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Molecular studies on the genus *Medora* H. et A. Adams, 1855 from Italy (Gastropoda Pulmonata Clausiliidae)

M. Stella Colomba[1*], Fabio Liberto[2], Agatino Reitano[3], Walter Renda[4], Giuseppe Pocaterra[5], Armando Gregorini[1] & Ignazio Sparacio[6]

[1]Università di Urbino "Carlo Bo", Dipartimento di Scienze Biomolecolari, via Maggetti 22 (loc. Sasso) - 61029 Urbino (PU), Italy
[2]Strada Provinciale Cefalù-Gibilmanna n° 93 - 90015 Cefalù, Palermo, Italy
[3]Via Gravina 77 - 95030 Tremestieri Etneo, Catania, Italy
[4]via Bologna 18/a - 87032 Amantea, Cosenza, Italy
[5]Via XXV Aprile 19 - 40018 San Pietro in Casale, Bologna, Italy
[6]Via E. Notarbartolo 54 int. 13 - 90145 Palermo, Italy
[*]Corresponding author: mariastella.colomba@uniurb.it

ABSTRACT In Italy, the genus *Medora* H. et A. Adams, 1855 includes two species: *M. italiana* (Küster, 1847) and *M. dalmatina* (Rossmässler, 1835). In particular, populations ascribed to *M. italiana* were, and still are, the focus of several works aiming at better understanding the real taxonomic value of these entities and defining their presence on the Italian territory. In order to contribute to the improvement of the current knowledge on the organization of the genus at different taxonomic levels, several populations were investigated by analysing 16S rDNA, COI and ITS2 gene partial sequences. Phylogenetic reconstructions were obtained by the Maximum Likelihood algorithm. Although further studies are needed, preliminary data suggest that the genus *Medora* shows a much more complex and articulate differentiation than hypothesized so far.

KEY WORDS *Medora*; *Medora italiana* complex; 16S rDNA; COI; ITS2; molecular phylogeny.

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INTRODUCTION

The genus *Medora* H. et A. Adams, 1855 has a typical trans-Adriatic distribution (Gridelli, 1950; La Greca, 1964; La Greca, 1984; Vigna Taglianti et al., 1993; Parenzan, 1994) with different species scattered in the North-Western Balkan peninsula, Italian peninsula and Sicily (Giusti et al., 1986; Reitano et al., 2007; Nordsieck, 2007; 2009; 2012; Bank, 2011). Regarding Italy, Nordsieck (1970) considered *M. italiana* (Küster, 1847) of the Central-Southern Apennines distinct from *M. albescens* (Menke, 1830) of the Balkan peninsula, as the two taxa differ by a few anatomical characters, mainly by the insertion mode of the diverticulum of the bursa copulatrix. In addition, he assigned to *M. italiana* the following subspecies: *M. i. italiana* (locus typicus: Piedimonte Matese, Caserta, Campania), *M. i. punctulata* (Küster, 1850) (locus typicus: Monte Tiriolo, Catanzaro, Calabria), *M. i. garganensis* (A.J. Wagner, 1918) (locus typicus: Pulsano, Monte Sant'Angelo, Foggia, Apulia), *M. i. milettiana* Giusti, 1967 (locus typicus: Monte Miletto, Campobasso, Molise) and *M. i. kobelti* Nordsieck, 1970 (locus typicus: Gola di Romagnano, Potenza, Basilicata).

A subsequent revision of the genus *Medora* in Italy and Yugoslavia was published by Giusti et al. (1986) based on morphological features, anatomical characters and allozymes (i.e. variant forms of an

enzyme coded by different alleles at the same locus). According to these authors, the statistical analysis of conchological characters allowed to recognize two species, *M. albescens* with two subspecies and *M. dalmatina* (Rossmässler, 1835) with three subspecies; the anatomical analysis of genitalia was of poor diagnostic value distinguishing only two species, *M. albescens* and *M. dalmatina*, without any elements at the subspecific level; and, finally, allozymes allowed to discriminate between two species with numerous subspecies: *M. albescens* with five subspecies: *albescens*, *almissana* (Küster, 1847) and *clissana* (Brancsik, 1897), in ex-Yugoslavia, *italiana* and *punctulata*, in Italy, and *M. dalmatina* with three subspecies, *dalmatina*, *orthopleura* (Westerlund, 1878) and *drasnicensis* H. Nordsieck, 1970, all in Croatia, Bosnia and Herzegovina. In conclusion, Giusti et al. (1986) suggested that: 1) it was not possible to distinguish *M. italiana* from *M. albescens*; 2) *Medora albescens* was widespread in the Balkan peninsula with several subspecies and in Italy with two subspecies: *M. a. italiana* in the central part of Italy, and *M. a. punctulata* showing a disjunctive distribution with some populations in Northern and Central Apennines and others in Calabria; and 3) the examined population of Gargano resulted indistinguishable from that of Ospo (Slovenia) and probably was of anthropic origin (see Giusti et al., 1986: 260). However, the authors also suggested that several subspecies of *Medora* could be considered good species according to the electrophoretic analysis results, but due to the absence of morphological characters discriminating among different taxa they preferred to lump them all togher (Giusti et al., 1986: 260, 316).

