

First record of *Hemidiaptomus (Gigantodiaptomus) superbus* (Schmeil, 1895) in Italy, with notes on distribution and conservation status (Copepoda, Calanoida, Diaptomidae)

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ABSTRACT

Hemidiaptomus (Gigantodiaptomus) superbus (Schmeil, 1895) was found in a temporary pool in the Pineta di Classe coastal pine-wood (Ravenna, northern Italy). This is the first official record of a species belonging to the subgenus *Gigantodiaptomus* of Italian fauna, and it widens the known distribution of this rare diaptomid to the Mediterranean basin. In order to supply data on this rare and poorly known species, original drawings for the Italian population have been provided, and some overlooked morphological features have been highlighted. In light of the growing evidence of the existence of a number of cryptic species in the Diaptomidae family, and considering the variability observed in the ornamentation of the fifth pair of female legs, the ribosomal DNA marker 16S was used to compare the specimens collected in Italy with those from the terra typica of the species. Molecular data confirmed the strict conspecificity of the two populations being studied. The environmental parameters relating to the new Italian site are described and compared with the few data currently available in the literature. A careful review of all the published data dealing with *H. (G.) superbus* has been performed, and the paucity of recent records is stressed, together with the opportunity to support dedicated conservation measures for the protection of this species.

Key words: temporary ponds, zooplankton, biogeography, biodiversity, 16S rDNA

1. INTRODUCTION

The Palaearctic diaptomid genus *Hemidiaptomus* Sars 1903 includes 19 species, occurring in temporary water bodies from Mongolia to Spain and Morocco (Dussart & Defaye 2002; Stepanova 2005a). These species are currently divided into three subgenera, i.e. *Hemidiaptomus* s. str. Sars 1903, *Gigantodiaptomus* Kiefer 1932, and *Occidodiaptomus* Borutzky 1991, all of which are mostly parapatric and present in limited co-occurrence areas (Kiefer 1978; Dussart & Defaye 2002; Marrone *et al.* 2010). The subgenus *Gigantodiaptomus*, a taxon considered of genus rank by some authors (Stepanova 2005b; Walter 2010), includes five species occurring in Asia and central Europe. Of these, *Hemidiaptomus (Gigantodiaptomus) superbus* (Schmeil 1895) has proved to be the rarest and most enigmatic species of the group. In spite of its widespread distribution, which covers central/eastern Europe and the Caucasus, few records are currently available and most of these are old reports lacking iconography and in need of confirmation.

Hemidiaptomus (G.) superbus has been described by Schmeil (1895) as '*Diaptomus superbus*', based on specimens collected from temporary ponds in the 'Biederitzer Busch bei Magdeburg' (Germany) and the draw-

ings published one year later (Schmeil 1896). Kiefer (1932a, 1932b) transposed it to the subgenus *Gigantodiaptomus* of the genus *Hemidiaptomus*, whilst stressing peculiarities of *H. (G.) superbus*, which are not typical of the other species of that subgenus. This attribution is nowadays widely accepted (e.g., Dussart & Defaye 2002) with the only exception of Einsle (1993), who has assigned the species to the subgenus *Hemidiaptomus* s. str., and Stepanova (2005a), who has raised the subgenus *Gigantodiaptomus* to genus level.

Its description having been documented, the species was sighted in Denmark (Jensen 1905 in: Tollinger 1911), the Ukraine (Belousov, 1908), France (Beuchamp 1918), Algeria (Roy 1924), Croatia and Serbia (Krmpotić 1925; Ternjej & Stankovic 2007; I. Ternjej, pers. comm.), Azerbaijan (Weisig 1931), Germany (Elster 1931, Gillandt & Martens, 1975; Gillandt *et al.* 1983), Iran (Brehm 1937), and Poland (Błędzki 2008) (Fig. 1). Based on the absence of setae on the distal part of the endopodites of the fifth pair of female legs, the *H. (G.) superbus* populations found in the Caucasus were considered by Brehm (1937) as a different variety of the European *H.(G.) superbus*, and the author gave it the name of '*Hemidiaptomus superbus* var. *hyrcanensis*'. This taxon, accepted by Löffler (1961) and Kiefer (1978), was later synonymised with *H. (G.) superbus* by Dussart & Defaye (2002).

