two *H. ingens* clades ($D = 0.181$; $\theta_{\text{SiculoTunisianClade}} = 0.0299$; $\theta_{\text{CorsoApulianClade}} = 0.0386$; $D > 4\theta$), while did not for the two alleged *H. gurneyi* clades ($D = 0.2036$; $\theta_{\text{CentralMediterraneanClade}} = 0.0986$; $\theta_{\text{EasternMediterraneanClade}} = 0.0061$; $D < 4\theta$).

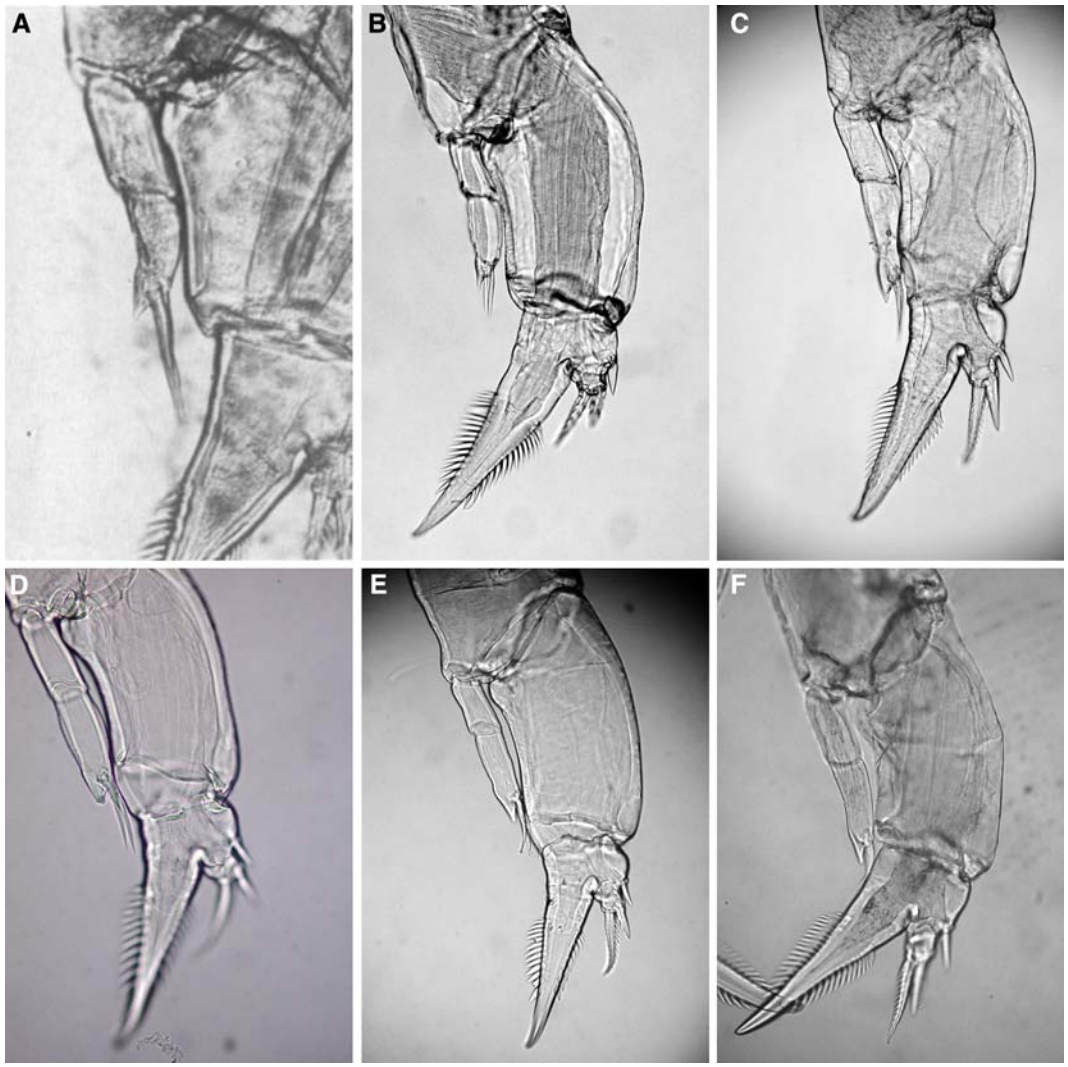

Fig. 2 Morphology of the female right P5 of *H. gurneyi* s.l. **A** Gamla, Golan Heights (from: Dimentman & Por, 1985); **B** Moshav Ishrash (Israel); **C** Oued El Amor (Tunisia); **D** Giannades (Corfu); **E** Lesina (Gargano, Peninsular Italy); **F** Monte Carcaci (Sicily). Oued El Amor is the *locus typicus* of *H. gurneyi* s.s.