After nearly ten years, Manganelli et al. (1995) reported for the Italian peninsula only two species without subspecies: *M. albescens* and, as first record for Italy, *M. dalmatina* whose presence, stressed by Forcart (1965) and Alzona (1971), had been ruled out by Nordsieck (1970) and Giusti et al. (1986). Later on, Nordsieck (2007) stressed once again that *M. albescens* is a Balkan taxon (sub *M. macascarensis albescens*) and confirmed for Italy, as stated previously (Nordsieck, 1970), *M. italiana* with the subspecies mentioned above. Reitano et al. (2007) described another additional subspecies, *M. italiana peloritana* Reitano, Liberto et Sparacio, 2007 from Monte Veneretta, Messina (NE Sicily). Colomba et al. (2008) carried out a preliminary study on molecular genetics of Italian populations of *Medora*, highlighting a good differentiation of the populations studied, especially the Southern ones. Bank (2011) reported for Italy *M. italiana* with the five subspecies cited above, while not citing *M. dalmatina*. Recently, Nordsieck (2012) described a new subspecies of *M. dalmatina*: *M. dalmatina pollinensis* Nordsieck, 2012 from Southern Italy, namely Basilicata and Calabria (S. Lorenzo Bellizzi, Cerchiara di Calabria, Gole di Raganello (near Civita), Orsomarso and Papasidero) and, according to unpublished preliminary molecular data (Hausdorf, unpubl.), anticipated that not only *M. italiana* would be a distinct species from *M. macascarensis* (Sowerby, 1828) but also that *M. i. garganensis* should be elevated to the species level. Finally, Cianfanelli et al. (2012) reported *M. dalmatina pollinensis* from Campicello, Laino Castello and Papasidero (Cosenza, Calabria) in alluvional debris.

The present study was carried out to characterize, by molecular analyses, the genus *Medora* in Italy, with particular attention to the *M. italiana* complex aiming at understanding the real taxonomic value of the populations ascribed to it and better defining the presence of this group in the Italian territory. To this purpose, we studied nearly all taxa currently considered subspecies of *M. italiana* including *M. i. italiana* from Piedimonte Matese, *M. i. punctulata* from Monte Consolino and Monte Tiriolo, *M. i. peloritana* from Monte Veneretta, *M. i. garganensis* from San Marco in Lamis (Foggia), *M. i. milettiana* from Monte Miletto, plus several *M. italiana* populations from Leonessa (Rieti), Posta (Rieti), Rubbiano (Ascoli Piceno) and Gualdo Tadino (Perugia). Finally, a few additional populations of other species of the genus *Medora* were included, i.e., *M. dalmatina pollinensis* from Civita, Orsomarso and Papasidero (Cosenza), and *M. macascarensis* (= *albescens*) from Makarska (Croatia).

Choosing molecular markers is certainly a crucial point when studying molecular evolution. Generally, mitochondrial genes are mostly employed as markers in phylogenetic studies of closely related species. This is because mitochondrial genes show considerable advantages including: (i) abundance in the tissues, (ii) easiness in being manipulated, (iii) inheritance in single copy; (iv) high mutation rates, which makes them particularly useful in this type of analysis. With regard to the cytochrome oxi-

dases, just because of the activities they perform, the functional products of these genes are highly conserved. This feature associated with high rates of mutation, especially in third position, makes both COI and COII extremely well suited to investigate the phylogenetic relations among taxa separated in relatively recent times. Mitochondrial genes, however, represent only a small part of the entire genome, hence, to ensure a more thorough and detailed phylogenetic analysis, they are often studied with nuclear genes (typically 18S, 5.8S and 28S rDNAs or the internal transcriptional spacers, ITS1 and ITS2). On this basis, we decided to investigate the phylogenetic relationships among the Italian taxa of the genus *Medora* by comparing ribosomal partial sequences of mitochondrial (16S rDNA and cytochrome oxidase c subunit I, COI) and nuclear (second internal transcriptional spacer, ITS2) genes, in order to shed some light on this interesting group whose systematics is still unclear and (in some respects) controversial.

