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Systematics and biogeography of the genus *Besdolus* Ricker, 1952 (Plecoptera, Perlodidae): molecules do not match morphology

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Abstract

The Central-Southern European genus *Besdolus* was reinstated and revised by Zwick and Weinzierl (1995), and includes five species: *B. imhoffi* (Pictet), *B. ventralis* (Pictet), *B. bicolor* (Navás), *B. ravizzarum* Zwick & Weinzierl, and *B. illyricus* Kovács & Zwick. Overall, these species are rarely collected and have apparent relictual distributions. From the ecological point of view, *B. bicolor*, *B. ravizzarum* and *B. illyricus* seem to be more orophilic whereas *B. imhoffi* and *B. ventralis* are associated to lowland rivers. These species are sensitive to the environmental perturbations and are endangered taxa, threatened with extinction. Species identifications are difficult using available morphological characters. We sequenced a fragment of the mitochondrial gene COI to better understand the systematics and biogeography of this genus and to evaluate the molecular intra- and interspecific distances. Specific boundaries, species relationships, degree of isolation and molecular similarity are also presented. The molecular data do not fully support the validity of the five species. Molecular distances between *B. bicolor* and *B. ventralis* and between *B. imhoffi* and *B. illyricus* are similar to what has been previously reported for conspecific stonefly taxa. In this study, the results of the molecular approach are not congruent with the traditional morphological arrangement. Biogeographically, we hypothesize that a Central European stem species dispersing westward and southward diverged into two lineages, then differentiated on the three European main peninsulas.

Key words: Plecoptera, Perlodidae, *Besdolus*, cytochrome oxidase subunit-1, evolutionary rates, mtDNA, phylogeny, stoneflies

Introduction

The genus *Besdolus* has a Central-Southern European distribution, and was originally established as a new subgenus of *Isogenus* by Ricker (1952) and later included by Stark *et al*. (1986) in *Dictyogenus* Klapálek, 1904. The genus *Besdolus* was reinstated and revised by Zwick and Weinzierl (1995) and presently includes five species, *B. imhoffi* (Pictet, 1841), *B. ventralis* (Pictet, 1841), *B. bicolor* (Navás, 1909), *B. ravizzarum* Zwick & Weinzierl 1995, and *B. illyricus* Kovács & Zwick 2008. Overall, these species are considered rare and have apparent relictual distributions (Zwick & Weinzierl, 1995). *Besdolus bicolor* is known from historical records from Central Spain (a few sites in the Guadalajara, Albacete, Madrid and Teruel provinces) and from Andalusia (one site). Currently, this species occurs only in two mountain systems of Andalusia ("Sierra de Alhama, Tejera y Almijara"; "Sierra de Cazorla, Segura y Las Villas" (Tierno de Figueroa *et al*., 2003, updated). *Besdolus imhoffi* was once abundant in Central Europe (Switzerland, Germany, Austria, and Belgium) and in the former Yugoslavia (Zwick & Weinzierl, 1995). *Besdolus imhoffi* was not collected for decades but recently rediscovered at one site in Central Europe (Uffinger stream, Ammer Basin, Germany) and in several sites in Croatia (Popijac & Sivec, 2009; Kovács & Murányi, 2008). *Besdolus ravizzarum* occurs in a small portion of the Italian northern Apennines, where it has a scattered distribution in the same drainage basin and at three sites in France (Var, Haute Provence and Haute-Garonne provinces, from 2 males, 5 females and 4 larvae collected from 1942 to 1976). *Besdolus ventralis* was

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described from a female holotype. The old material referred to this species (one record each for Switzerland, Germany and Macedonia) was collected at the beginning of the last century, in 1916 (2 males), 1913 (1 female) and 1916/1918 (3 females) respectively. *Besdolus ventralis* probably exhibited a distribution pattern overlapping with *B. imhoffi* and has been recently collected only from the Carpathian basin (two sites in Austria, one site in Hungary), and Greece (one site) (Kovács & Ambrus, 2001; Kovács *et al*., 2004; Kovács & Zwick, 2008). The last known species, *B. illyricus* was recently described by Kovács & Zwick (2008) from Montenegro and is known from three streams in Montenegro and in four sites in Albania (Kovács & Murányi, 2008). *Besdolus* species have never been found living in sympatry.

