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Overlooked cryptic endemism in copepods: Systematics and natural history of the calanoid subgenus *Occidodiaptomus* Borutzky 1991 (Copepoda, Calanoida, Diaptomidae)

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ABSTRACT

Our comprehension of the phylogeny and diversity of most inland–water crustaceans is currently hampered by their pronounced morphological bradytely, which contributed to the affirmation of the "Cosmopolitanism Paradigm" of freshwater taxa. However, growing evidence of the existence of cryptic diversity and molecular regionalism is available for calanoid copepods, thus stressing the need for careful morphological and molecular studies in order to soundly investigate the systematics, diversity and distribution patterns of the group.

Diaptomid copepods were here chosen as model taxa, and the morphological and molecular diversity of the species belonging to the west-Mediterranean diaptomid subgenus *Occidodiaptomus* were investigated with the aim of comparing the patterns of morphological and molecular evolution in freshwater copepods. Three species currently lumped under the binomen *Hemidiaptomus* (*Occidodiaptomus*) *ingens* and two highly divergent clades within *H.* (*O.*) *roubaui* were distinguished, thus showing an apparent discordance between the molecular distances recorded and *Occidodiaptomus* morphological homogeneity, and highlighting a noteworthy decoupling between the morphological and molecular diversity in the subgenus.

Current *Occidodiaptomus* diversity pattern is ascribed to a combined effect of ancient vicariance and recent dispersal events. It is stressed that the lack of sound calibration points for the molecular clock makes it difficult to soundly temporally frame the diversification events of interest in the taxon studied, and thus to asses the role of morphological bradytely and of accelerated molecular evolutionary rates in shaping the current diversity of the group.

1. Introduction

Until recently, most inland-water microcrustaceans were considered widespread species whose distribution was mostly regulated by ecological factors. The "Cosmopolitanism Paradigm", based on the observation of the scarce morphological differentiation among presumed conspecific populations along wide areas and of the potentially high capacities of microcrustaceans for passive dispersal, was advocated to explain this hypothesised pattern (see references in: Bohonak and Jenkins, 2003). The model assigned a fundamental role to the resting stages, which are apt to be passively transported by wind, vertebrates or flying insects and that could thus maintain the genetic homogeneity of the species through an extensive gene flow across their distribution ranges (e.g. Mayr, 1963; Bilton et al., 2001). Conversely, growing morphological and molecular evidence has shown that a significant part of the currently considered widely-distributed morphospecies, i.e. those taxa defined on morphology alone, are in fact groups of closely related species with a more restricted distribution (e.g. Reid, 1998; Lee, 2000; Thum and Derry, 2008, and references therein); these last are sometimes "true" sibling species, completely indistinguishable based on morphology alone. However, there are cases in which the presumed cryptic species are morphologically distinguishable if based on morphological characters which were to date overlooked ("pseudo-sibling species" *sensu* Knowlton, 1993), thus attesting the dearth of morphological characters of taxonomic utility which afflicts some microcrustacean groups.

The idea of the existence of an important realised gene flow among inland water microcrustacean populations has been recently challenged by the "Monopolization Hypothesis" (De Meester et al., 2002) according to which a combination of founder effect, rapid local adaptation, and resilience due to large egg banks acts against the establishment of "newcomers", thus restricting the extent of actual gene flow in spite of the occurrence of possible frequent dispersal events (the "dispersal-gene flow paradox").

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In order to correctly understand the speciation patterns, diversity and biogeography of inland water microcrustaceans, a thorough morphological and molecular revision of the existing taxa is thus needed. We are here focusing on the copepod taxon *Occidodiaptomus* Borutzky 1991, a subgenus belonging to the family Diaptomidae, the calanoid family which dominates the lentic inland water bodies of the Palaearctic region, a key-taxon for lacustrine and pond ecosystems. The species of the family are characterised by limited distribution ranges, which are significantly constrained by the legacies of historical biogeographical events (e.g. Leibold et al., 2010). However, genetic studies proved that our comprehension of the real distribution patterns of the diaptomid species is currently hampered by a gross underestimating of the diversity of the group (e.g. Boileau, 1991; Thum and Derry, 2008; Thum and Harrison, 2009; Makino and Tanabe, 2009; Marrone et al., 2010).

In the Mediterranean region, about one hundred diaptomid species belonging to fourteen genera are currently reported (Dussart and Defaye, 2002). Among these, the subgenus *Occidodiaptomus* (it belongs to the genus *Hemidiaptomus sensu* Borutzky et al., 1991) is endemic to this area and is currently considered to include the species *H.* (*O.*) *roubaui, H.* (*O.*) *ingens* and *H.* (*O.*) *maroccanus* (Table 1). The morphology-based taxonomy of the whole *Hemidiaptomus* genus is controversial, and there is neither agreement on the taxonomical rank to be attributed to the taxon *Occidodiaptomus* nor on the relationships of the species belonging to it (e.g. Kiefer, 1973; Marrone and Naselli-Flores, 2004; Stepanova, 2005; Marrone et al., 2011).

In the frame of the growing evidence of the overlooked diversity of microcrustaceans, and in the light of the controversial ideas on the composition, rank and distribution of the taxon *Occidodiaptomus* (e.g. Stepanova, 2005; Marrone et al., 2010), we investigated the taxonomy, distribution and phylogeography of the species of the subgenus *Occidodiaptomus* with the following aims: (i) testing the monophyly and the taxonomical rank of *Occidodiaptomus* within the family Diaptomidae, (ii) investigating the phylogenetic relationships among the taxa belonging to the subgenus *Occidodiaptomus*, and (iii) proposing a hypothesis on the phylogeography of the group. In fact, in order to make sound inferences on the systematics and phylogeny of the members of the subgenus *Occidodiaptomus*, it is first necessary to check for the monophyly of the taxon itself, and then probe the coupling of morphological and molecular data on the inter-taxa phylogenetic relationships and on the hypothesised natural history of the group.

These aims are pursued here by implementing both molecular analyses and a cladistic analysis of morphological characters. Analyses were performed following a hierarchical taxonomical approach: (i) conservative nuclear ribosomal DNA genes (18S and 28S), which are widely used to resolve relationships at the genus- and family-rank in copepods (e.g.: Bucklin et al., 2003; Braga et al., 1999; Wyngaard et al., 2010; Blanco-Bercial et al., 2011), were used to investigate the taxonomical rank to be assigned to the taxon *Occidodiaptomus* in the frame of the family Diaptomidae, (ii) species grouping based on a matrix of morphological characters was compared to that obtained through molecular analyses, (iii) fragments of the mitochondrial genes 16S and Cyt-b were used to check for the monophyly of *Occidodiaptomus* and to investigate the relationships among the taxa belonging to it. Finally, the COI gene was chosen as an additional mitochondrial marker for further testing the monophyly of the clades singled out with the other mitochondrial markers in the only *H. ingens* s.l. (see Section 4); the COI is a widely used marker for species identification in the frame of the "barcoding of life" project and it proved to be a suitable marker for species identification in crustaceans (Hebert et al., 2003; Costa et al., 2007, and references therein).

2. Material and methods

2.1. Taxon sampling and identification

Occidodiaptomus samples were collected throughout the distribution range of the subgenus. Particular attention was paid to collect samples from the *terra typica* of each species and subspecies described in the group (Table S1). In order to test the taxonomical arrangement proposed by Stepanova (2005), six taxa belonging to the other *Hemidiaptomus* subgenera (i.e. *Hemidiaptomus* s.str. Sars and *Gigantodiaptomus* Kiefer) and nine species belonging to other diaptomid genera were included in the molecular analyses. *Calanipeda aquaedulcis* Kritchagin 1873, a representative of the family Pseudodiaptomidae, was chosen as outgroup in some of the analyses because this family is closely related to the family Diaptomidae (Bradford-Grieve et al., 2010; Figueroa, 2011). The complete list of studied material and its occurrence localities is reported in Table S1.

Sampling was carried out with 125 μm mesh-sized hand- and towing-nets. Specimens were fixed *in situ* with 80–95% ethanol, sorted under a stereomicroscope and stored in 95% ethanol at 4 °C. If available, ten males and ten females from each population were prepared according to Huys and Boxshall (1991) to be identified according to Kiefer (1978), Borutzky et al. (1991), and Einsle (1993).

2.2. Cladistic analysis of the morphological characters

Thirty-four morphological characters traditionally considered to be taxonomically informative were selected for analysis; these

Table 1
Currently known distribution of the species belonging to the subgenus *Occidodiaptomus* (genus *Hemidiaptomus*, Calanoida, Diaptomidae) *sensu* Borutzky et al. (1991). Sources: 1: Dussart and Defaye, 2002; 2: Champeau, 1971; 3: Caramujo and Boavida, 2010; 4: N. Rabet, *unpubl. data*, 5: Ramdani, 1988; 6: Ruffo and Stoch, 2005; 7: Mouelhi et al., 2000; 8: Sahuquillo and Miracle, 2010; 9: Marrone and Naselli-Flores, 2004; 10: Alfonso and Belmonte, 2011; 11: Kiefer, 1957; 12: Gurney, 1909; 13: Gauthier, 1928; 14: Alonso, 1998; 15: Richard, 1888; 16: Kiefer, 1954; 17: Kiefer, 1973; 18: Marrone et al., 2010.