MATERIALS AND METHODS

Specimens

Sixty-six (66) specimens were fixed in 70-90% ethanol. Particularly, fourty-six *M. italiana* from Piedimonte Matese (Caserta, Campania), Monte Consolino, Stilo (Reggio Calabria, Calabria), Monte Veneretta (Messina, Sicily), Monte Tiriolo (Catanzaro, Calabria), Monte Miletto (Campobasso, Molise), San Marco in Lamis (Foggia, Apulia), Leonessa (Rieti, Latium), Posta (Rieti, Latium), Rubbiano (Montefortino, Fermo, Marche) and Gualdo Tadino (Perugia, Umbria); fifteen *M. dalmatina pollinensis* from Papasidero, Orsomarso and Civita (Cosenza, Calabria); and five *M. macascarensis* from Makarska (Croatia) (Table 1). Each population was labelled respectively as PMA, CON, PEL, TIR, MIL, GAR, REA, RIE, RUB, GT, PAP, RSO, CIV and MCR, using consecutive numbers for specimens (up to five per site). Samples were stored at -20 °C in test tubes. For each individual, the entire animal was used for total DNA extraction (by Wizard Genomic DNA Purification Kit, Promega). For each population, para-voucher specimens - sensu Groenenberg et al. (2011) i.e. different specimens than the ones used for DNA analysis, but from the same sample or population - were stored by Dr. Colomba (University of Urbino). 16S rDNA fragments (283-292 bp) were amplified by the primers MED16S_F (5'-ACTGTGCAAAGGTAGCATAA-3') and MED16S_R (5'-CCAACATCGAGGTCACAA-3') (present paper); COI amplicons (616-658 bp) were obtained by the universal internal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') as in Folmer et al. (1994); ITS2 fragments (445-531 bp) were amplified by the primers ITS2_F (5'-ACATTGAAC ATCGACATCTTGA-3') and ITS2_R (5'-CTCCGCTTAGTAATATGCTTAA-3') (present paper). The three molecular loci were amplified in 50 µl reactions by the following (slightly different) PCR protocols: (i) 95 °C for 5 min; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (30 cycles), 72 °C for 10 min (16S rDNA and ITS2); (ii) 95 °C for 5 min; 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min (35 cycles), 72 °C for 10 min (COI). To remove primers and unincorporated nucleotides, the amplified products were purified by the Wizard SV gel and PCR Clean-up kit (Promega). Sequencing of the purified PCR products was carried out using automated DNA sequencers at Eurofins MWG Operon (Germany). Sequence chromatograms of each amplified fragment were browsed visually for reading mistakes by the sequencer. All sequences were visualized with BioEdit Sequence Alignment Editor 7 (Hall, 1999), aligned with the ClustalW option included in this software and double checked by eye. Nucleotide diversity within groups (π = Pi and π_{JC} Pi corrected according to Jukes and Cantor) and nucleotide divergence between groups corrected according to Jukes and Cantor (Dxy_{JC}), using the full set of all sequences were computed with DnaSP 5 (Librado & Rozas, 2009). Phylogenetic analyses were conducted in MEGA 5 (Tamura et al., 2011) using the Maximum Likelihood algorithm. The best-fit evolution models of nucleotide substitution resulted Tamura 3-parameter + gamma distribution for 16S rDNA and COI; and Kimura 2-parameter + gamma distribution for ITS2. Support for nodes was calculated by the Bootstrap method (1000 replicates) and expressed as percentages. *Muticaria syracusana* and *Muticaria neuteboomi* 16S rDNA, COI and ITS2 partial sequences (GenBank IDs: HQ696866-HQ696869, AY382117) were used as outgroup to