Ecologically, *B. bicolor*, *B. ravizzarum* and *B. illyricus* are more orophilic whereas *B. imhoffi* and *B. ventralis* are associated with the epipotamon. These species are sensitive to environmental perturbations and are considered endangered and threatened with extinction (see for instance Fenoglio *et al*., 2010).

Using morphological characters to identify the species of *Besdolus* is problematic (Zwick & Weinzierl, 1995), and identification is often based on geographical proximity. The objective of this study was to provide a better understanding of the systematics and biogeography of the genus *Besdolus* and to evaluate genetic isolation and molecular intra- and interspecific distances using cytochrome c oxidase subunit 1 (COI). The use of DNA sequencing has been used previously to elucidate the systematics and biogeography of Plecoptera (Fochetti *et al*., 2009).

Material and methods

Taxon sampling and specimen collection. Specimens, collector, traditional attribution and source of sampling species are reported in Table 1 with acronyms used in this paper. We analysed nymphs of 10 *Besdolus* populations (almost half of the known populations) for a total of 22 specimens, preserved in 99% ethanol. Nymphs were all collected from streams that were inhabited by a single known taxon of *Besdolus*. We collected and used sequences from two populations of *Perlodes microcephalus* (Pictet) and *Perla* sp. sequences taken from GenBank as outgroups.

DNA extraction, amplification and sequencing. Total genomic DNA was extracted from single entire individuals using the Easy-DNA™ Kit (Invitrogen Co., Carsbad, USA or Qiagen, Hilden, Germany) following manufacturer procedures. A region of the mitochondrial gene encoding the COI was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*. 1994) obtaining a 658 bp sequence. Primers were used in PCR with 1 µg of total DNA, using GoTaq®Green Master Mix (Promega) in an Eppendorf MiniCycler™ model PTC-150-16 (MJ Research, USA). Purification and sequencing (one strand) were performed by Macrogen Inc. (Seoul, Korea). All sequences have been deposited at GenBank (see Table 1 for accession numbers).

Sequence and phylogenetic analyses. Chromatograms were manually adjusted with Chromas Lite 2.01 (Copyright© 1998-2005, Technelysium Pty Ltd); the obtained sequences were analysed for similarity in BLAST (Altschul *et al*., 1997). Amino acid sequences were initially aligned by ClustalW2 using default parameters (Thompson *et al*., 1994) and manually adjusted with MEGA 4.0 (Tamura *et al*., 2007). Selected sequences of *Perla* sp. from GenBank (HM880064, HM880063, HM880062, HM880061) and sequences from two distinct populations of *P. microcephalus* (see Table 1) were used as outgroups. MEGA 4.0 (Tamura *et al*., 2007) was used to build phylogenetic trees using Neighbour Joining [NJ (Saitou & Nei, 1987] and Maximum Parsimony [MP (Farris, 1970)] assumptions. Bootstrap support for MP trees was calculated using 10000 bootstrap replicates. The evolutionary distances were computed using by default the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Treefinder (Copyright © 1997–2008, Gangolf Jobb; Jobb *et al*., 2004) was used to build the Maximum Likelihood phylogenetic tree, using an exhaustive search strategy (branch-and-bound) (Felsenstein, 1981). In all approaches, gaps and missing data were eliminated from the dataset, obtaining a final dataset of 591 positions (available on request from the first author).

A model of evolution was chosen (HKY) after evaluation by MrModeltest 2.1 (Nylander, 2004) using a likelihood ratio test and the Akaike information method. Bootstrap support was calculated for ML trees using 10000 bootstrap replicates under the same assumptions, performing heuristic searches with stepwise addition of taxa, 10 random-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping.

TABLE 1. Locality and collecting data of the specimens, acronyms used in the text and figures, traditional taxonomic attribu-

tion and Genbank Accession numbers of the sequences.