Species	Distribution	Sources	Notes
H. (*O.*) *roubaui* (Richard, 1888)	Portugal, Spain, France, Sardinia, Morocco?	1, 2, 3, 4, 5, 6, 7, 14, 11, 15, 17	The species was erroneously reported for Corsica and Peninsular Italy. Its actual presence in Morocco is in need of confirmation
			The subspecies *H. roubaui lauteborni* was described based on a French population and later found to be synonymous of the nominal subspecies
H. (*O.*) *ingens* (Gurney, 1909)	Spain, Algeria, Tunisia, Corsica, Sicily, Peninsular Italy	1, 2, 8, 9, 10, 11, 12, 13, 17, 18	The subspecies *H. ingens inermis* was described for Algeria and later posed in synonymy with the nominal subspecies. A recent study (18) supports the existence of independent lineages of species rank within the binomen *H. ingensIts*
H. (*O.*) *maroccanus* (Kiefer, 1954)	Southern Spain, Morocco	1, 7, 14, 16, 17	Its presence in southern France, reported by 1 and 9, is erroneous

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are related to the chaetotaxy and ornamentation of male and female first antennae, the shape of female genital segment and of the male urosome, and the shape and ornamentation of the male fifth pair of swimming legs (Table S2). Twelve *Hemidiaptomus* taxa (11 species and 2 subspecies) were included in the analysis, and some species belonging to different diaptomid genera were used as outgroups (Table S1). All the taxa were studied on original material with the only exception of *Hemidiaptomus brehmi*, *H. tracicus* and *H. dischensis*, for which no samples were available but for which reliable drawings are available in the literature (Kiefer, 1978; Petkovski, 1983).

Both bi- and multi-status characters were used in the cladistic analysis. These were all considered unweighted and unordered as we are currently missing sound information on the ancestral state of all the studied characters but the antennular chaetotaxy (Huys and Boxshall, 1991; Boxshall and Huys, 1998). The morphological matrix was analysed under the Maximum Parsimony (MP) optimality criterion using the software PAUP (Swofford, 2003); a 50% majority rule consensus tree was produced with the branch-and-bound search (maxtrees = 100).

2.3. DNA extraction, amplification and sequencing

Prior to DNA extraction, specimens were carefully cleaned under the stereomicroscope and soaked in double-distilled water for 2–3 h. DNA was then extracted using whole specimens and following the "DNEasy – Animal Tissue Kit" (QIAGEN) protocol or a phenol–chloroform extraction following the procedure described in Korn et al. (2006).

18S and 28S nuclear ribosomal genes and three mitochondrial genes (16S, Cyt-b, and COI) were amplified using different primer pairs and thermal cycling conditions (Table S3).

For 18S and 28S, the PCR mix consisted of 17.25 μl double-distilled water, 2.5 μl Buffer 10×, 1.8 μl $MgCl_2$ solution (25 mM), 0.2 μl dNTPs (20 mM), 0.25 μl of each primer (10 μM), 0.25 μl *Taq* Polymerase 5u/μl, and 1.5 μl of DNA template, for a total volume of 25 μl.

For all mitochondrial fragments, the PCR mix consisted of 17.32 μl double-distilled water, 2.5 μl Buffer 10×, 2 μl $MgCl_2$ solution (25 mM), 0.36 μl dNTPs (10 mM of each), 0.43 μl of each primer (10 μM), 0.28 μl *Taq* Polymerase 5u/μl and 0.6 μl of DNA template, for a total volume of 25 μl.

After PCR, 5 μl of each PCR product were separated by electrophoresis on a 2% agarose gel at 70 V for 1 h and visualised with a UV Transilluminator. When PCR products showed a clear and single band of the correct expected length, they were purified using the Exo-SAP-IT kit and sequenced with an ABI 3130xL (Applied Biosystem) sequencer. The forward primers were used for direct sequencing of the PCR product, and the sequences' quality was checked with the Applied Biosystems Sequence Scanner v1.0 software. Only sequences with continuous reads of high quality bases (defined as those bases with a QV score greater than 20) were used. When the sequences were not of sufficient quality, the complement/reverse sequences were obtained additionally. Mega5 (Tamura et al., 2011) was used to translate in amino acids the sequences of protein-coding genes (i.e. Cyt-b and COI) in order to check for the possible presence of frameshifts or premature stop codons, which would indicate the presence of sequencing errors or pseudogenes.

2.4. Sequence alignment and analyses

Three datasets were analysed in the frame of this study. A first dataset included the 28S and 18S sequences of both *Hemidiaptomus* s.l. and non-*Hemidiaptomus* taxa, and it was used to test the hypothesis that the currently considered *Hemidiaptomus* subgenera might in fact deserve the rank of independent genera. A second dataset ("16S and Cyt-b") including 16S and Cyt-b sequences was used to investigate the monophyly of the subgenus *Occidodiaptomus*, and to test for the possible presence of cryptic species. Finally, a third dataset including COI sequences ("COI") was used to further test the consistency of the different lineages observed within *H. ingens* s.l. by the analyses performed on the "16S and Cyt-b" dataset and by a previous preliminary study (Marrone et al., 2010).

Chromatograms were imported and edited with Chromas Lite 2.01 (Technelysium Pty. Ltd.) and aligned with BioEdit (Hall, 1999). The sequences were deposited in GenBank (Table S4 – ***it will be realised upon acceptance of the ms***).

The ILD test (Farris et al., 1995), implemented in PAUP as Partition Homogeneity Test, was performed in order to test whether two or more fragments could be combined in a unique dataset. According to Cunningham (1997), when $P > 0.01$ the partitions are not incongruent, and thus it is possible to put them in a single dataset (see also: Barker and Lutzoni, 2002).

Bayesian (BA), Maximum Parsimony (MP), and Maximum Likelihood (ML) analyses were performed as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), PAUP* 4.0b10 (Swofford, 2003), and PhyMl v.3 (Guindon and Gascuel, 2003), respectively. As a measure of branch support, bootstrap values were calculated with 1000 replicates in the MP and ML trees. Nodes support of BA trees were evaluated by their posterior probabilities. For Maximum Likelihood and Bayesian analyses, the best evolutionary model for each dataset was selected using mrModeltest (Nylander, 2004).

2.5. Nuclear dataset

The two nuclear fragments could be combined to be analysed together as the ILD test showed that there was no discordance in their phylogenetic signal (20 OTUs; 1000 reps.; $P = 0.239$). The combined dataset was thus analysed under MP and ML, and using BA.

The best-fit model for the combined dataset was a Generalised Time-Reversible model plus Invariant sites plus Gamma distributed model (GTR + I + G), selected by AIC; parameter values can be obtained from the first author upon request.

2.6. Mitochondrial datasets

The "16S and Cyt-b" dataset includes fragments of Cyt-b and 16S sequences for three outgroup taxa (*Diaptomus cyaneus*, *D. serbicus*, and *Metadiaptomus chevreuxi*) and 53 *Hemidiaptomus* specimens, belonging to the three *Hemidiaptomus* subgenera *sensu* (Borutzky et al., 1991). The ILD test showed that the 16S and Cyt-b fragments presented no discordance in the phylogenetic signal (56 OTUs; 1000 reps.; $P = 0.236$), and that they could then be combined in a single sequence. The best-fit model for the combined dataset was a Generalised Time-Reversible model plus Invariant sites plus Gamma distributed model (GTR + I + G), selected by AIC; parameter values can be obtained from the first author upon request.

The Likelihood Ratio Test was carried out on this dataset in order to check for the possibility of using a molecular clock for the dating of some nodes. This was done by estimating the likelihood for the molecular trees under the best evolutionary model suggested by MrModeltest, both imposing a molecular clock and after having removed this assumption.

The additional mitochondrial fragment of the COI gene was amplified in all the *H. ingens* s.l. specimens available and in two outgroups (i.e. *Hemidiaptomus superbus* and *Metadiaptomus chevreuxi*). No other taxa belonging to the subgenus *Occidodiaptomus* could be included in the analyses as direct sequencing of COI from *H.* (*O.*) *roubaui* and *H.* (*O.*) *maroccanus* individuals yielded poor sequence with multiple peaks, suggesting the presence of COI pseudogenes as already observed in other diaptomid calanoids by Thum and Harrison (2009).