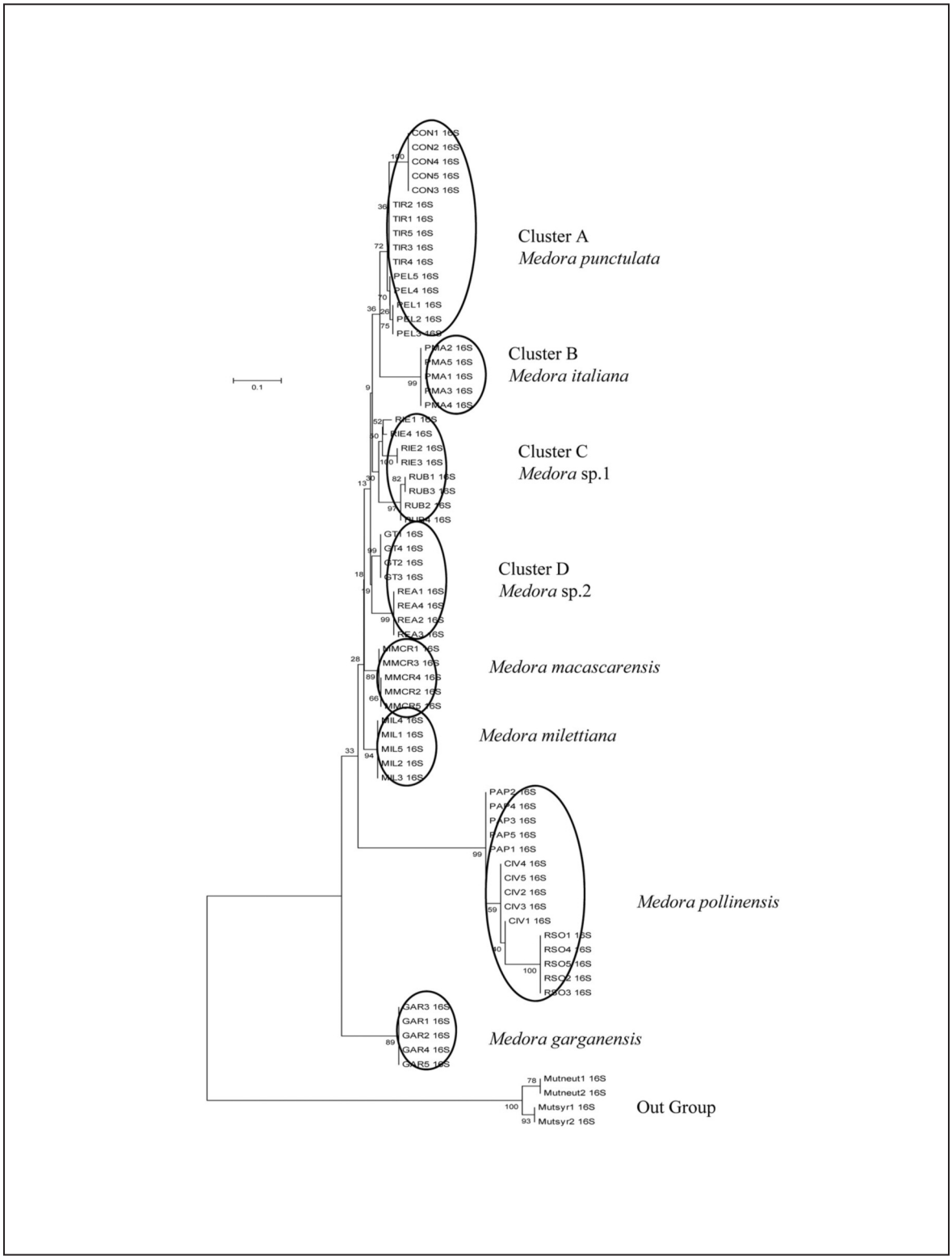

Figure 1. Consensus tree for 16S rDNA gene partial sequences showing the evolutionary history inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model + Gamma distribution (G= 0.28, 5 categories). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap percentages are shown on the nodes.