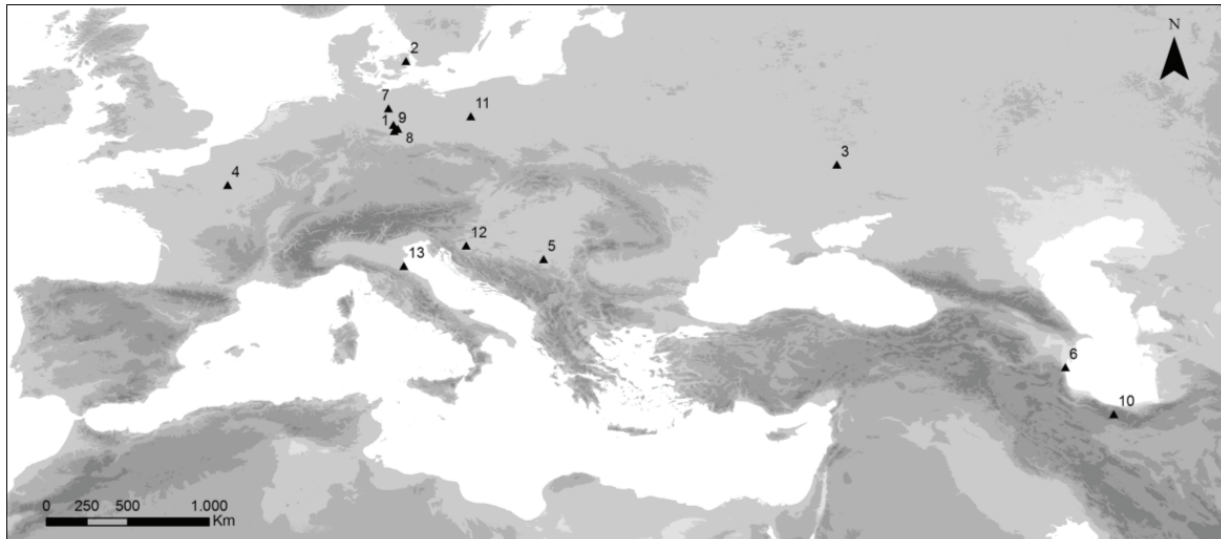


Fig. 1. Occurrence localities of *H. (G.) superbus*. Sources: **1:** Schmeil, 1895; **2:** Jensen, 1905; **3:** Belousov, 1908; **4:** Beuchamp, 1918; **5:** Krmptović, 1925; **6:** Weisig, 1931; **7:** Gillandt & Martens, 1975; **8, 9:** Elster, 1931; **10:** Brehm, 1937; **11:** Błędzki, 2008; **12:** I. Ternjej, pers. com.; **13:** Present work. The record of the species for Algeria (Roy, 1924) is most likely erroneous (see text) and thus has not been reported on the map.

Unfortunately, most of the reports of the species were published without drawings, which precludes checks as to their reliability e.g., *Hemidiaptomus (G.) superbus* was officially recorded in the Reghaïa forest, near Algiers (Roy, 1924), a site where extensive surveys carried out in subsequent years led to the finding of the only *Hemidiaptomus (Occidodiaptomus) ingens* (Gauthier 1928). Furthermore, Roy & Gauthier (1927) did not include *H. (G.) superbus* in their work on the copepod fauna of Algeria, thus implying that the authors themselves deemed that the previous record of the species in Algeria was erroneous. Currently, reports of the species are considered doubtful (Dussart 1989), or have not been reported at all (Mouelhi *et al.* 2000; Hamaidi *et al.* 2010) in the Reviews dealing with the copepods of Algeria and the Maghreb.