Species	Locality, date, collector, identifier	Specimens, acronym	GenBank accession number
Besdolus ravizzarum	Curone stream (Piedmont, ITALY), 6/IV/2010, m 470. 44°46'12,78'' N; 9°09'16,12''E (Fenoglio & Bo leg. and det.)	4, BRA_Curo	JN034537; JN034538; JN034539; JN034540
B. ravizzarum	Chero stream (Emilia, ITALY), 9/III/2010, m 300. 44°48'40,77"N; 9°44'32,81"E (Fenoglio & Bo leg. and det.)	2, BRA_Cher	JN034541; JN034542
B. ravizzarum	Trebbia stream (Emilia, ITALY), 8/III/2010, m 340. 44° 42'59,13"N; 9°22'58,86"E (Fenoglio & Bo leg. and det.)	1, BRA_Treb	JN034543
B. ravizzarum	Vobbia stream (Liguria, ITALY), 1/IV/2010, m 475. 44°36'40,42'' N; 9°00'51,79''E (Fenoglio & Bo leg. and det.)	3, BRA_Vobb	JN034544; JN034545; JN034546
B. ravizzarum	Staffora stream (Lombardy, ITALY), 8/III/2010, m 490. 44°47'30,63" N; 9°13'38,75"E (Fenoglio & Bo leg. and det.)	2, BRA_Staf	JN034547; JN034548
B. imhoffi	Grosse Lauter at Lauterach, (Baden-Württemberg, GERMANY), 5/V/1999 (Teslenko & Zwick leg. and det.)	1, BIM	JN034556
B. ventralis	Rába River, (Magyarlak, HUNGARY), 8/IV/2010 (Kovács leg. and det.)	3, BVE	JN034557; JN034558; JN034559
B. illyricus	Donja Polja: Zoljski ljevak (MONTENEGRO) (locus typicus), 12/IV/2010 (Kovács leg. and det.)	3, BIL	JN034553; JN034554; JN034555
B. bicolor	River Borosa, (Sierra de Cazorla, Segura y las Villas, Jaén province, SPAIN), 4/II/2009, m 690. U.T.M. 30 S X: 0512498; Y: 4207025 (Tierno de Figueroa leg. and det.)	2, BBI_Boro	JN034560; JN034561
B. bicolor	River Cacín, (Sierra de la Almijara, Fornes, Granada province, SPAIN), 18/XII/2008, m 860. UTM: 30S 423488 4088316, (Tierno de Figueroa leg. and det.)	1, BBI_Caci	JN034562
Perlodes microcephalus	Parma stream (Emilia, ITALY), 9.III.2010, m 554. 44°28'57,97"N; 10°05'36,52"E; Nure stream (Emilia, ITALY), 9/III/2010, m 639. 44° 38'30, 44"N; 9°29'44,67" E (Fenoglio & Bo leg. and det.)	4, PMI_Parm; PMI_Nure	JN034549; JN034550; JN034551; JN034552
Perla sp.		4	HM880064; HM880063; HM880062; HM880061

Results

Sequences and molecular divergence. A total of 30 COI sequences, 591 bp long, were obtained (including outgroups). We found a total of 17 haplotypes out of 22 *Besdolus* specimens analysed. Where more sequences were available within a species, intraspecific distances ranged from 0.000 to 0.083. In particular, divergence within *B. ravizzarum* ranged from 0.000 to 0.026 (n = 12), within *B. illyricus* from 0.002 to 0.033 (n = 3), within *B. ventralis* from 0.003 to 0.033 (n = 3), within *B. bicolor* from 0.009 to 0.083 (n = 3) (Table 2). Interspecific distances for *B. bicolor*/*B. ventralis* and *B. imhoffi*/*B. illyricus* are similar to the ranges for intraspecific comparisons (from 0.009 to 0.091). The remaining interspecific comparisons ranged from 0.12 (BRA_Staf2– BBI_Boro1) to 0.175 (BIL 3–BBI_Boro2). Intergeneric distances varied from 0.213 to 0.262 (*Perlodes* vs *Besdolus*), from 0.250 to 0.292 (*Perla* vs *Besdolus*) and from 0.257 to 0.292 (*Perla* vs *Perlodes*).

TABLE 2. Pairwise composite molecular distances (Maximum Composite Likelihood method) based on COI sequences comparison, for 22 specimens and 5 species of the genus *Besdolus*. Acronyms as in Table 1.