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The ILD test was used to test whether the COI fragments could be combined with the *H. ingens* Cyt-b and 16S fragments included in the "16S and Cyt-b" dataset. The test revealed significant differences in incongruence length ($P = 0.001$; 1000 replicates), thus the COI data set should not be united to that of the Cyt-b and 16S fragments for a combined analysis. However, at the light of the existing criticisms about the ILD test (e.g. Barker and Lutzoni, 2002; Dowton and Austin, 2002; Ramirez, 2006), the COI dataset was analysed both separately and united with the 16S and Cyt-B fragments. The best-fit model for the "COI-only" dataset was a Hasegawa–Kishino–Yano plus Invariant sites plus Gamma distributed model (HKY + I + G), while the best-fit model for the "16S, Cyt-B and COI" dataset was a Generalised Time-Reversible plus Invariant sites plus Gamma distributed model (GTR + I + G) (parameter values can be obtained from the first author upon request).

All the mitochondrial datasets were analysed under MP and ML, and using BA.

2.7. Singling out the cryptic species

Following De Queiroz's "unified species concept" (De Queiroz, 2007), we consider the "lineages evolving separately from others" as species. The "4x rule" (Birky et al., 2010) was applied to molecular mitochondrial distance matrices within and among the detected allegedly intraspecific clades, this was done based both on uncorrected *p*-distance and ml-distance calculated with PAUP 4.0b10 (Swofford, 2003) in order to check if the observed intraspecific clades could be considered separate evolving lineages, i.e. independent species *sensu* De Queiroz (2007), or if the inter-clades distances were just ascribable to random drifts or other transient effects. The implementation of the 4x rule on sexual organisms is based on the assumption that the studied mitochondrial genes mirror the behaviour of the nuclear genome. However, it considers that mitochondrial genes of two allopatric lineages will become reciprocally monophyletic earlier than nuclear ones, thus providing the earliest evidence of the occurrence of an allopatric speciation event (Marrone et al., 2010; Baird et al., 2011; Kieneke et al., 2012).

In the frame of this study, the 4x rule was applied to the *Occidodiaptomus* taxa which showed a stronger structuring in the phylogenetic reconstructions, i.e. *H.* (*O.*) *ingens* s.l. and *H.* (*O.*) *roubaui*.

3. Results

3.1. Cladistic analysis of the morphological characters

Out of 34 unordered and unweighted morphological characters, 28 were parsimony-informative. No cases of intra-taxon morphological variability were observed in the studied taxa, as in the frame of the morphological analysis we considered the two different "forms" of *Hemidiaptomus ingens* s.l. as different subspecies, i.e. *H. ingens ingens* and *H. ingens inermis*, which are morphologically constant at the intra-population level (Table 2) (see also: Marrone and Naselli-Flores, 2004; Marrone et al., 2010). Conversely, and in accordance with the current taxonomical arrangement, neither *H. roubaui* or *H. maroccanus* exhibited any intraspecific morphological variation.

The 50% majority rule consensus tree based on 73 equally parsimonious trees (74 steps; CI: 0.5676; HI: 0.4324; RI: 0.7091) showed a topology (Fig. 1), which is in general good accordance with the taxonomical schemes proposed by Kiefer (1978) and Borutzky et al. (1991). The taxa currently ascribed to the genus *Hemidiaptomus* s.l. form a monophyletic group, where *Occidodiaptomus* is the best resolved taxon. The species belonging to the other subgenera *sensu* (Borutzky et al., 1991) constitute, in fact, two coherent groups with the exception of *H.* (*G*) *superbus*: this last species, currently considered to belong to the subgenus *Gigantodiaptomus* (but see Marrone et al., 2011), clusters together with a group including the *Hemidiaptomus* species currently ascribed to the subgenus *Hemidiaptomus* s.s. Conversely, the close relationship of *H.* (*G.*) *amblyodon* and *H.* (*G.*) *hungaricus* is confirmed.

Within the subgenus *Occidodiaptomus*, *H. maroccanus* is the most divergent species, while *H. ingens* s.l. and *H. roubaui* are more closely related.

3.2. Nuclear markers

The tree topologies based on BA (Prset statefreqpr = dirichelet (1,1,1,1); Lset nst = 6 rates = 9), ML (evol mod.: GTR + I + G), and MP (Tree length = 302; CI = 0.8113; HI = 0.1887; RI = 0.8100) of a 2206 bp long concatenated fragment of the nuclear ribosomal genes 18S and 28S are congruent. The consensus tree, rooted on the pseudodiaptomid calanoid copepod *Calanipeda aquaedulcis*, is reported in Fig. 2. The trees grouped together taxa which are ascribed to currently recognised genera. Conversely, they failed to recover the monophyly of the different *Hemidiaptomus* subgenera *sensu* Borutzky et al. (1991). On the other hand, the clear structuring of the two *Diaptomus* s.l. subgenera (i.e. *Diaptomus* s.s. vs. *Chaetodiaptomus*), and the close proximity of *Copidodiaptomus* and *Eudiaptomus*, two closely allied genera (Kiefer, 1968), are in good accordance with the current taxonomical arrangement.

3.3. Mitochondrial markers

The topologies of the trees based on the "16S and Cyt-b" dataset obtained with the various analyses (BA, ML, MP, see Fig. 3 legend for details) were largely congruent, and described a clear characterisation of the three currently recognised subgenera as described by Borutzky et al. (1991), the only exception being *H.* (*G.*) *superbus* (Fig. 3). This last species did not group with any other *Hemidiaptomus* s.l. species in ML and BA trees (Fig. 3A), and clustered with *Hemidiaptomus* s.s. instead of with the other *Gigantodiaptomus* species according to the MP tree (Fig. 3B). Moreover, while the ML and BA trees (Fig. 3A) failed to indicate the relationships between these three "higher rank clades" and represented them with an unresolved polytomy, the MP tree (Fig. 3B) showed a monophyletic *Occidodiaptomus* clade and a monophylum of *Gigantodiaptomus* + *Hemidiaptomus* s.s. Within the species belonging to the subgenus *Occidodiaptomus*, *Hemidiaptomus* (*O.*) *maroccanus* was identified as the sister species of "*H. roubaui* + *H. ingens*" in both the reconstructions; this species presented low mitochondrial divergences among Spanish and Moroccan populations. Conversely, both *Hemidiaptomus roubaui* and *H. ingens* showed a remarkable mitochondrial polymorphism. It is possible to single out some clusters within each of these alleged species: *H. roubaui* showed quite a clear differentiation between its northernmost ("Northern clade", from Northern Spain, France and Sardinia) and southernmost ("Southern clade", from southern Spain and Portugal) populations (cf. Table S1 for the code and collection locality of each specimen). Conversely, the clustering of *H. ingens* specimens did not mirror a clear geographical pattern. The three main clades singled out within *H. ingens* s.l. were hereby named *H. ingens* "1", *H. ingens* "2", and *H. ingens* "3" (cf. Fig. 3).

The "COI-only" and "16S, Cyt-b and COI" trees of *H. ingens* alone presented the same topology at the major nodes, and corroborated that three main clades are present within *Hemidiaptomus ingens* s.l., although some differences in the branching pattern within each clade among the BA, ML and MP reconstructions are present (Fig. 4). These clades are well supported and in perfect accordance with those evidenced in the "16S and Cyt-b" dataset although some minor differences in the relationships among the populations

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Table 2
Occurrence sites for the morphological subspecies and mitochondrial lineages scored within *H.* (*O.*) *ingens*. The coordinates of the sites are reported in Table S1.

Site	Location	Morphological subspecies[a]	Mt-lineage[b]
Margio di Gallitello	Sicily (Italy)	*H. ingens ingens*	*H. ingens* "1"
Gorgo di Baglio Cofano	Sicily (Italy)	*H. ingens ingens*	*H. ingens* "1"
Garaet Sejenane	Gouvernorat de Bizerte (Tunisia)	*H. ingens ingens*	*H. ingens* „1"
Garaet El Khala	Gouvernorat de Nabeul (Tunisia)	*H. ingens ingens*	*H. ingens* "1"
Garaet Raoued	Gouvernorat de Ariana (Tunisia)	*H. ingens ingens*	*H. ingens* "1"
Patula Mancina	Apulia (Italy)	*H. ingens inermis*	*H. ingens* "2"
Masseria Paludi	Apulia (Italy)	*H. ingens inermis*	*H. ingens* "2"
Contrada Silva	Apulia (Italy)	*H. ingens inermis*	*H. ingens* "2"
Tre Padule de Suartone	Corsica (France)	*H. ingens inermis*	*H. ingens* "2"
Santa Manza	Corsica (France)	*H. ingens inermis*	*H. ingens* "2"
Bassa del Cavall	Comunitat Valenciana (Spain)	*H. ingens inermis*	*H. ingens* "2"
Lavajo de Abajo	Comunitat Valenciana (Spain)	*H. ingens inermis*	*H. ingens* "3"

[a] Sensu Kiefer (1973).
[b] See text, and Figs. 4 and 5.