Given the unreliability of some of the older records, all those relating to *Hemidiaptomus (G.) superbus*, which lack adequate iconography, should be confirmed. Thus, the presence of the species can only be considered certain for Germany (Schmeil 1895; Elster 1931, Gillandt & Martens 1975; Gillandt *et al.* 1983) and the southern coast of the Caspian Sea (Azerbaijan and Iran) (Weisig 1931; Brehm 1937; Löffler 1961). No record of the species has been published for the last seventy years, with the exception of a single population from Peverstorf, Germany (Gillandt & Martens 1975; Gillandt *et al.* 1983). *H. (G.) superbus* is currently unanimously considered as one of the rarest Palearctic diaptomid species (Dussart 1967, 1989; Kiefer 1978; Einsle 1993). By means of an extensive faunal survey, the aim of which was to update the checklist and distribution of Italian inland water copepods, various specimens of *H. (G.) superbus* were collected in a temporary pool near Ravenna (northern Italy).

2. MATERIALS AND METHODS

The study material was collected on 6 June 2010 in four temporary pools in the Pineta di Classe near Ravenna, Italy. The sampled sites are shallow, clear-water temporary pools, which are located in a mature coastal pinewood. The bioclimatic characterization of the area is based on the classification formulated by Rivas-Martinez (1995). Water temperature, pH and specific conductivity were registered with a MM40 Crison multimeter. Microcrustacean samples were collected by means of a 200 µm mesh-sized hand net and fixed *in situ* with 80% ethanol. Calanoid copepods were isolated, dissected under the stereomicroscope and identified to the species level, according to Kiefer (1978), Borutzky *et al.* (1991) and Einsle (1993). Co-occurring Cladocera, Cyclopoida, and Ostracoda were identified according to Margaritora (1985), Alonso (1996), Meisch (2000), Einsle (1993), as updated following the recent literature. Drawings of selected specimens (total length, females: 4200 µm; males: 3400 µm) were made with a Zeiss® Standard 18 compound microscope, which is equipped with a camera lucida, after tissue cleaning with in hot lactic acid. Preserved specimens and mounted slides have been stored in the authors' collection.

Various specimens of *Hemidiaptomus (G.) superbus* and environmental data from a temporary pond near the town of Breitenhagen (Germany) have been included in the study as comparative material. This site (coordinates UTM-WGS84 32U: 5756674 N; 703109 E; Altitude: 53 m a.s.l.) was sampled on 4 April 2008, its location being the same river basin of the type locality of the species (i.e., the Elbe River basin), and approximately 30 km from its *locus typicus*. The site was rich in water

macrophytes, water temperature was 12.1 °C, and specific conductivity was 1430 $\mu\text{S cm}^{-1}$ on the sampling date. Adult specimens of *Hemidiaptomus (G.) superbis* and *Hemidiaptomus (G.) amblyodon* (Marenzeller 1837) specimens were collected at the same time.

In light of the growing evidence of the presence of a cryptic species among diaptomid calanoid copepods (e.g., Thum & Harrison 2009) and within the genus *Hemidiaptomus* in particular (Marrone *et al.* 2010) and in order to check for the molecular divergence between the populations being studied, a fragment of the 16S ribosomal mtDNA was amplified from a single *H. (G.) superbis* specimen per population. Prior to DNA extraction, specimens were carefully cleaned and soaked in double-distilled water for 3 hours. DNA was then extracted using whole specimens and the "DNEasy Animal Tissue Kit" (QIAGEN). A partial sequence of the 16S ribosomal DNA subunit was then amplified using the "Skisto-1" (5' -TGG TAA GGT AGC ATA ATA AT- 3') e Skisto-2 (5' -CCG GTT TGA ACT CAG ATC ATG T -3') primers, as described by Thum & Harrison (2009).