	BRA_Cher1	BRA_Cher2	BRA_Staf1	BRA_Staf2	BRA_Treb	BRA_Vobb1	BRA_Vobb2	BRA_Vobb3	BRA_Curo1	BRA_Curo2	BRA_Curo3	BRA_Curo4	PMI_Nure1	PMI_Nure2
BRA_Cher1														
BRA_Cher2	0.007													
BRA_Staf1	0.007	0.010												
BRA_Staf2	0.009	0.012	0.002											
BRA_Treb	0.009	0.009	0.002	0.003										
BRA_Vobb1	0.012	0.015	0.009	0.010	0.010									
BRA_Vobb2	0.019	0.026	0.019	0.021	0.021	0.023								
BRA_Vobb3	0.007	0.014	0.007	0.009	0.009	0.012	0.023							
BRA_Curo1	0.015	0.022	0.012	0.014	0.014	0.021	0.019	0.015						
BRA_Curo2	0.014	0.017	0.014	0.015	0.015	0.019	0.026	0.021	0.024					
BRA_Curo3	0.009	0.015	0.005	0.007	0.007	0.014	0.017	0.009	0.012	0.016				
BRA_Curo4	0.007	0.010	0.000	0.002	0.002	0.009	0.019	0.007	0.012	0.014	0.005			
PMI_Nure1	0.217	0.225	0.216	0.213	0.218	0.229	0.235	0.223	0.223	0.228	0.220	0.216		
PMI_Nure2	0.245	0.253	0.246	0.243	0.248	0.253	0.262	0.254	0.254	0.251	0.251	0.246	0.042	
PMI_Parm1	0.242	0.250	0.243	0.241	0.246	0.253	0.259	0.251	0.251	0.251	0.248	0.243	0.067	0.077
PMI_Parm2	0.220	0.228	0.219	0.216	0.221	0.233	0.233	0.226	0.226	0.226	0.224	0.219	0.014	0.035
BIL1	0.131	0.133	0.127	0.125	0.129	0.134	0.138	0.133	0.133	0.136	0.129	0.127	0.218	0.230
BIL2	0.133	0.135	0.129	0.127	0.131	0.136	0.140	0.136	0.136	0.138	0.131	0.129	0.220	0.232
BIL3	0.158	0.160	0.158	0.156	0.161	0.162	0.149	0.161	0.159	0.161	0.159	0.158	0.239	0.238
BIM	0.136	0.138	0.131	0.129	0.133	0.138	0.138	0.138	0.138	0.136	0.134	0.131	0.231	0.244
BVE1	0.128	0.135	0.128	0.126	0.130	0.133	0.137	0.133	0.134	0.135	0.128	0.128	0.204	0.209
BVE2	0.130	0.137	0.128	0.126	0.130	0.133	0.137	0.133	0.134	0.133	0.128	0.128	0.207	0.211
BVE3	0.151	0.157	0.151	0.149	0.153	0.156	0.160	0.155	0.157	0.153	0.151	0.151	0.220	0.223
BBI_Boro1	0.124	0.128	0.122	0.120	0.124	0.127	0.133	0.128	0.130	0.124	0.124	0.122	0.212	0.211
BBI_Boro2	0.159	0.166	0.161	0.159	0.164	0.167	0.171	0.164	0.168	0.169	0.161	0.161	0.238	0.240
BBI_Caci	0.122	0.126	0.124	0.122	0.126	0.129	0.135	0.127	0.132	0.126	0.126	0.124	0.212	0.212
Perla_sp.	0.255	0.263	0.253	0.255	0.255	0.267	0.272	0.258	0.261	0.267	0.250	0.253	0.256	0.292
Perla_sp.	0.258	0.261	0.255	0.258	0.253	0.270	0.274	0.261	0.263	0.270	0.253	0.255	0.259	0.295
Perla_sp.	0.258	0.261	0.255	0.258	0.253	0.270	0.274	0.261	0.263	0.270	0.253	0.255	0.259	0.295
Perla_sp.	0.258	0.261	0.255	0.258	0.253	0.270	0.274	0.261	0.263	0.270	0.253	0.255	0.259	0.295