Discussion

Pitfalls of the morphological identification

Investigated *H. amblyodon* and *H. gurneyi* s.s. specimens show a constant morphology and a perfect agreement with the descriptions of the species currently available in the literature (e.g., Kiefer, 1978; Stella, 1984; Borutzky et al., 1991; Einsle, 1993). In contrast, the single morphological character currently used to discriminate the nominal subspecies of *H. gurneyi* from its Israeli subspecies *H. gurneyi canaanita* proved to be uniform among all the studied populations of *H. gurneyi s.l.*, including the topotypical one (Oued El Amor pond, Tunisia). The Israeli subspecies should in fact be characterized by the endopodite of the fifth pair of legs presenting "*a slightly curved and medially constricted second segment, which ends in a spiniform processus. Therefore, the two terminal setae are displaced to a subapical position. Also, the surface of this segment bears a very delicate covering of scales*" (Dimentman & Por, 1985, p. 94), a description which is in perfect agreement with the characteristics of the

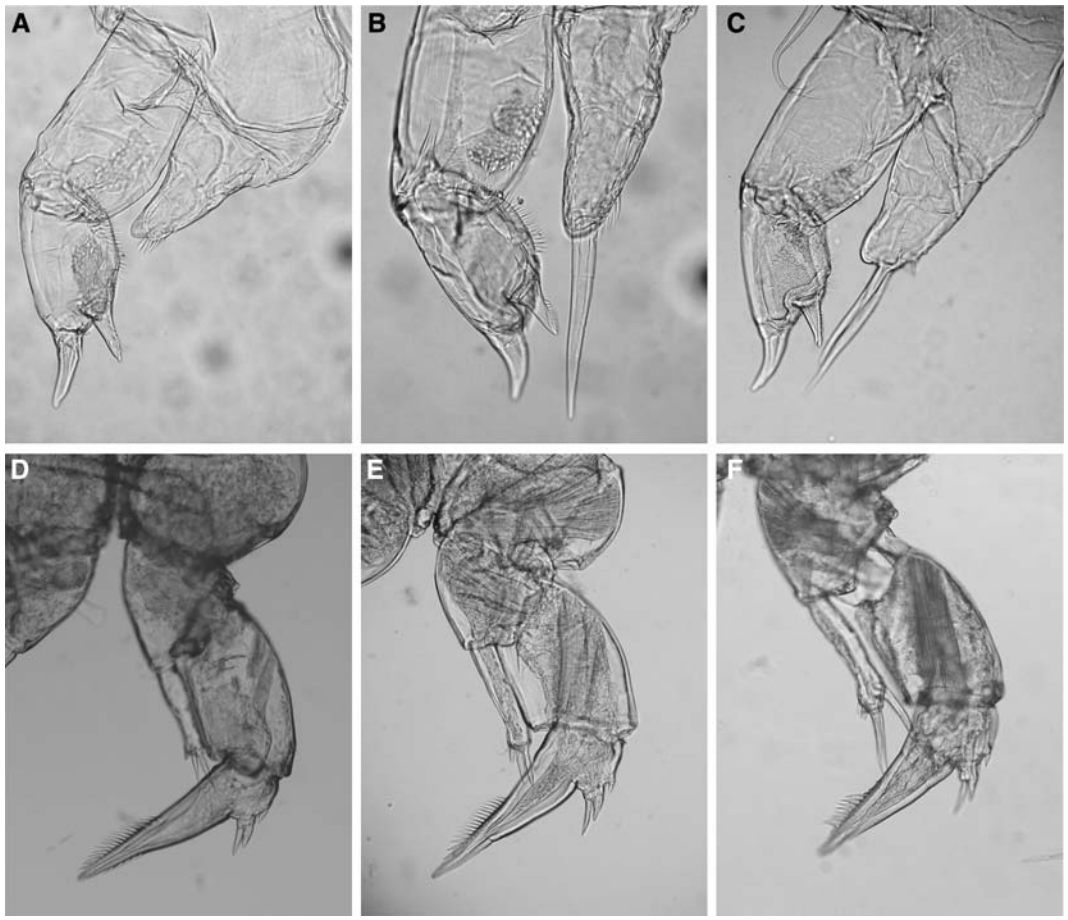

Fig. 3 Morphological variability in the sexual legs (P5) of *H. ingens*. **A**–**C**: male left P5 (**A**: Sandonaci, Peninsular Italy; **B**: Sejenane, Tunisia; **C**: Monte Cofano, Sicily) **D**–**F**: female left fifth pair of legs, dorsal view (**D**: P. Mancina, Peninsular Italy; **E**: P. de Suartone, Corsica; **F**: Monte Cofano, Sicily)