The PCR mix consisted of 3.5 μL of Buffer 10 \times (THERMOSCIENTIFIC), 3 μL of 25 mM MgCl_2 , 0.35 μL of dNTP 10 mM, 1 μL of each 10 μM primer, 0.4 μL of Red Hot *Taq* Polymerase 5U/ μL (THERMOSCIENTIFIC), 1 μL of DNA template, and 23.7 μL of double-distilled water, producing a total reaction volume of 35 μL . The amplification consisted of an initial denaturation step of 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 50 seconds, 42 °C for 50 seconds, and 72 °C for 1 minute, followed by a final extension at 72 °C for 8 minutes. The PCR products were separated by electrophoresis on 2% agarose gel at 70 V for 1 h and visualized with a UV Transilluminator. Amplified bands were cut, purified from agarose gel (using the Qiaquick Gel Extraction Kit) (QIAGEN) and sequenced in 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 BioEdit (Hall 1999); sequence divergence was calculated with Mega ver. 4 (Tamura *et al.* 2007). The sequences have been deposited in GenBank (Accession numbers: HQ687215 and HQ687216).

3. RESULTS AND DISCUSSION

A population of *Hemidiaptomus (G.) superbis* has been located in Italy approximately three decades after the last published record of the species (Gillandt *et al.* 1983). The morphology of the Italian specimens correlates well with the description of the species currently available in the literature (Kiefer 1978; Borutzky *et al.* 1991; Einsle 1993). However, the presence of a small chitinous hump on the dorsal side of the second segment of the exopodite of fifth, right, male leg (Fig. 2H), which was present in all the studied male specimens,

has not to date been reported in the published drawings. Within the *Gigantodiaptomus* subgenus, this chitinous outgrowth is reported to occur in all the species with the only exception of *H. (G.) hungaricus* (Kiefer 1978; Stepanova 2005a).

Furthermore, the length of the apical setae on the endopodite of fifth female pair of legs (Fig. 2B) is slightly shorter than that reported in the available drawings of German specimens (Gillandt & Martens 1975; Kiefer 1978; Einsle 1993). This characteristic is noteworthy as the 'hyrcanensis' variety of *H. (G.) superbis* was described as lacking these setae in the specimens originating from the Caucasus (Brehm 1937). It thus seems that a certain variability exists in the ornamentation of the fifth pair of female legs, which also concerns the presence of a transversal row of spinules on the apical part of the second exopodal segment of the fifth leg: these spinules are present in the German and Italian specimens (e.g., Fig. 2B) but they are absent in the specimens from the Balkans (Krpmotić 1925).

A 350 base-pairs long fragment of the ribosomal DNA subunit 16S was successfully amplified and sequenced. The uncorrected p-distance between the amplified sequences is just 0.9% (Fig. 3), while it often accounts for 14.9-23% among congeneric diaptomid species and even among distinct presumed intraspecific clades (Thum & Harrison 2009). Thus, molecular data confirm the conspecificity of the two populations under study and the existence of a certain intraspecific variability in the ornamentation of the fifth pair of female legs.

The co-occurring microcrustacean fauna, collected in the Pineta di Classe pools (Tab. 1), consists of species which are widespread in Italy and often related to slightly brackish water bodies (Marrone *et al.* 2006a; Stoch 2006; pers. obs. of the authors). *Hemidiaptomus (G.) superbis* was collected in only one out of the four sampled sites (i.e. 'pool A') on the sampling date but large diaptomids, probably *H. (G.) superbis*, had also been observed in the other pools in May 2010 (D. Miserochi, personal observation). Coordinates, water temperature, pH and the specific conductivity of the sampled pools are reported in table 2. The pools were a few square metres in width on the sampling date and the only observed macrophytes were *Alisma lanceolata* With. and *Galium palustre* L.

According to Rivas-Martinez (1995), both the Italian and the German areas, where *H. (G.) superbis* occur, lie in the temperate-oceanic bioclimatic area. However, all the other *Hemidiaptomus* species occurring in Italy, i.e. *Hemidiaptomus (Hemidiaptomus) gurneyi*, *H. (Occidodiaptomus) ingens* s.l., and *H. (O.) roubaui* (Ruffo & Stoch 2006; Marrone *et al.* 2006b; Marrone *et al.* 2010; Alfonso & Belmonte 2011) occur in areas characterised by a Mediterranean pluviseasonal, oceanic bioclimate. Unfortunately, the scarce and anecdotal data currently available for *Hemidiaptomus (G.) superbis* do not clarify the ecological preferences of the species.