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TABLE 2. (continued)

	PMI_Parm1	PMI_Parm2	BIL1	BIL2	BIL3	BIM	BVE1	BVE2	BVE3	BBI_Boro1	BBI_Boro2	BBI_Caci	Perla_sp.	Perla_sp.	Perla_sp.	Perla_sp.
BRA_Cher1																
BRA_Cher2																
BRA_Staf1																
BRA_Staf2																
BRA_Treb																
BRA_Vobb1																
BRA_Vobb2																
BRA_Vobb3																
BRA_Curo1																
BRA_Curo2																
BRA_Curo3																
BRA_Curo4																
PMI_Nure1																
PMI_Nure2																
PMI_Parm1																
PMI_Parm2	0.063															
BIL1	0.252	0.218														
BIL2	0.255	0.221	0.002													
BIL3	0.272	0.234	0.032	0.033												
BIM	0.261	0.220	0.009	0.010	0.037											
BVE1	0.235	0.205	0.133	0.136	0.152	0.143										
BVE2	0.237	0.207	0.133	0.136	0.154	0.143	0.003									
BVE3	0.218	0.215	0.153	0.155	0.167	0.157	0.030	0.033								
BBI_Boro1	0.237	0.213	0.135	0.137	0.156	0.140	0.010	0.014	0.041							
BBI_Boro2	0.264	0.238	0.155	0.157	0.175	0.160	0.069	0.071	0.091	0.075						
BBI_Caci	0.237	0.213	0.142	0.144	0.163	0.147	0.019	0.022	0.048	0.009	0.083					
Perla_sp.	0.295	0.257	0.259	0.262	0.289	0.266	0.250	0.255	0.264	0.250	0.275	0.258				
Perla_sp.	0.298	0.260	0.262	0.264	0.292	0.268	0.252	0.257	0.266	0.252	0.278	0.260	0.002			
Perla_sp.	0.298	0.260	0.262	0.264	0.292	0.268	0.252	0.257	0.266	0.252	0.278	0.260	0.002	0.000		
Perla_sp.	0.298	0.260	0.262	0.264	0.292	0.268	0.252	0.257	0.266	0.252	0.278	0.260	0.002	0.000	0.000	