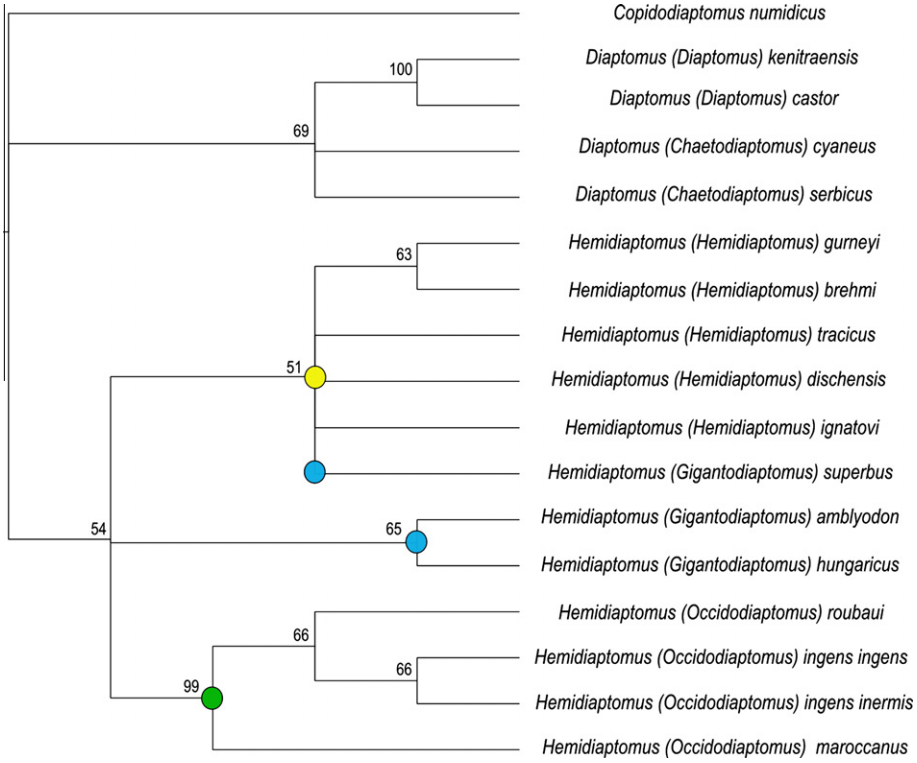

Fig. 1. Fifty percent majority rule consensus MP tree of selected diaptomid species based on morphological characters. Numbers at nodes are percentages of the 73 equally parsimonious trees that contained the taxon in that group/position. Circles stress the *Hemidiaptomus* subgenera according to Borutzky et al. (1991) (yellow: *Hemidiaptomus* s.s.; Azure: *Gigantodiaptomus*; Green: *Occidodiaptomus*). The grouping of *H.* (*G.*) *superbus* with *Hemidiaptomus* s.s. species is not in accordance with current diaptomid systematics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

belonging to each single clade were found in the different analyses (data available on request).

3.4. Molecular clock and timing

The likelihood ratio test (LRT) was used to test the null hypothesis that the "16S and Cyt-b" dataset evolved under a molecular clock. Using 56 individuals, the difference in log-likelihoods between tree reconstruction with and without a clock enforced was 49 (−6525.97650 + 6575.39114). The double of this value is more than the χ^2 value of 72.1532 (df: 54, alfa = 0.05; $p < 0.0002$) indicating that the likelihood values for the reconstructions were significantly different. Clock-like divergence was thus rejected. Furthermore, no fossil records, and thus possible calibration points for the molecular clock, are available for diaptomid copepods. However, in order to infer at least a rough estimate of the temporal frame in which the splitting of the main evolutionary lineages within the genus *Hemidiaptomus* s.l. may have occurred (cf. Gomez et al., 2002) two standard evolutionary rates for crustacean mitochondrial DNA (2.6% my^{-1}, the fastest, and 0.9% my^{-1}, the slowest one: Dooh et al., 2006; Thum and Harrison, 2009; Milligan et al., 2011 and references therein) were applied to distances between the sequences of the studied species, as shown in Table 3.

3.5. Singling out the cryptic species

The 4x rule was applied to the two mitochondrial datasets separately. In the light of the high level of inter-clades differences observed within *Hemidiaptomus ingens* s.l., and in accordance with the guidelines of the 4x rule (Birky et al., 2010), the distance

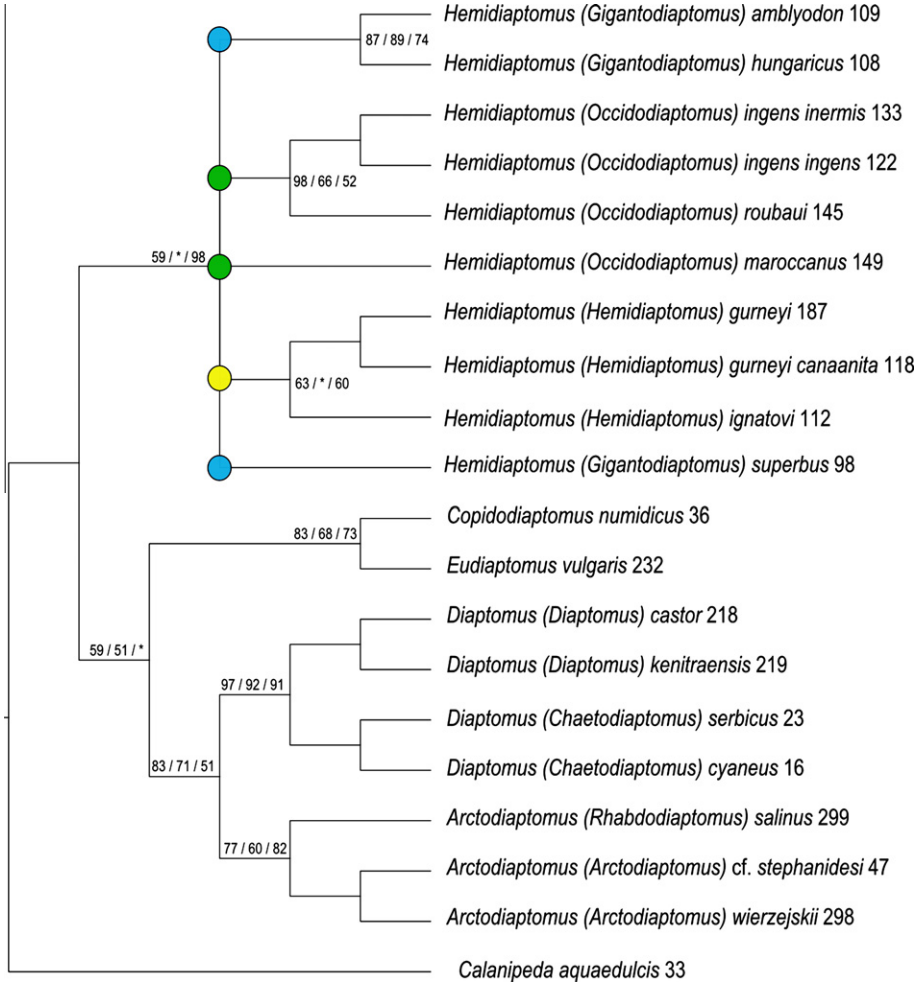

Fig. 2. Phylogenetic reconstruction of five Diaptomidae genera based on a 2206 bp long concatenated fragment of the nuclear ribosomal genes 18S and 28S. Support at nodes is represented as BA posterior probability/ML bootstrap/MP bootstrap. "*" indicates bootstrap support lower than 50. The node support for ML and MP trees is based on 1000 bootstrap replicates. ML analysis is based on the GTR + I + G evolutionary model. Circles stress clades including species belonging to the different *Hemidiaptomus* subgenera (yellow: *Hemidiaptomus* s.s.; Azure: *Gigantodiaptomus*; Green: *Occidodiaptomus*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corrected for multiple hits (ml-dist) was used when calculating the inter-clades average distances.

The results of the three analyses carried out on *H. ingens* s.l. were concordant in showing that the inter-clades distances (*K*) were much higher than 4Θ, based on both the mitochondrial datasets (Table 4). The three *H. ingens* s.l. clades evidenced by the mitochondrial markers (Figs. 3 and 4) thus meet the assumptions of the 4x rule and are to be considered independently evolving lineages, i.e. species *sensu* De Queiroz (2007). Conversely, the 4x rule applied to *H. roubaui* ("16S and Cyt-b" dataset only), showed *K* values much lower than 4Θ, thus not supporting the existence of independently evolving lineages within this taxon (data available from the authors on request).