female P5 in all the investigated populations (Fig. 2). It is thus clear that this character does not allow a discrimination of the populations of the two alleged *H. gurneyi* subspecies based on morphology, and that a re-evaluation of the taxonomical status of the Israeli populations is needed.

In *H. ingens*, the variability of the chaetotaxy of the apical part of the endopodite of the male left P5 was early observed by several authors (e.g., Roy & Gauthier, 1927), leading to the description of the subspecies *H. ingens inermis* (Kiefer, 1954). However, later investigations led Kiefer himself to deem the possible absence of setae on the endopodite of the male left P5 as "abnormal morphologies" or "atavisms" which are likely lacking of any taxonomical value (Kiefer, 1973). Accordingly, the subspecies *H. ingens inermis* was thereafter considered a junior synonym of *H. ingens* s.s. in the subsequent studies (including Kiefer, 1978, and Dussart & Defaye, 2002). Thus, at the current status of the knowledge, no sound morphological features which may allow to distinguish between the two alleged *H. ingens* subspecies are known.

The genetic structure of the species

Hemidiaptomus amblyodon is the most widespread and common *Hemidiaptomus* species in central Europe and western Asia, while it has never been recorded west of the Pyrenees, south of the Alps, and in the western Balkans. Its exclusive occurrence in areas which was faunally depleted during the Pleistocene glaciations (Schmitt, 2007) testifies for a recent range expansion of the species from some still