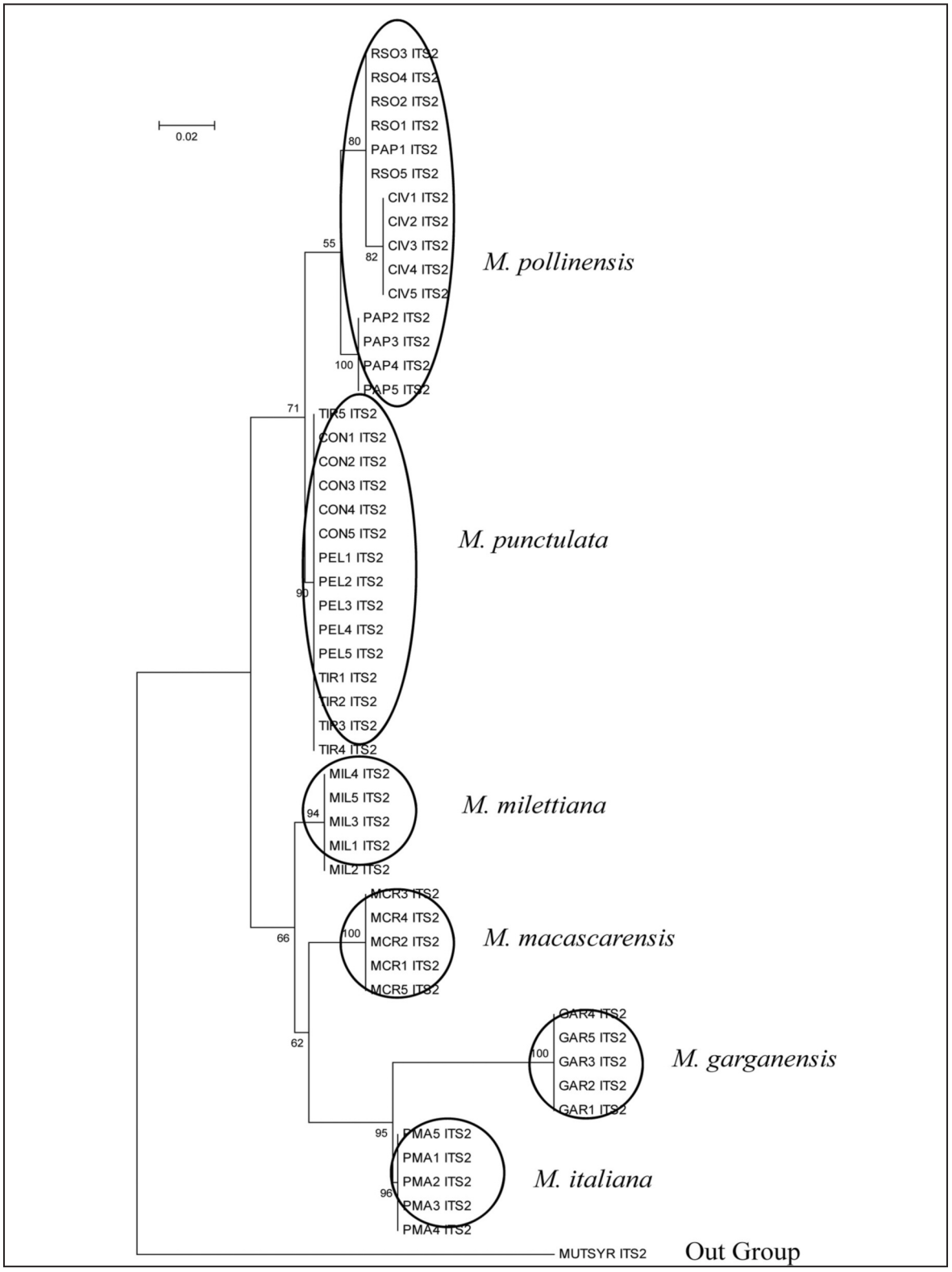

Figure 2. Consensus tree for ITS2 gene partial sequences obtained by using the Maximum Likelihood method based on the Kimura 2-parameter model + Gamma distribution (G= 0.46, 5 categories) The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap percentages are shown on the nodes.