4. Discussion

4.1. Systematics

The use of conservative nuclear markers such as 18S and 28S is widely applied when investigating the monophyly and reciprocal relationships of higher taxa in copepods, while it is known to have loose resolution for the discrimination of infra-generic taxa (Braga et al., 1999; Thum, 2004; Blanco-Bercial et al., 2011; Figueroa, 2011). In the frame of this study, the tree topologies based on the nuclear ribosomal DNA fragments support the monophyly of the genus *Hemidiaptomus* (Fig. 2), while its subgenera are not clearly resolved, thus supporting the hypothesis that they should not be considered as independent genera, but at most an infra-generic taxonomical rank has to be assigned to the taxa *Hemidiaptomus* s.s., *Gigantodiaptomus* and *Occidodiaptomus*.

Furthermore, the morphological and mitochondrial phylogenetic trees (Figs. 1 and 3) are in good accordance with grouping the *Hemidiaptomus* s.l. species in three clades; these mirror the taxonomical arrangement of the genus proposed by Borutzky et al. (1991), with clearly-defined monophyletic "*Occidodiaptomus*", "*Hemidiaptomus* s.s." and "*Gigantodiaptomus*" taxa of subgeneric rank. The only outlier is *Hemidiaptomus* (*Gigantodiaptomus*) *superbus*, which clustered with the *Hemidiaptomus* s.s. species (Figs. 1 and 3B) or did not group with any of the known subgenera (Fig. 3A). This was partly expected, and some doubt on the affinities of this extremely rare *Hemidiaptomus* species were already raised (Einsle, 1993; Marrone et al., 2011). Our results are thus not in accordance with the re-arrangement of the genus *Hemidiaptomus* proposed by Stepanova (2005), which assigned to *Occidodiaptomus* the rank of an independent genus including two subgenera (i.e. *Occidodiaptomus* s.s. and *Balcanodiaptomus*); furthermore, the species belonging to the subgenus *Balcanodiaptomus*

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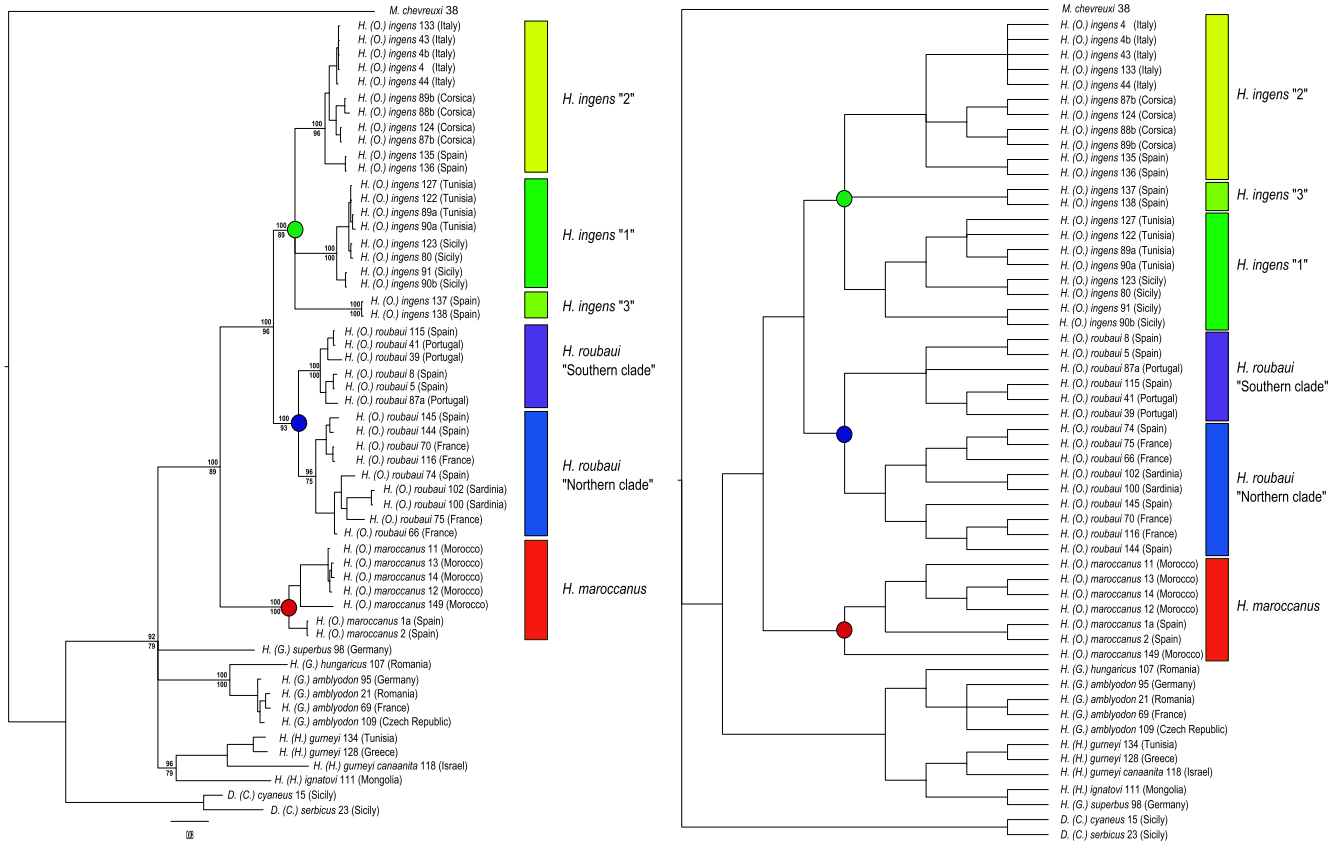

Fig. 3. Phylogenetic reconstructions of *Hemidiaptomus* species based on the "16S and Cyt-b" dataset. Bayesian tree (Prset statefreqpr = dirichlet (1,1,1,1); Lset nst = 6 rates = invgamma) (A), node support is shown as posterior probability (upper value) and ML bootstrap value based on 1000 replicates (lower value). Strict consensus MP tree (B) based on 52 equally parsimonious trees (Tree length = 1316; CI = 0.4240; HI = 0.5760; RI = 0.7879). Circles in colour stress the MRCA of the each alleged species within the *Occidodiaptomus* subgenus (Green: *H.* (*O.*) *ingens*; Blue: *H.* (*O.*) *roubaui*; Red: *H.* (*O.*) *maroccanus*). Rectangles in colour stress the molecular clades within each alleged species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proposed by Stepanova (2005) constituted a paraphyletic group both based on morphology and on molecular markers and should thus be rejected.

The monophyly of the subgenus *Occidodiaptomus* is supported by both the morphological and mitochondrial data and six major clades can be singled out within it based on the mitochondrial trees (Fig. 3). The first includes *Hemidiaptomus* (*Occidodiaptomus*) *maroccanus* specimens from Morocco and southern Spain; this species is characterised by morphological and molecular consistency throughout its distribution range.

Hemidiaptomus roubaui specimens are grouped in two major geographically-segregated but morphologically-indistinguishable clades, a "Southern clade" including the populations from south-western Spain and Portugal, and a "Northern clade" including the north-easternmost populations (northern Spain, France, and Sardinia) (Fig. 3). The output of the 4x rule based on the "16S and Cyt-b" dataset did not support the independent species rank for the two clades of *Hemidiaptomus roubaui*, which has then to be considered a single, molecularly highly-structured species. However, further studies on ecological or reproductive isolation might provide further insights on the relationships among these two different clades.

Three clades with unresolved relationships can be singled out within *Hemidiaptomus* (*Occidodiaptomus*) *ingens* s.l. based on the "16S and Cyt-b" dataset (Fig. 3). Two of these (i.e. "*H. ingens* 1" and "*H. ingens* 2") were anticipated by Marrone et al. (2010) based on Cyt-b sequences only, the third one ("*H. ingens* 3") includes a single population from north-eastern Spain which was not included in the previous work. Although the ILD test showed that the COI sequences ("COI dataset") could not be combined with the 16S and Cyt-b sequences in a single dataset, the tree topologies based on the COI only (Fig. 4) are in accordance with the "16S and Cyt-b" and the "16S, Cyt-b and COI" datasets in singling out the three clades within *H. ingens* s.l.

Out of the three clades, "*H. ingens* 1", occurring in Tunisia and Sicily, can be readily identified based on the morphology of the male specimens: it is the only *Hemidiaptomus* species whose males bear 1–3 strong setae at the distal part of the endopodite of the left P5; conversely, the clades "*H. ingens* 2", occurring in Spain, Corsica and southern Italy, and "*H. ingens* 3", occurring in a single Spanish locality, are lacking this feature and they are morphologically indistinguishable. The relationships among these three clades are unresolved to poorly-resolved based on all the mitochondrial datasets.