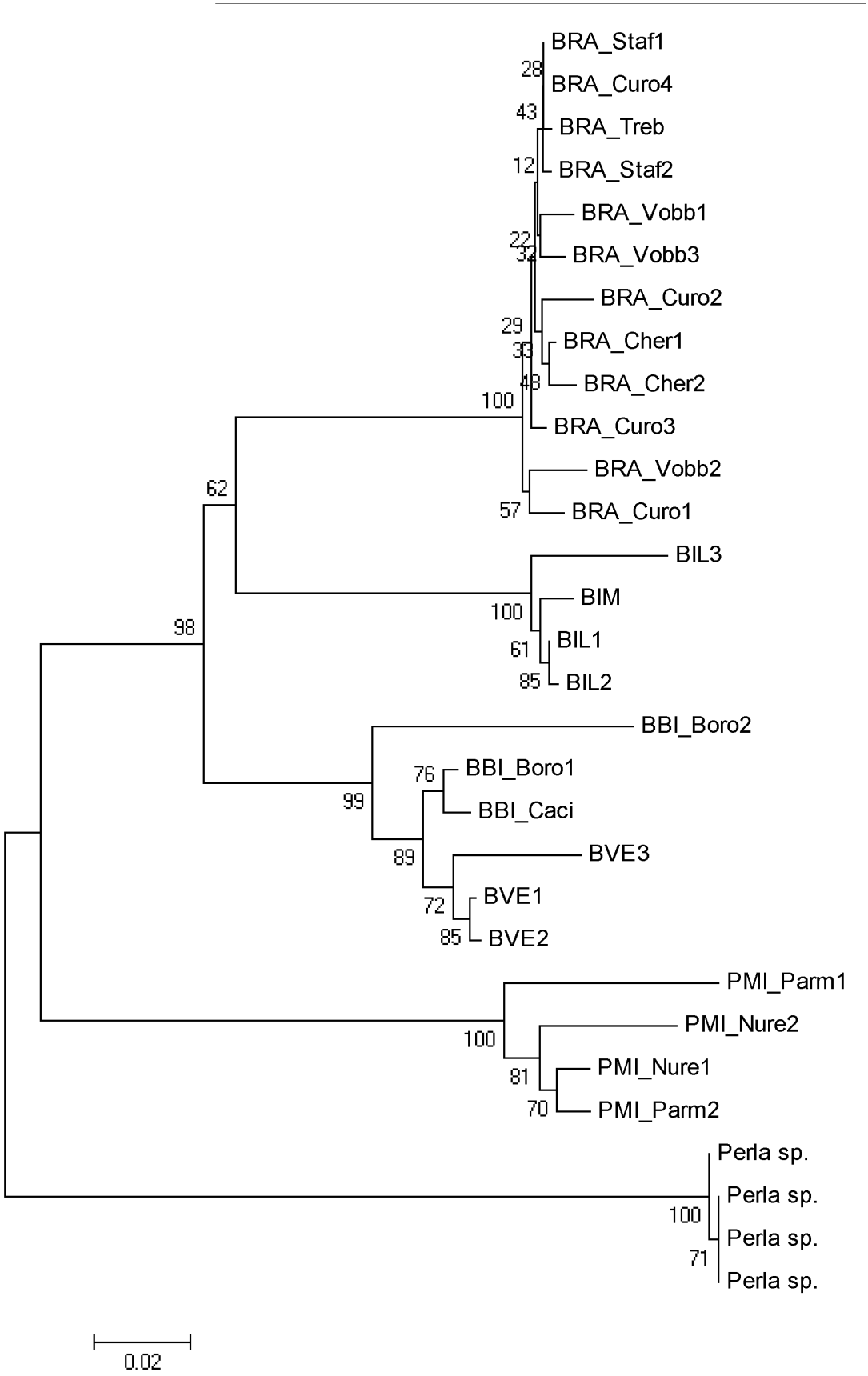

FIGURE 1. Phylogenetic reconstruction obtained using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Acronyms as in Table 1.