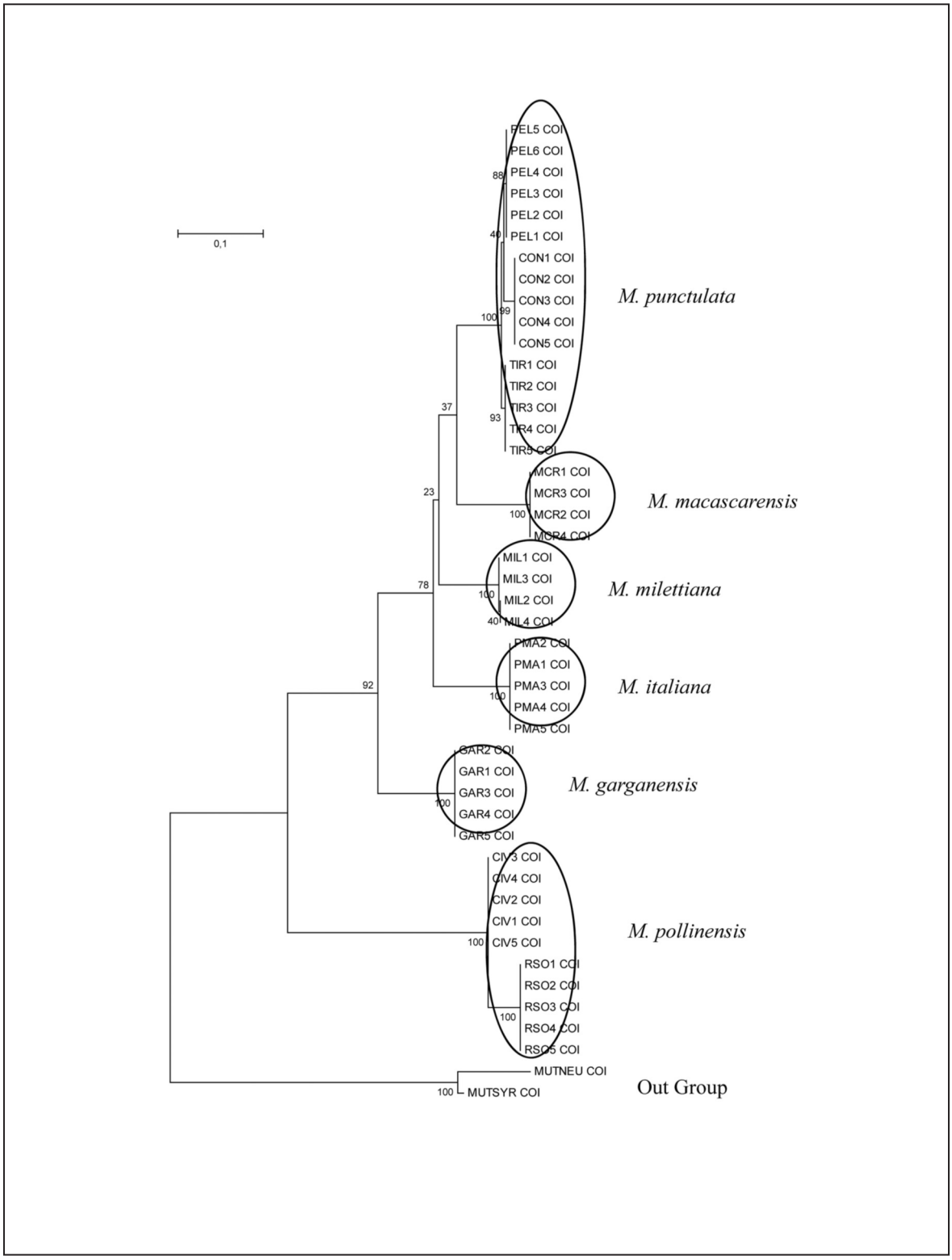

Figure 3. Consensus tree for COI gene partial sequences obtained by using the Maximum Likelihood method based on the Tamura 3-parameter model + Gamma (G= 0.17, 5 categories) The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap percentages are shown on the nodes.

root the phylogenetic trees. Sequences generated in this study were released in GenBank (IDs: KC833899-KC834014, KC853238-KC853281).

RESULTS

Mitochondrial genes (16S rDNA and COI) showed a phylogenetic signal strong enough to distinguish unambiguously all taxa and mostly resolve their phylogenetic relations; in addition, ITS2 was particularly useful to separate all taxa at the specific level. In order to be able to better decipher the phylogenetic framework, particular attention has been devoted to genetic distances. Differences between DNA sequences are frequently reported as p distance (i.e. proportion, calculated as number of nucleotide differences divided by the total number of sites). Unfortunately, p is a rather rough estimate of the actual number of evolutionary changes occurred, since the same site may have undergone more than one mutations over time. Hence to try to correct the risk of underestimation, divergence between two sequences is therefore not simply measured as a percentage of different nucleotides, but, taking account of possible events of multiple substitutions and back-substitutions, is calculated using suitable mathematical models which consider the stochastic nature of gene mutation. In this study we used the Jukes and Cantor correction which assumes equal base frequencies and equal mutation rates.

All populations under study were analysed for the 16S rDNA marker. As shown in figure 1, the topology of the maximum likelihood consensus tree revealed several clearly distinct clusters: "*pollinensis*" including the populations of Papasidero, Orsomarso and Civita; "*garganensis*" with the population of San Marco in Lamis; "*milettiana*" with the population of Monte Miletto; "*macascarensis*" with the population of Makarska; and, finally, the "*Medora italiana* complex" which comprises a few sub-clusters. These latters, indicated with alphabet letters, are respectively, cluster A including the populations of Monte Tiriolo, Monte Consolino and Monte Veneretta; cluster B with the