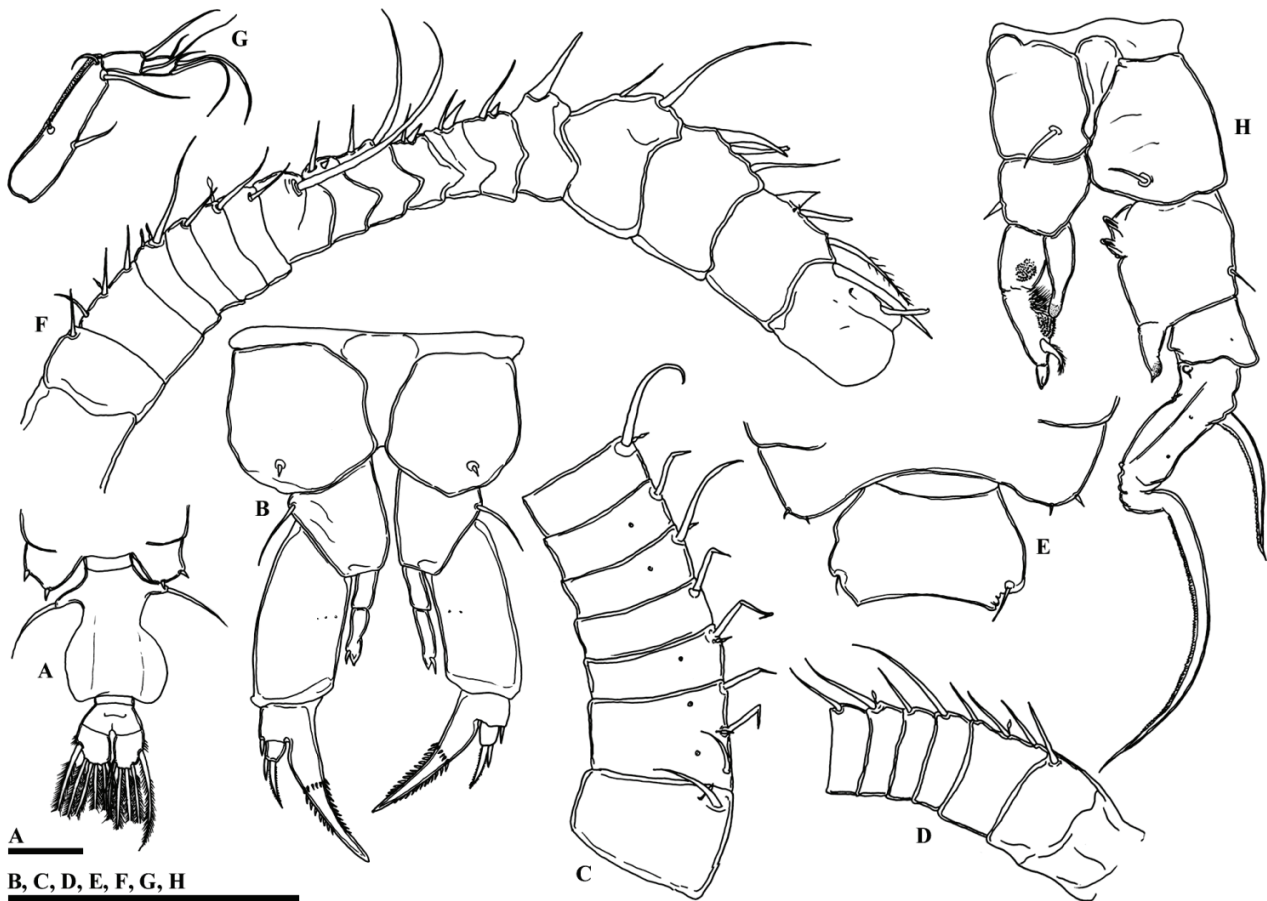


Fig. 2. A-C: *Hemidiaptomus superbis*, female. A: somites 4-5 and urosome; B: fifth pair of legs; C: antennula, segments 1-7. D-H: *H. superbis*, male. D: left antennula, segments 1-6; E: somite 5 and genital segment; F: right antennula, segments 1-17; G: right antennula, last three segments; H: fifth pair of legs. Scale bars: 500 µm.