Based on the mitochondrial datasets, mean sequence divergence between *H. ingens* s.l. clades is always higher than the divergence within clades, with no overlap of values (Fig. 5), a criterion often used to delimit cryptic species (Hebert et al., 2003; Baird et al., 2011). Furthermore, the application of the 4x rule to the "16S and Cyt-b" dataset and to the "COI" dataset in *H. ingens* s.l. (Table 4) confirmed these three clades as independently evolving lineages, i.e. species *sensu* De Queiroz (2007). The subgenus *Occidodiaptomus* thus appears to be composed of five taxa of species rank in contrast to the three taxa known for the group to date. *Hemidiaptomus* (*O.*) *maroccanus* and *H.* (*O.*) *roubaui* are confirmed as good species, whose identification can be soundly performed based on morphology alone. Conversely, three species are currently concealed within *Hemidiaptomus* (*Occidodiaptomus*) *ingens*, two of them being not distinguishable based on traditional morphology.

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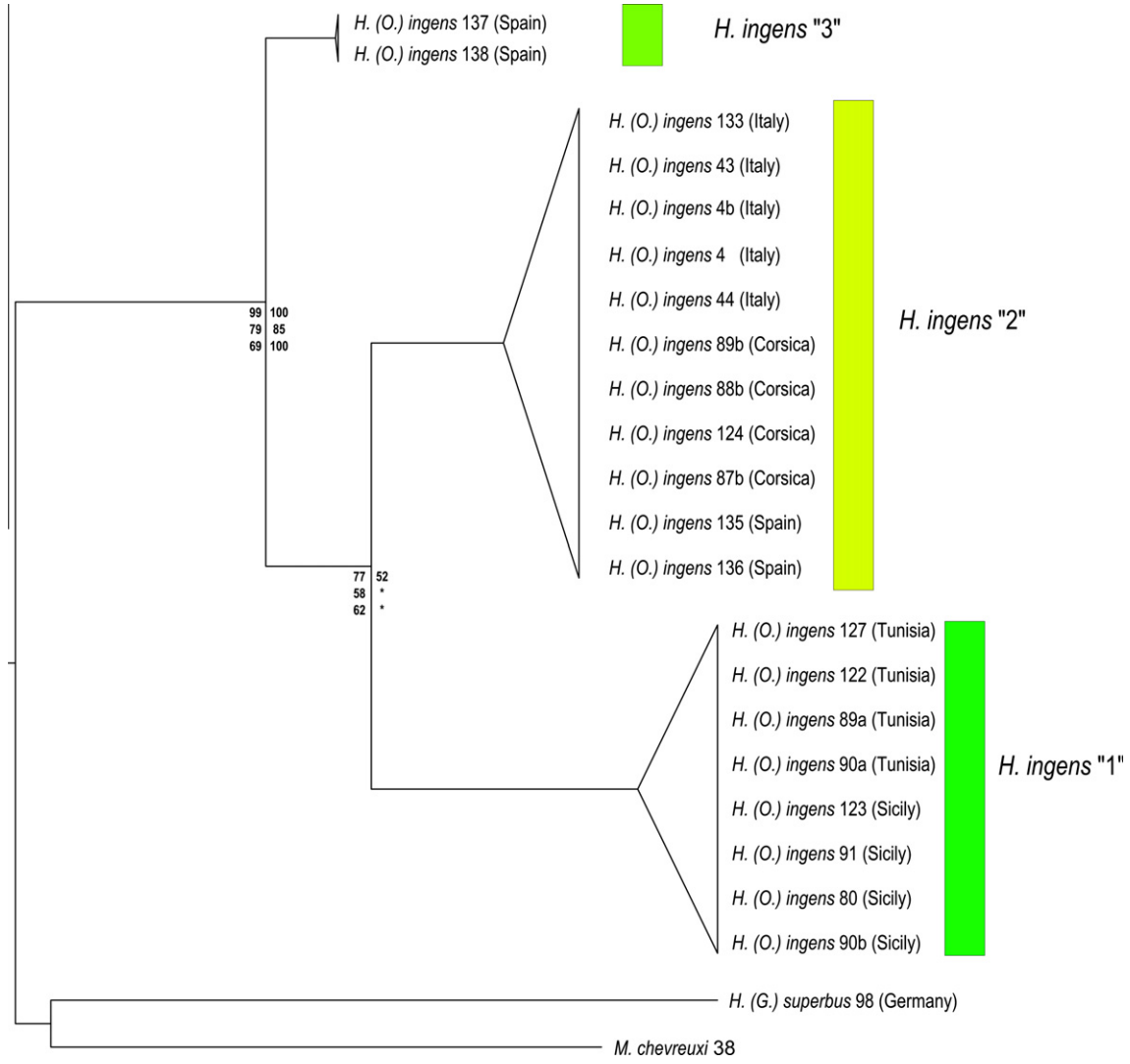

Fig. 4. Phylogenetic hypothesis based based on a 639 bp fragment of the COI (“COI-only” dataset). Bayesian (Prset statefreqpr = dirichlet (1,1,1,1); Lset nst = 2 rates = gamma), ML (HKY + I + G), and MP (Tree length = 484; CI = 0.7066; HI = 0.2934; RI = 0.8629) trees. Node support is reported left of the nodes, and it is shown as posterior probability (up), ML bootstrap value based on 1000 replicates (middle), and MP bootstrap based on 1000 replicates (down). The node support based on the BA (Prset statefreqpr = dirichlet (1,1,1,1); Lset nst = 6 rates = invgamma), ML (GTR + I + G) and MP (Tree length = 951; CI = 0.7182; HI = 0.2818; RI = 0.8503) analyses of the 1314 bp fragment of the “16S, Cyt-b and COI” dataset is reported right of the same nodes. “*” indicates node support lower than 50.

Within *Hemidiaptomus ingens* s.l., the Sicilian–Tunisian clade (“*H. ingens* 1”) includes some populations collected a few kilometres apart from the type locality of *Hemidiaptomus ingens* (Gurney, 1909) and it is morphologically consistent with the original description of *H. ingens* given by Gurney (1909); it can thus be considered as “*Hemidiaptomus ingens*” s.str. However, it is currently impossible to give a formal name to the *H. ingens* “2” and “3” clades because the morphology of the subspecies “*H. ingens inermis*” (Kiefer, 1954) is consistent with both clades, and no molecular data are nowadays available for the topotypical *H. ingens inermis* population or for nearby Algerian populations.

4.2. Phylogeography of the group

The ‘Monopolization Hypothesis’ (De Meester et al., 2002) provides a theoretical basis to explain the extraordinarily high genetic distances observed between presumed conspecific and geographically close *Occidodiaptomus* populations in spite of the high potential for passive dispersal over wide geographical distances due to the production of resting eggs. The current distribution of the species and the pattern of molecular diversity of the subgenus *Occidodiaptomus* (Fig. 6) allow to draw a working hypothesis on the phylogeography of the taxon. This scenario would locate the “ancestral area” of the subgenus, i.e. its ancestral range of distribution, on the Iberian plate. There are three facts which support this hypothesis: (i) the basal split between the clade of *H. maroccanus* and that of “*H. roubaui* + *H. ingens* s.l.” is likely to be located in southern Iberia; (ii) the Iberian Peninsula hosts four of the five species currently known for the subgenus, and the single species missing there (i.e. *H. ingens* s.s.) occurs in northern Tunisia, that is on the former Numidian microplate of Iberian origin; and (iii) no species belonging to different *Hemidiaptomus* subgenera occurs south of the Pyrenees.

Based on the dating of the cladogenetic events based on the application of standard molecular clocks (Table 3), the subgenus *Occidodiaptomus* could have differentiated from *Hemidiaptomus* s.str. and *Gigantodiaptomus* due to the Pyrenean orogeny, which occurred about 35 mya (Meulenkamp and Sissingh, 2003) causing the onset of an insurmountable geographical barrier to the dispersal for these primarily lowland diaptomid species. The diversification within the subgenus is then to be ascribed to Oligocene and Miocene vicariance events (Table 3B and Fig. 7), likewise related to the palaeogeography of the area. An early split between the

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Table 3
Divergence times (in MY before present) estimated according to the faster (2.6% *per* MY, above the diagonal) and slower (0.9% *per* MY, below the diagonal) molecular evolution rates currently known for crustaceans. (A) Comparison among subgenera; (B) comparison among the evolutionary lineages singled out within the subgenus *Occidodiaptomus*.

	Hemidiaptomus s.s.	*Gigantodiaptomus*	*Occidodiaptomus*
A			
Hemidiaptomus s.s.		15.71	20.39
Gigantodiaptomus	45.38		19.22
Occidodiaptomus	58.92	55.52	

	H. ingens "3"	*H. ingens* "2"	*H. ingens* "1"	*H. roubaui*	*H. maroccanus*
B					
H. ingens "3"		6.98	7.55	8.19	15.11
H. ingens "2"	20.17		6.13	7.81	13.56
H. ingens "1"	21.83	17.72		7.77	12.75
H. roubaui	23.66	22.56	22.46		12.90
H. maroccanus	43.66	39.18	36.86	37.28	

Table 4
Application of the "4x rule" to *Hemidiaptomus ingens* s.l. mitochondrial lineages. (A) Four times the nucleotide polymorphism within each clade (4Θ); (B) K among clades based on the ml-distance. Lower triangle: "16S and Cyt-b" dataset; upper triangle: "COI" dataset. See text for details.