N	ID	scientific name	Localities	Latitude Longitude	exx.
1	MCR	*M. macascarensis*	Croatia, Makarska, Kotisina, 350 m slm, IV.2008, G. Pocaterra	43°17'29" N 17°02'48" E	5
2	RUB	*Medora* sp. 1	I, Marche, Fermo, Montefortino Rubbiano, 580 m slm, IV.2009, G. Pocaterra	42°55'43.60" N 13°18'31.18" E	4
3	RIE	*Medora* sp. 1	I, Lazio, Rieti, Posta, Fiume Velino, Romualdo, 604 m slm, X.2010, A. Hallgass & G. Pocaterra	42°28'39"N 13°05'08"E	4
4	GT	*Medora* sp. 2	I, Umbria, Perugia, Gualdo Tadino, La Rocchetta, 600 m slm, IV.2009, G. Pocaterra	43°13'42.58" N 12°48'13.39" E	4
5	REA	*Medora* sp. 2	I, Lazio, Rieti, Leonessa, Monti Reatini, 1107 m slm, 9.IX.2009, A. Hallgass	42°31'52.65" N 12°57'03.08" E	4
6	MIL	*M. milettiana*	I, Molise, Campobasso, Campitello Matese, Monte Miletto, 1620 m slm, X.2008, A. Hallgass	41°27'41.23" N 14°22'43.95" E	5
7	PMA	*M. italiana*	I, Campania, Caserta, Piedimonte Matese, Gole del Torano, 274 m slm, 7.VIII.2008,W. Renda & G. Pocaterra	41°21'45.23" N 14°22'46.53" E	5
8	GAR	*M. garganensis*	I, Puglia, Foggia, San Marco in Lamis, 5.VI.2008, M. Perilli		5
9	CIV	*M. pollinensis*	I, Calabria, Cosenza, Civita, Gole del Raganello, 340 m slm, 13.VII.2008, W. Renda	39°49'45.80" N 16°19'00.69" E	5
10	PAP	*M. pollinensis*	I, Calabria, Cosenza, Papasidero, Valle del Lao, 192 m slm, 13.IX.2008, W. Renda	39°52'00.05" N 15°54'04.19" E	5
11	RSO	*M. pollinensis*	I, Calabria, Cosenza, Orsomarso, Valle dell'Argentino, 131 m slm, 13.IX.2008, W. Renda	39°48'01.36" N 15°54'26.91" E	5
12	TIR	*M. punctulata*	I, Calabria, Catanzaro,Tiriolo, Monte Triolo, 720 m slm, 21.II.2009, W. Renda	38°57'04.58" N 16°31'04.59" E	5
13	CON	*M. punctulata*	I, Calabria, Reggio Calabria, Stilo, Monte Consolino, 666 m slm, 3.VII.2008, W. Renda	38°28'54.11" N 16°27'53.57" E	5
14	PEL	*M. punctulata peloritana*	I, Sicily, Messina, Castelmola, Monte Veneretta, 750 m slm, 26.IX.2008, A. Reitano & W. Renda	37°52'10.72" N 15°16'02.50" E	5

Table 1. *Medora* populations collected in Croatia and Italy.

	CIV	CON	GAR	GT	MIL	MCR	MNEU	MSYR	PAP	PEL	PMA	REA	RIE	RSO	RUB	TIR
CIV																
CON	16%															
GAR	16%	13%														
GT	14%	7%	13%													
MIL	14%	9%	11%	5%												
MCR	14%	8%	11%	6%	6%											
MNEU	27%	27%	26%	25%	25%	24%										
MSYR	26%	25%	27%	25%	26%	24%	5%									
PAP	2%	16%	15%	14%	14%	14%	27%	26%								
PEL	15%	4%	13%	5%	7%	6%	25%	23%	14%							
PMA	17%	8%	15%	9%	9%	10%	26%	26%	18%	8%						
REA	14%	9%	12%	5%	7%	7%	26%	26%	14%	7%	9%					
RIE	16%	9%	13%	6%	7%	7%	25%	25%	15%	6%	8%	7%				
RSO	4%	18%	18%	17%	17%	17%	28%	28%	4%	18%	18%	17%	19%			
RUB	15%	10%	12%	6%	6%	7%	27%	26%	15%	7%	11%	8%	6%	18%		
TIR	15%	3%	12%	5%	6%	6%	25%	23%	14%	1%	7%	7%	6%	17%	7%	

Table 2. Matrix showing the number of base differences per site (expressed in %) from averaging over all 16S rDNA partial sequence pairs between groups.

	CIV	CON	GAR	MCR	MIL	MNEU	MSYR	PEL	PMA	RSO	TIR
CIV											
CON	17%										
GAR	17%	12%									
MCR	16%	10%	12%								
MIL	17%	10%	11%	10%							
MNEU	19%	19%	19%	19%	19%						
MSYR	19%	20%	18%	19%	20%	7%					
PEL	17%	1%	12%	9%	10%	19%	20%				
PMA	16%	10%	13%	11%	10%	21%	21%	11%			
RSO	3%	18%	18%	17%	18%	21%	20%	18%	17%		
TIR	17%	2%	12%	9%	10%	19%	20%	1%	11%	18%	

Table 3. Matrix showing the number of base differences per site (expressed in %) from averaging over all COI partial sequence pairs between groups.