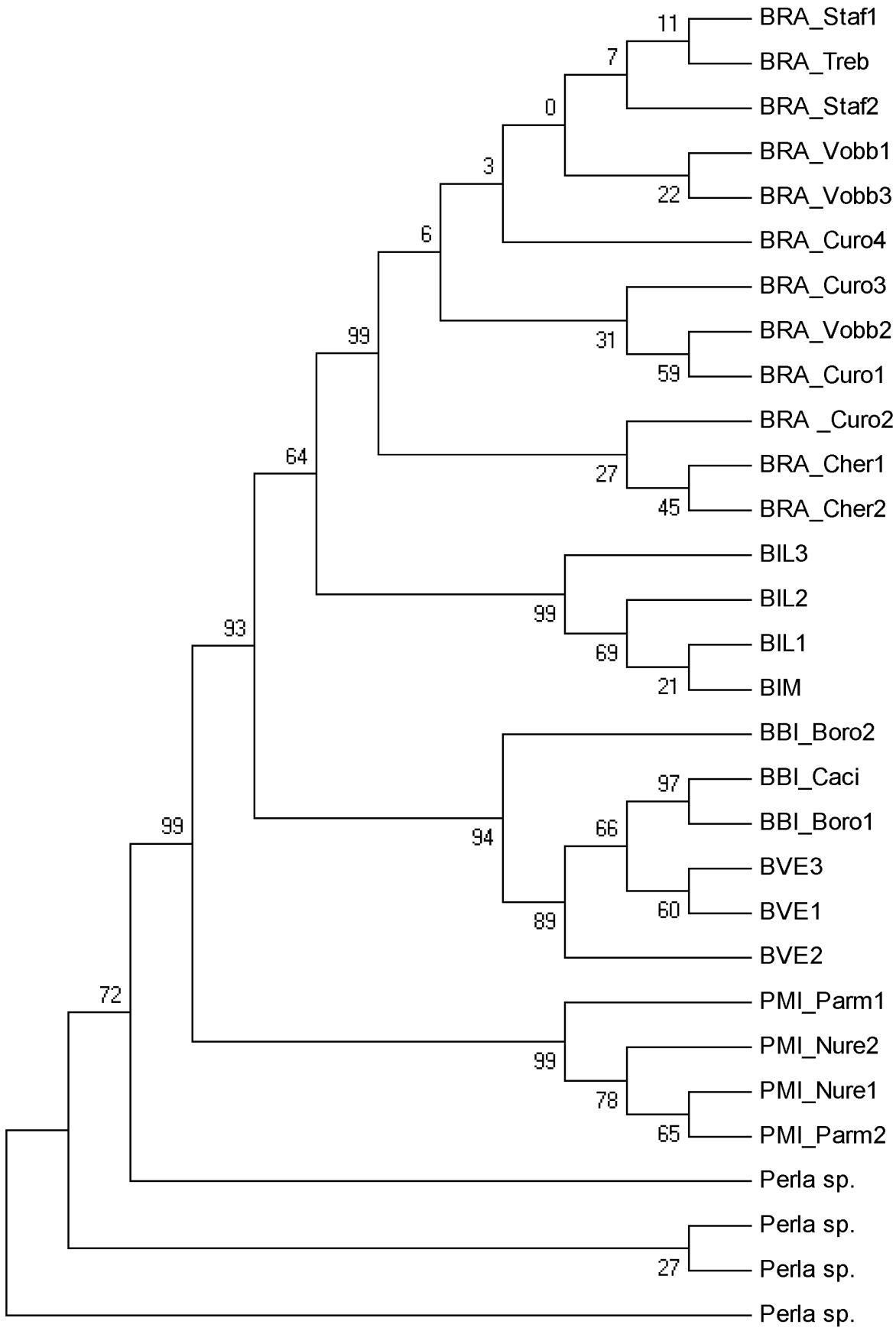

FIGURE 2. Maximum likelihood (ML) phylogram of the recovered haplotypes. Numbers are the support of the nodes by bootstrapped ML (10000 replicates; HKY model of evolution). Acronyms as in Table 1.

Phylogenetic analysis. All approaches were congruent in discriminating three main clades within the genus *Besdolus*. Under MP assumption 16 most parsimonious trees were found (length = 441 steps, not shown here). The consistency index was 0.67 and the retention index was 0.9. NJ found an optimal tree, with a sum of the branch length = 0.7664 (Fig. 1); main branches are statistically well supported. ML tree is shown in Fig. 2. Its overall topology is almost overlapping that of NJ tree (and that, not shown, of MP tree); the only difference regards the two populations of *P. microcephalus* that cluster with *Perla* sequences in the ML tree before joining the *Besdolus* species while in the NJ tree they are isolated from *Perla* sequences.

In all approaches *B. ventralis* joined *B. bicolor*, *B. illyricus* joined *B. imhoffi* and then *B. ravizzarum* was added to this latter group.

Discussion

The COI molecular arrangement obtained in this study was not completely congruent with the current recognized species of *Besdolus* based on morphology. In fact, molecular distances between *B. bicolor* and *B. ventralis* (from 0.010 to 0.091) and those between *B. imhoffi* and *B. illyricus* (< 0.037) were similar to what has been previously reported for conspecific stonefly taxa (Fochetti et al., 2009). In *B. ravizzarum*, intraspecific distances were always <0.026. Interspecific molecular distances were sometime similar to those considered intraspecific in stoneflies. For example, in the comparison of the species pair *B. bicolor*/*B. ventralis*, the maximum interspecific value was 0.091, and in the comparison *B. imhoffi*/*B. illyricus* it was 0.037 (see Table 2). The only specimen of *B. imhoffi* analyzed was molecularly closer to specimens BIL1 and BIL2 of *B. illyricus* than they are to the other *B. illyricus* specimens BIL3. Similar, a specimen of *B. bicolor* from Borosa River (BBI_Boro2), was more distant from the other *B. bicolor* specimens than they are from *B. ventralis*.