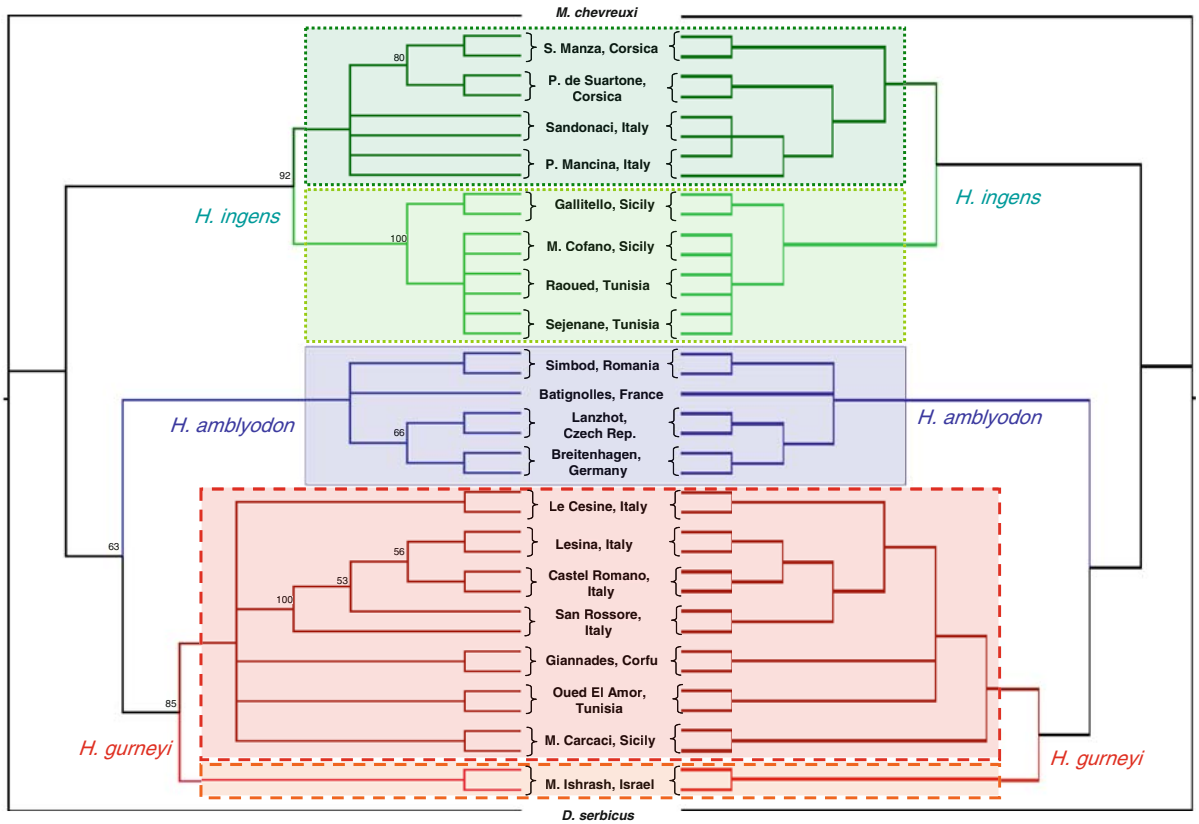

Fig. 4 Neighbor Joining tree based on ML-corrected distance (HKY + I + G) (*left*), and strict consensus MP tree based on six equally parsimonious trees (490 steps; CI: 0.5796; HI: 0.4204; RI: 0.8952) (right). Number at nodes of the NJ tree refer to statistical support for 10,000 bootstrap replicates, nodes with less than 50 of bootstrap support are represented as unresolved polytomies. The two clades singled outwithin *H. ingens.* and *H. gurneyi* are represented with dotted and dashed lines, respectively; *H. amblyodon* samples are represented with a solid line

unidentified extra-Mediterranean refugia. This process could have begun at most 10,000 years ago, with the beginning of the last postglacial period, when *H. amblyodon* recolonized central and northern Eurasia tracking the receding glacial ice sheets. The investigated populations, collected over a geographical range of more than 2,000 km of map distance, show shallow molecular divergences and not a clear geographical structure; this pattern is likely to be ascribed to a rapid expansion of the species not hindered by barriers to gene flow as often observed in several animal groups (Steward & Lister, 2001; Schmitt, 2007).

Hemidiaptomus gurneyi is the westernmost representative of the subgenus *Hemidiaptomus* s.s. It is currently considered a polytypic taxon, whose nominal subspecies occurs in the central Mediterranean countries and in the Balkans, and the subspecies *H. gurneyi canaanita* only in Israel (Dimentman & Por, 1985). The morphological study of specimens from the type locality (Oued El Amor pond, close to Tabarka, Tunisia), Sicily, Peninsular Italy, Corfu, and from the *terra typica* of *H. gurneyi canaanita* (Israel) showed no differences in the alleged differential characters between subspecies (Fig. 2), which, therefore, lack of systematic value.

The phylogenetic pattern shows a certain differentiation of a "Central Mediterranean" versus an "Eastern Mediterranean" group (Appendix 2—Supplementary material & Fig. 4) but, in the light of the high nucleotide polymorphism of the "Central Mediterranean group" ($\theta_{\text{CentralMediterraneanClade}}$) and of the results of the "4x rule" ($D < 4\ \theta_{\text{CentralMediterraneanClade}}$), the mean inter-groups distance "D" is not big enough to consider these two groups as reciprocally monophyletic; waiting for further, more detailed, studies *H. gurneyi canaanita* can thus be maintained as an

eastern Mediterranean subspecies of *H. gurneyi* currently impossible to be told apart from the nominal taxon based on the morphological characters used in the systematics of the genus. Furthermore, lacking records of *H. gurneyi* s.l. from Peninsular Greece and Turkey (Dussart & Defaye, 2002), the disjointed distribution pattern of *H. gurneyi* may suggest the presence of a process of allopatric speciation which is still in progress.

Hemidiaptomus ingens is currently considered a monotypic species, as its two previously described subspecies *H. ingens provinciae* Petit & Schachter and *H. ingens inermis* Kiefer were later synonymized, respectively, with *Hemidiaptomus roubaui* and *Hemidiaptomus ingens* s.s. (Kiefer, 1973, 1978; Dussart & Defaye, 2002). Unfortunately, it was impossible to collect and include in the analyses of topotypical specimens of the nominal form (Oued Tindja pond, close to Bizerte, Tunisia) and of its presumptive subspecies *H. ingens inermis* (pond in La Reghaia forest, close to Alger, Algeria) but the observed shallow intra-clade and the deep inter-clades sequence divergences are compatible with a separation of the species in two independent, even though strictly related, evolutionary lineages. The two clades observed within *H. ingens* s.l. are in fact well supported by phylogenetic analyses (Fig. 4) and they pass the "4x rule" criterion ($D > 4\theta$), although they cannot be told apart based on the morphological characters currently considered in the systematics of the genus *Hemidiaptomus*. A noteworthy morphological variability has been observed in some characters as the male and female sexual legs (e.g., Fig. 3, and Marrone & Naselli-Flores, 2004), and an in-depth morphological study is currently underway with the aim of singling out possible constant differential characters between the two lineages.