Tab. 1. List of crustaceans occurring in the sampled pools on 6 June 2010.

Taxa	Pool A ER003	Pool B ER004	Pool C ER005	Pool D ER006
Copepoda				
Calanoida				
Diaptomidae				
<i>Hemidiaptomus (Gigantodiaptomus) superbis</i> (Schmeil, 1895)	X			
<i>Mixodiaptomus kupelwieseri</i> (Brehm, 1907)	X	X	X	X
Copepoda				
Cyclopoida				
<i>Cyclops divergens</i> (Lindberg, 1936)	X	X	X	X
<i>Megacyclops viridis</i> (Jurine, 1820)	X	X		
<i>Diacyclops bisetosus</i> (Rehberg, 1880)	X	X	X	X
<i>Diacyclops lubbocki</i> (Brady, 1869)	X	X		X
Branchiopoda				
Anomopoda				
Daphniidae				
<i>Scapholeberis rammneri</i> (Dumont & Pensaert, 1983)		X		
<i>Ceriodaphnia laticaudata</i> (P.E. Müller, 1867)		X	X	
<i>Daphnia (Daphnia) curvirostris</i> (Eylmann, 1887)		X	X	X
Ostracoda				
Podocopa				
Candonidae				
Unidentified larvae				
Cyprididae				
<i>Eucypris virens</i> 'complex' (Jurine, 1820)	X		X	
<i>Heterocypris salina</i> (Brady, 1868)		X		X

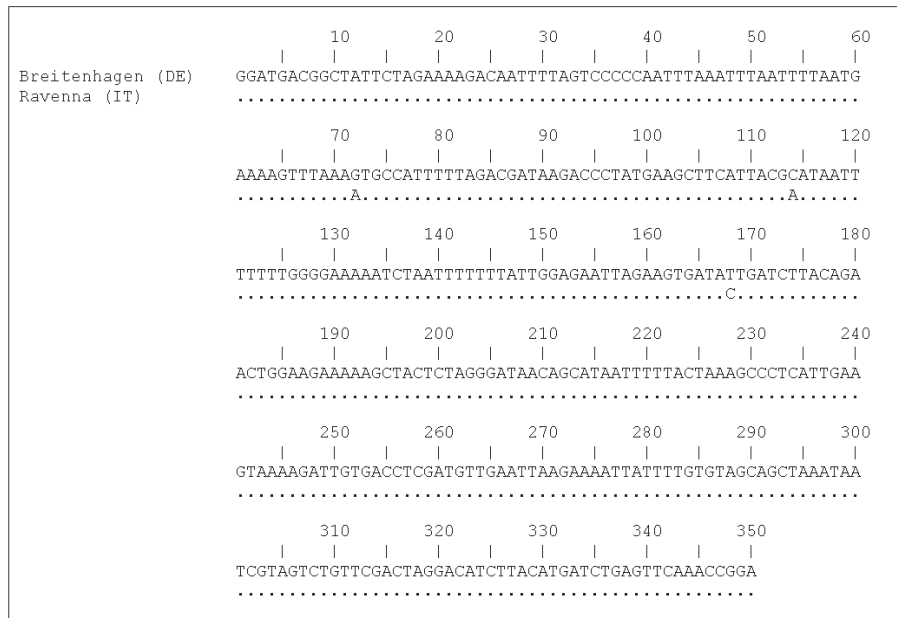


Fig. 3. Compared 16S rDNA nucleotide sequences for the Pineta di Classe (Ravenna, Italy) and Breitenhagen (Salzlandkreis, Germany) populations.