In a study of the species and populations of the stonefly genus *Tyrrhenoleuctra* using the COI gene, the magnitude of interspecific comparisons was always > 0.1, whereas for intraspecific comparisons values were always very low (< 0.024) (Fochetti *et al*. 2009). Assuming a rough specific boundary of approximately D = 0.1, the specific distinctions of *B. bicolor*/*B. ventralis* and *B. imhoffi*/*B. illyricus* are not fully supported.

Aubert (1952) already indicated that the original description of *B. bicolor* by Navás (1909) fits *B. ventralis*. Zwick & Weinzierl (1995) considered that the only species known to occur in Spain was the endemic *B. bicolor*, and these authors distinguished it from *B. ventralis*. If the molecular data presented herein will be confirmed by a more comprehensive study, *B. bicolor* may be considered a synonym of *B. ventralis*. Similarly, a presumed synonymy between *B. illyricus* and *B. imhoffi* can be suggested. In the original description by Kovács & Zwick (2008), the presumed affinities of the new species were not extensively discussed. The only comparison indicated " ...The new species differs from all congeners in its short lateral stylets whose blunt tips are largely embedded in the surface of the cowl and project little, even when the epiproct is erect. In all other species, the lateral stylets end in freely projecting spines or claws of specific shape" (Kovács & Zwick 2008, p. 184). In this regard, a molecular analysis of the populations not studied here and an analysis of morphological variability is needed, since our molecular data indicate that *B. illyricus* may be a synonym of *B. imhoffi*.

Zwick & Weinzierl (1995, p. 14) hypothesized that, based on a possible existence of a morphocline, *B. ravizzarum* could be either a subspecies or conspecific with *B. bicolor*. Ignoring the species boundaries as discussed above, molecular data indicate that, despite being morphologically similar, *B. bicolor* and *B. ravizzarum* are two well-isolated and distinct species. Zwick & Weinzierl (1995) report that *B. ravizzarum* is very similar to *B. ventralis* exhibiting specific characters only in details of the male genitalia, egg chorion, and larval setation. Based on COI gene, *B. ravizzarum* is distinct from *B. ventralis* and is more closely related to *B. illyricus*/*B. imhoffi*, whereas *B. ventralis* is closer to the presumed *B. bicolor*. These results show that in the present case the outcomes from the molecular approach do not fully match previously identified morphological distinctions.

Molecular divergence rates and biogeography. A 2% substitution rate per m.y. is usually used in insects and other arthropods for COI but the rate is controversial, varying from 0.4 to 9, with common values around ~ 1-2 (see for instance Caccone & Sbordoni, 2001; Farrell, 2001; Ketmayer *et al*., 2003; Quek *et al*., 2004; Forgie *et al*. 2006; Sota & Hayashi 2007). Stoneflies are considered to have very low evolutionary rates, either when using nuclear markers (allozymes) or mitochondrial DNA (Fochetti 1991, 1994; Fochetti *et al*. 2004, 2009). Fochetti *et al*. (2009) using the same COI gene, estimated molecular evolutionary rates ranging from 0.24 to 0.7 per million

years (my) in interspecific comparisons in *Tyrrhenoleuctra*. In the current study, if a rate 2% of divergence equals one million years, an estimate of 60,000/87,500 years is obtained for interspecific comparisons (D range from 0.12 to 0.175). Using the lower evolutionary rates found in stoneflies, the estimates would range from 171,500 to 700,000 years. However, we did not perform a likelihood-ratio test (Huelsenbeck & Rannala 1997) to test the null hypothesis that there was no difference in evolutionary rates among different lineages (i.e. existence of a molecular clock). In both cases, the cladogenetic events of speciation in the genus *Besdolus* would have occurred in the middle Pleistocene and may be partially related to Pleistocene glaciation events. Therefore, it could be hypothesized that a Central European stem species dispersing westward and southward diverged into two lineages, then differentiating on the three European main peninsulas. The colonisation of the Iberian Peninsula by one of the two lineages (*B. ventralis*/*bicolor*) could have happened considerably later after the colonization of the Italian Peninsula by the other lineage. That may explain why *B. ventralis*/*bicolor*, according to our data, have not fully diverged as separated species. Such a biogeographical reconstruction has been hypothesized, for instance, in the fish genus *Squalius* (Sanjur *et al*. 2003).

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