Comparison among the observed patterns

The three investigated species show a sharply different degree of genetic structuring. In particular, *H. amblyodon* shallow divergences are in good agreement with the current taxonomy of the species, and show that it may be reliably identified based on morphology. In contrast, molecular divergences observed within *H. gurneyi* and *H. ingens* are unexpectedly high, and suggest the possibility that under these binomens are in fact included complexes of cryptic, or currently just unrecognized, evolutionary independent lineages, and that a re-evaluation of the meaning and taxonomic rank of the previously described subspecies is needed. In *H. ingens*, the observed divergence between presumed conspecific clades is 5.6–7.2 times greater than those observed within each clade; unfortunately, the presence of a single population of the *H. gurneyi* "Eastern Mediterranean clade" in present analysis prevents from getting a clear picture of the intra-clade versus inter-clades divergences in this species. These results are somehow less definite than those on Nearctic diaptomids, where differences between presumed intraspecific clades about as 18 times larger than within each clade were observed (Thum & Derry, 2008), however, it sharply appears that under the binomen "*H. ingens*" are in fact included at least two closely related but independent evolutionary units, likely of specific level, which were to date not singled out based on morphology or were with some uncertainties considered belonging to different subspecies. Furthermore, in this species, the average pairwise *p*-distance between the supposedly conspecific clades is of the same order of magnitude as that observed between congeneric species currently ascribed to different subgenera (Appendix 4—Supplementary material), thus giving strength to the hypothesis of their independent specific status.

As expected in accordance with the general pattern of "northern purity *vs.* southern richness" observed in most Palaearctic taxa (Hewitt, 2004), the species currently distributed in the Mediterranean area (i.e., *H. gurneyi* and *H. ingens*) exhibit a much stronger molecular structure than *H. amblyodon*, a species currently occurring in areas which were faunally depleted during the last ice ages. The populations of the first two species, in fact, have likely experienced several events of isolation and expansions during the Plio-Pleistocene climate fluctuations, which may have allowed the birth and the survival of local haplotypes in a number of different refugia scattered along the circum-Mediterranean countries and the physiographically variegated European peninsulas. Conversely, *H. amblyodon* likely survived the Plio-Pleistocene upheaval just in a single northern extra-Mediterranean refugial area, or at most in a few neighboring areas, from where it rapidly re-colonized central Europe and western Asia at the beginning of the Holocene. The strong bottleneck experienced by the species, the absence of isolated populations and

the rapid colonization of the deglaciated regions during the last 10,000 years, did not allow the survival of well characterized and divergent local lineages (cf. Grant & Bowen, 1998).

Dating the separation of the intra-specific lineages

Unfortunately, no fossil records are available for diaptomid copepods, and the more ancient Diaptomidae remains currently known are copepod spermatophores and fossil egg sacks dubiously attributed to *Diaptomus castor* from late Quaternary sediments of Greenland and Denmark (Bennike, 1998); lacking other fossil evidences, a specific calibration of the molecular clock for diaptomids has not been realized to date. However, molecular clock calibrations for crustacean mtDNA range from 0.9 to 2.6% sequence divergence *per* million years (Thum & Harrison, 2009, and references therein). We thus applied these extreme rates to the cyt *b* divergences observed in the studied species to propose a rough temporal frame in which the separation of the main intra-specific lineages may have occurred. This way, the divergence between the main *H. gurneyi* and *H. ingens* presumed intra-specific lineages should be dated back to the Miocene: the two *H. gurneyi* clades should be separated from about 7.8–22.5 million years, while the two *H. ingens* clades from about 6.9–20 million years. As pointed out by Thum & Harrison (2009), the possibility that mtDNA clock in diaptomids could tick at a faster rate when compared with that of other crustaceans has also to be considered, and further studies aimed at investigating the natural history of diaptomid copepods are definitely needed to clarify this point.

However, our results give further evidences of the high potential for copepod speciation with no or little morphological changes (e.g.,: Lee, 2000; Grishanin et al., 2006; Thum & Derry, 2008; Thum & Harrison, 2009), which could be due both to accelerated mitochondrial DNA evolutionary rates in copepods, to extremely slow rates of morphological evolution in this diverse crustacean group, or to a concerted action of the two processes, and it stresses the need of a revision of the most controversial Palaearctic diaptomid genera with a combined molecular and morphological approach.

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