Tab. 2. Coordinates, water temperature, pH and water conductivity of the sampled pools on 6 June 2010.

	Coordinates (UTM-WGS84)	Water Temperature (°C)	Specific conductivity ($\mu\text{S cm}^{-1}$)	pH
Pool A – ER003	4913408 N; 33 283404 E	24	1433	8.04
Pool B – ER004	4913413 N; 33 283411 E	25	864	7.89
Pool C – ER005	4913411 N; 33 283426 E	25.2	1329	7.94
Pool D – ER006	4913418 N; 33 283442 E	24.1	768	7.95

However, a rather high degree of specific conductivity was recorded for both the Italian and German sites, which is quite unusual for most *Hemidiaptomus* species (e.g., Gauthier 1928; Alonso 1998; Marrone *et al.* 2006a).

The scattered geographical and temporal distribution of the records of occurrence of *H. (G.) superbis* might be ascribed to three different reasons, which are not mutually exclusive: (i) the species could be naturally rare in nature; (ii) the distribution barycentre of *H. (G.) superbis* might indeed occur in relatively unknown regions of south-eastern Europe and the Caucasus, thus being present in western Europe only with few, short-living populations which episodically colonise sites along the westernmost fringe of the species' distribution area; and (iii) the species could have experienced a total collapse in recent decades, having been replaced by other, more generalist, copepod species. The latter hypothesis is supported by the fact that, compared to nine records published between 1895 and 1937 (Schmeil 1895; Jensen 1905; Belousov 1908; Beauchamp 1918; Roy 1924; Krmpotić 1925; Weisig 1931; Elster 1931; Brehm 1937), only two records of *Hemidiaptomus (G.) superbis* were published after 1895 and both of them regarded the same area near the town of Pevestorf, Germany (Gillandt & Martens 1975;

Gillandt *et al.* 1983). The occurrence of the species near Breitenhagen (Germany), which was reported by Elster (1931), has been confirmed in the ambit of this study; however, it was not possible to find *H. (G.) superbis* in the *locus typicus* of the species (Schmeil, 1895) in the Magdeburg area, which is the other site reported by the same author (Elster 1931). Furthermore, the European distribution area of *H. (G.) superbis* overlaps with that of the more common and widespread congeneric species *Hemidiaptomus (G.) amblyodon* (Dussart & Defaye 2002), which could have replaced the *H. (G.) superbis* in various sites. This process might be ongoing, e.g. these two *Hemidiaptomus* species currently co-occur in the Breitenhagen pond, both syntopically and synchronically, while Elster (1931) reported for the same site the occurrence of *H. (G.) superbis* only.

It is necessary to confirm the current presence of the species for most of the countries where it has been recorded, and it would be desirable to check the historical and current occurrence of *H. (G.) superbis* throughout its known distribution area. Until more data regarding the distribution and consistency of the species in Europe are available, dedicated, protective measures of the few, confirmed known occurrence sites of the species (i.e., the pools in the Pineta di Classe in Italy, and the ponds near Breitenhagen and Pevestorf in Germany)

should be implemented, and *H. (G.) superbus* should be included on the national 'red lists' for invertebrates (e.g., Herbst 1982).

The presence of *Hemidiaptomus (Gigantodiaptomus) superbus* in Italy, which constitutes the first *Hemidiaptomus* record from the Padanian faunal province (Minelli *et al.* 2006), has increased to 26 the number of diaptomid species currently known to occur in Italy (Stoch 2006), and it confirms Italy as one of the European countries with the richest diaptomid copepod fauna (Dussart & Defaye 2002). This finding is rather unexpected since no other species belonging to the *Gigantodiaptomus* subgenus have been reported south of the Alps and Pyrenées (see Marrone *et al.* 2010 for a review). The Italian finding further stresses the considerable cognitive gaps which affect our knowledge relating to particular taxa, even in the assumed better-known countries in the west-Palaeartic area.

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