	16S and Cyt-b dataset		COI dataset	
	p-Dist	ml-Dist	*p*-Dist	ml-Dist
A				
1. *H. ingens* "1"	0.072187	0.085207	0.170092	0.204940
2. *H. ingens* "2"	0.125128	0.150050	0.164271	0.195284
3. *H. ingens* "3"	0.012519	0.012521	0.012519	0.012520

	1	2	3
B			
1. *H. ingens* "1"		0.351258	0.319081
2. *H. ingens* "2"	0.202728		0.208427
3. *H. ingens* "3"	0.263195	0.245874	

ancestor of *H. maroccanus* and that of the lineage "*H. roubaui* + *H. ingens* s.l." took place in the late Oligocene, with the separation of the populations inhabiting the Baetic–Rif system (i.e. the core of the area currently inhabited by *H. maroccanus*) and those located north of this area. Such a pattern is known for other taxa (e.g.: Veith et al., 2004; Pfenninger et al., 2010) and is to be ascribed to an early separation of the Baetic–Rif area from the rest of the Iberian plate.

Hemidiaptomus maroccanus roughly remained in the area where the species originated through a vicariance event which differentiated it from the other species of the subgenus. *Hemidiaptomus maroccanus* is, in fact, the most divergent species of the group both based on morphology (Fig. 1) and on nuclear and mitochondrial data (Figs. 2 and 3). Furthermore, the only morphological character for which the ancestral state is known for diaptomid copepods, i.e. the antennulary chaetotaxy, is present in its ancestral form in *H. maroccanus* (i.e. two setae on the 16th female antennular segment), while it is derived (i.e. a single seta on the 16th female antennular segment) in all the other species of the group, further supporting the early split of *H. maroccanus* from the other *Occidodiaptomus* species.

The common ancestor of the subgroup "*H. roubaui* + *H. ingens* s.l.", occurring in the central and northern part of the Iberian plate, was then subject to cladogenetic events related to the fragmentation of the Iberian plate and the consequent isolation of the populations inhabiting the drifting microplates. This way, the ancestors of *Hemidiaptomus ingens* s.l. would differentiate in the drifting microplates, while the ancestor of *H. roubaui* likely remained in the Ibero–Provençal area, from where it expanded its distribution to southern Iberia and central and northern France during the Pleistocene. The heavily structured molecular tree for this last species mirrors the complex situation of the Iberian peninsula during the Plio–Pleistocene glacial events, when the existence of a complex network of refugia and a varied physiography enhanced the formation and survival of isolated local lineages, as already known for other animal groups (Gomez and Lunt, 2006).

This scenario is nowadays blurred by local extinctions and recent dispersal events, which have to be advocated to explain (i) the absence of well-differentiated *Occidodiaptomus* lineages in Sicily or Sardinia, (ii) the presence of *H. roubaui* in Sardinia, and in central and northern France, areas which could have been colonised only during the last 10,000 years, i.e. after the last glacial event, and (iii) the presence of *Hemidiaptomus ingens* "2" in Apulia, which is unexpected in the frame of the biogeography of a

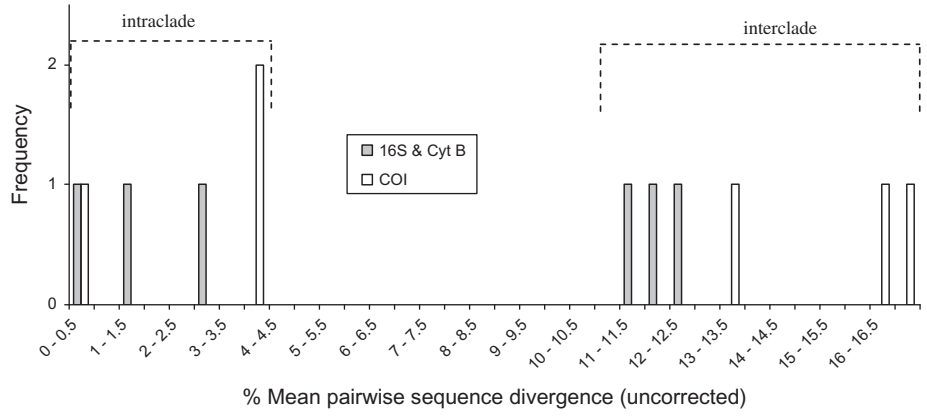

Fig. 5. Histogram showing frequency of mean uncorrected sequence divergences within and between *H. ingens* s.l. clades based on the "16S and Cyt-b" and "COI-only" mitochondrial datasets.

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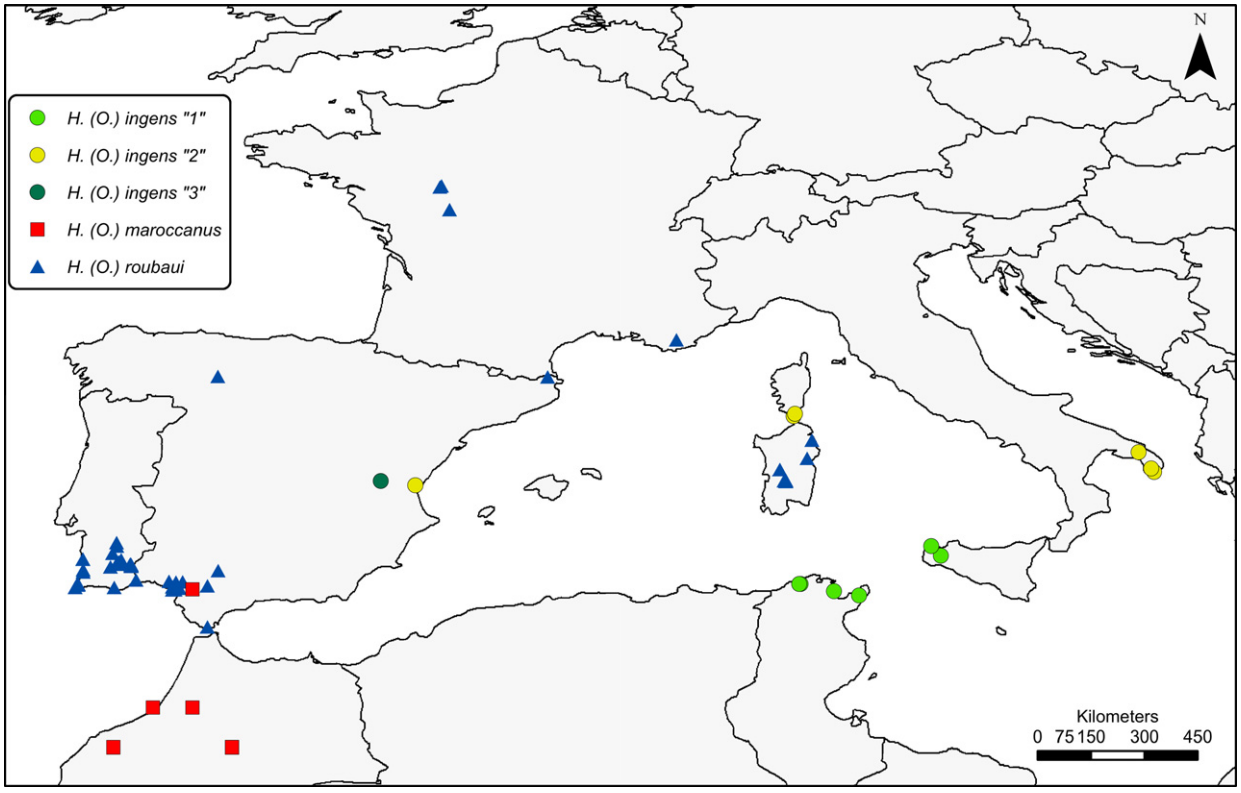

Fig. 6. Map of the Mediterranean area showing the sampled sites of the *Occidodiaptomus* species included in the analyses (see Table S1 for site details).

supposedly west-Mediterranean taxon. Furthermore, it is currently difficult to single out the distribution area for such a rare species as *H. ingens* "3", a taxon for which a single occurrence locality is currently known (Table 2).

The presence of *Hemidiaptomus roubaui* in Sardinia is particularly puzzling, as one would expect that a species belonging to *H. ingens* s.l. would occur there, possibly *H. ingens* "2", which occurs also in southern Corsica and in north-eastern Spain. Conversely, in spite of an intense sampling effort, *H. roubaui* is the only *Hemidiaptomus* species ever found in Sardinia, where it is rather common especially in the central and northern part of the island.

It is noteworthy that the high potential for passive dispersal of the *Occidodiaptomus* species seems to have not completely masked the original distribution pattern of the taxon as the dispersal events led to a successful colonisation only in those areas where no species of the group occurred, which is in good accordance with the Monopolization Hypothesis (De Meester et al., 2002), and in accordance with the existence of only extremely limited areas of sympatric co-occurrence of *Occidodiaptomus* species (cf. Table 1 and Fig. 6).

The current diversity and distribution of the species of the group is thus the result of a combination of ancient (Oligocene–Miocene) vicariance speciations and more recent (Pleistocene to Holocene) dispersals and colonisation events. Similarly complex diversity patterns are known for other invertebrate species (e.g.: Montreuil, 2008; Fochetti et al., 2009; Pfenninger et al., 2010) and these are considered typical for palaeo-Tyrrhenian taxa.

4.3. Some remarks on morphological and molecular evolutionary rates in copepods

According to the fastest and the slowest crustacean mitochondrial evolutionary rates known to date, the species differentiation within the subgenus *Occidodiaptomus* is to be dated in temporal frames comprised between 6 and 15 or 17 and 43 mya, respectively (Table 3). Although already described for other invertebrate species (e.g. Gomez et al., 2002), such an ancient dating of the diversification of morphologically-similar to undistinguishable taxa belonging to a single subgenus is noteworthy and it is in sharp contrast with the hypothesis of Pleistocene diversification which is often advocated for explaining the current diversity patterns in the Holoarctic region (e.g. Hewitt, 2000; Thum and Harrison, 2009). In such cases, the use of taxon-specific, properly calibrated, evolutionary rates instead of the "standard" ones would be recommendable.

However, in copepods the estimate of taxon-specific evolutionary rates is hampered by the nearly complete absence of fossil records to be used as reference points for the calibration of molecular clocks. To date, only few fossil copepods are known (e.g. Palmer, 1960; Selden et al., 2010), and the only known freshwater calanoid fossil remains are spermatophores and egg sacks from late Quaternary sediments (Bennike, 1998). Moreover, a noteworthy morphological conservatism is known for those few microcrustaceans which left fossil evidences, and modern species which are morphologically similar or even indistinguishable might in fact be separated by an ancient history of independent evolution: modern morphological resemblance does not necessarily mirror recent cladogenetic events (Taylor et al., 1996; Rocha-Olivares et al., 2001; Suno-Uchi et al., 2008; Thum and Harrison, 2009).

Although it has been routinely applied to a number of freshwater crustacean taxa (e.g. Taylor et al., 1996; Braga et al., 1999; Korn et al., 2006; Thum and Harrison, 2009; Zofkova and Timms, 2009; Ketmaier et al., 2012), the application of standard molecular evolutionary rates to freshwater microcrustaceans is to be used with caution, as it might overestimate the divergence times of the studied taxa. This is possibly due to the very short life span, fast generation time and peculiar life history of microcrustacean species, which might give many more chances to mutations to take place,

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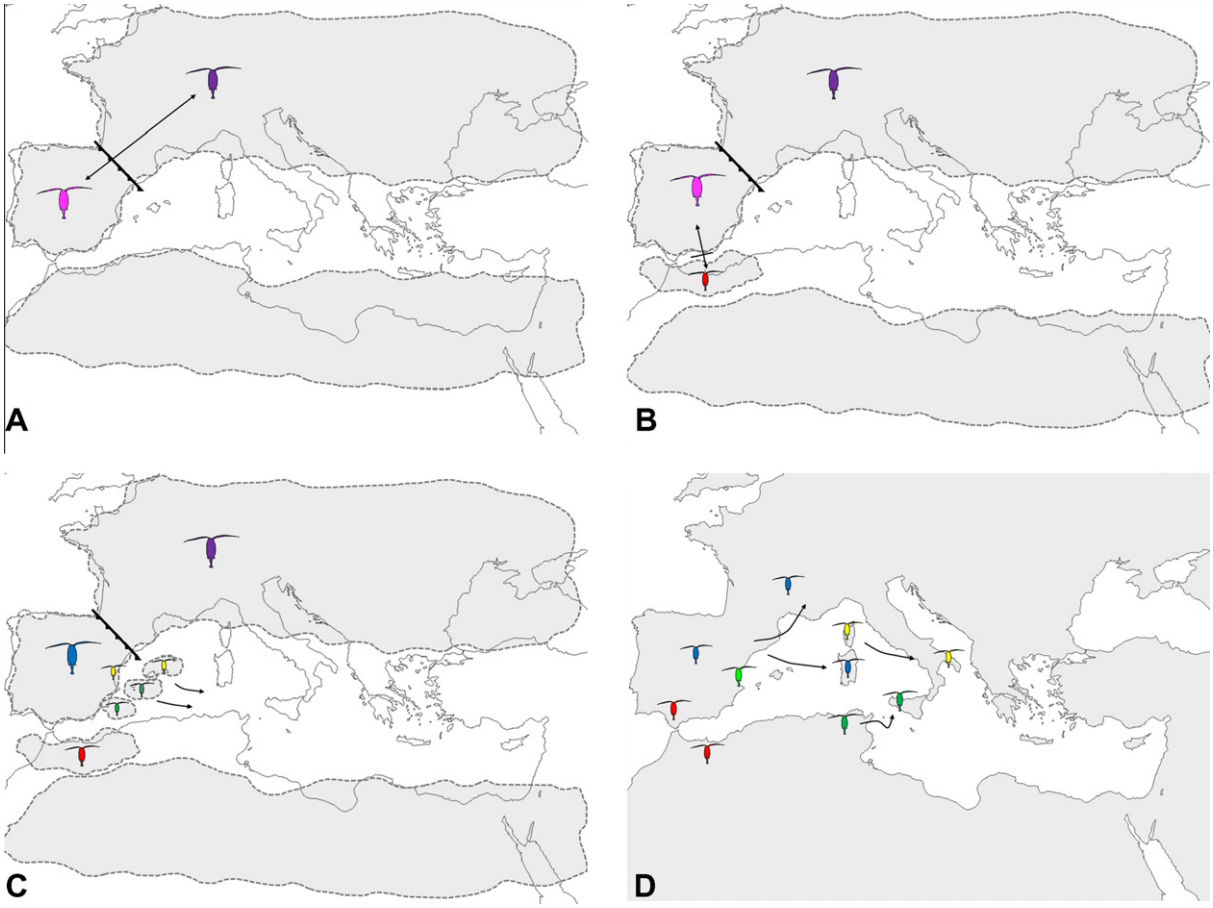

Fig. 7. Hypothesis on the origin and diversification the subgenus *Occidodiaptomus*. Grey: land above sea level. (A) Upper Oligocene Pyrenean orogeny and split of the ancestor of the *Occidodiaptomus* species from the ancestor(s) of the other *Hemidiaptomus* subgenera. (B) Late Oligocene separation of the Baetic–Rif area from the Iberian Plate, with the separation of *H.* (*O.*) *maroccanus* from the common ancestor of *H.* (*O.*) *roubaui* and *H.* (*O.*) *ingens* s.l. (C) Miocene fragmentation of the Iberian Plate, with the isolation of the populations inhabiting the drifting microplates. (D) Pleistocene to Olocene dispersal and colonisation of France and southern Italy. Different colours represent different lineages. Purple: *Hemidiaptomus* s.l.; lilac: ancestor of the subgenus *Occidodiaptomus;* red: *H.* (*O.*) *maroccanus*; blue: *H.* (*O.*) *roubaui*; green and yellow: different *H.* (*O.*) *ingens* s.l. lineages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

when compared to observations in decapods or other malacostracan groups currently used for the calibration of "standard" molecular evolutionary rates (e.g. Knowlton and Weigt, 1998; Ketmaier et al., 2003). Furthermore, the possibility that mutagenic effects might act more incisively in shallow and ecologically-variable habitats has also to be considered: Zofkova and Timms (2009) suggested that the mutagenic effect of high UV exposure accelerates rates of molecular evolution in a fairy shrimp inhabiting shallow temporary ponds.

This study stresses once more the high frequency of "cryptic speciation" events in copepods (e.g. Lee, 2000; Grishanin et al., 2005; Thum and Derry, 2008; Makino and Tanabe, 2009; da Costa et al., 2011) and the apparent decoupling of morphological and molecular evolutionary rates in this group. A new, exciting challenge for the future copepod research is to find a way to deal with this decoupling: new models on the rates of molecular evolution in microcrustaceans are to be developed and tested for inland water copepods, taking in account the different natural history of different taxa.

5. Data accessibility

All the crustacean and DNA samples are stored in FM's crustacean collection at the "Dipartimento di Biologia ambientale e Biodiversità" of the University of Palermo.

Sequences are deposited in GenBank (Table S4; ***it will be realised upon acceptance of the ms***).

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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.2012.09.016.

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