population of Piedimonte Matese; cluster C showing the populations of Posta and Rubbiano; and cluster D with the populations of Leonessa and Gualdo Tadino. Genetic distances range from 2% to 19% (Table 2). Based on tree topology, we believe that, within the *M. italiana* complex, clusters A (CON + TIR + PEL), B (PMA), C (RIE + RUB) and D (GT+ REA) are, respectively, separate species. When comparing PMA (cluster B) to the populations of cluster A, the genetic divergence is 7-8% (16S rDNA, Table 2) or 10-11% (COI, Table 3). Hence, *M. italiana* from Piedimonte Matese can, with reasonable certainty, be considered a separate species. Cluster A includes the populations of Monte Tiriolo (locus typicus of "*punctulata*"), Monte Consolino, and Monte Veneretta which, based on their genetic divergences (Table 2) can all be ascribed to the same species which we refer to as *M. punctulata*. In addition, both p distances and tree topology reveal a certain degree of internal differentiation which may suggest to consider them subspecies, as proposed by Reitano et al. (2007) for the taxon from Monte Veneretta ("*peloritana*" herein labelled PEL). Regarding the populations included within cluster C, indicated as *Medora* sp.1, taking into account the p distances between them (6.3%, for 16S rDNA), they could even belong to two distinct species. At the moment, however, we cannot give a definitive answer to the question which has to be analysed in-depth by larger samples and more detailed studies (currently in progress). Finally, cluster D, *Medora* sp. 2, encloses two populations that we consider conspecific (p distance for 16S rDNA, 5%). In this regard it is interesting to note that the populations referred to as RIE (cluster C) and REA (cluster D) are both from Latium, respectively from Posta (Rieti) and Leonessa (Rieti), thus suggesting that in this region two *Medora* species might occur in areas which are only 13 Km far. In conclusion then, the *M. italiana* complex would include *M. italiana*, *M. punctulata* and at least two other additional species at present reported as *Medora* sp.1 and *Medora* sp. 2. Finally, the Maximum Likelihood consensus tree seems to strongly support the specific rank of *milettiana*, *garganensis*, *macascarensis* and *pollinensis*.

As regards ITS2 and COI genes, although our data are only partial because not all populations have been studied yet, nevertheless, obtained results deserve attention as, although limited, seem to confirm the big picture outlined by the analysis of 16S rDNA sequences. Particularly, ITS2 proved very useful in discriminating taxa at the specific level. Indeed, the Maximum Likelihood analysis (Fig. 2) clearly distinguishes *pollinensis*, *milettiana*, *garganensis* and *macascarensis*, confirming their specific rank and, interestingly, separates *M. italiana* from Piedimonte Matese (PMA) from the populations of *M. punctulata* of Monte Consolino, Monte Tiriolo and Monte Veneretta (CON, TIR, PEL). The same pattern is shown by the Maximum Likelihood consensus tree based on COI partial sequences analysis (Fig. 3).

DISCUSSIONS AND CONCLUSIONS

The present work represents, as to our knowledge, the first attempt to conduct a full and thorough molecular analysis of the genus *Medora* from Italy. Our findings, though preliminary and subject to further study, however, allow us to suggest some interesting considerations both on the systematics of the genus and on the paleo-biogeographic events that, over time, originated the current distribution of *Medora* populations on the Italian territory (Fig. 4). First of all, it is interesting to note that all three genes (16S rDNA, COI and ITS2) confirm the specific rank of *M. italiana*, *M. milettiana*, *M. garganensis*, *M. punctulata* and *M. pollinensis*, underlining that the genus *Medora*, in Italy, includes much more species than reported so far. Given the distribution (trans-Adriatic) of *Medora* and the fact that the Italian populations are little differentiated morphologically (Giusti et al., 1986), it has been suggested that *Medora* specimens might have reached the Italian territories relatively recently. At the end of the Pliocene, the Apennines were already connected to the Alps and extended up to the Matese area with the more southerly regions fragmented in a vast archipelago (Sgrosso, 1998; Boccaletti et al., 2005). The Italian peninsula was therefore well separated from the Balkan peninsula, but with the first glaciation, the Adriatic Sea dried up almost completely allowing most Eastern species to passage to Italy. Moreover, glacial cycles occurred throughout the Pleistocene, joined to the major territorial extension of the Apennine and to the paleo-climatic phenomena relevant to this period, allowed a progressive spread and differentiation of these organisms in the Italian territory up to the most southern regions (i.e.

Calabria, with the populations attributable to *M. punctulata*). M. *punctulata peloritana* (from North-Eastern Sicily) represents, therefore, the southernmost population of the genus and, probably, the most recently differentiated. Our data confirm the affinity between *peloritana* and the populations from Calabria (see also Reitano et al., 2007; Nordsieck, 2012) and it is interesting to note that in all examined cladograms, *M. punctulata peloritana* is always distinct and sometimes (as in the case of 16S rDNA) also well separated from the Calabrian populations as a whole.

More complex could be instead the position of *M. garganensis* in relation to the geological vicissitudes of the "Abruzzi-Apulia paleobioprovince" which the Gargano is part of (cfr. Azzaroli, 1982; Ricchetti et al., 1992; Angelone, 2007). In fact, *M. garganensis* might represent one of the earliest populations settled in Italy, or at least have had an evolutionary process distinct from that of the other populations since the Gargano area underwent long periods of isolation.

The populations reported for Italy as *M. dalmatina* (Manganelli et al., 1995) were described by Nordsieck (2012) as a distinct subspecies: *M. dalmatina pollinensis*. Notably, as already evidenced by Giusti et al. (1986), the character of the initial portion of the spiral fin forked can be observed in

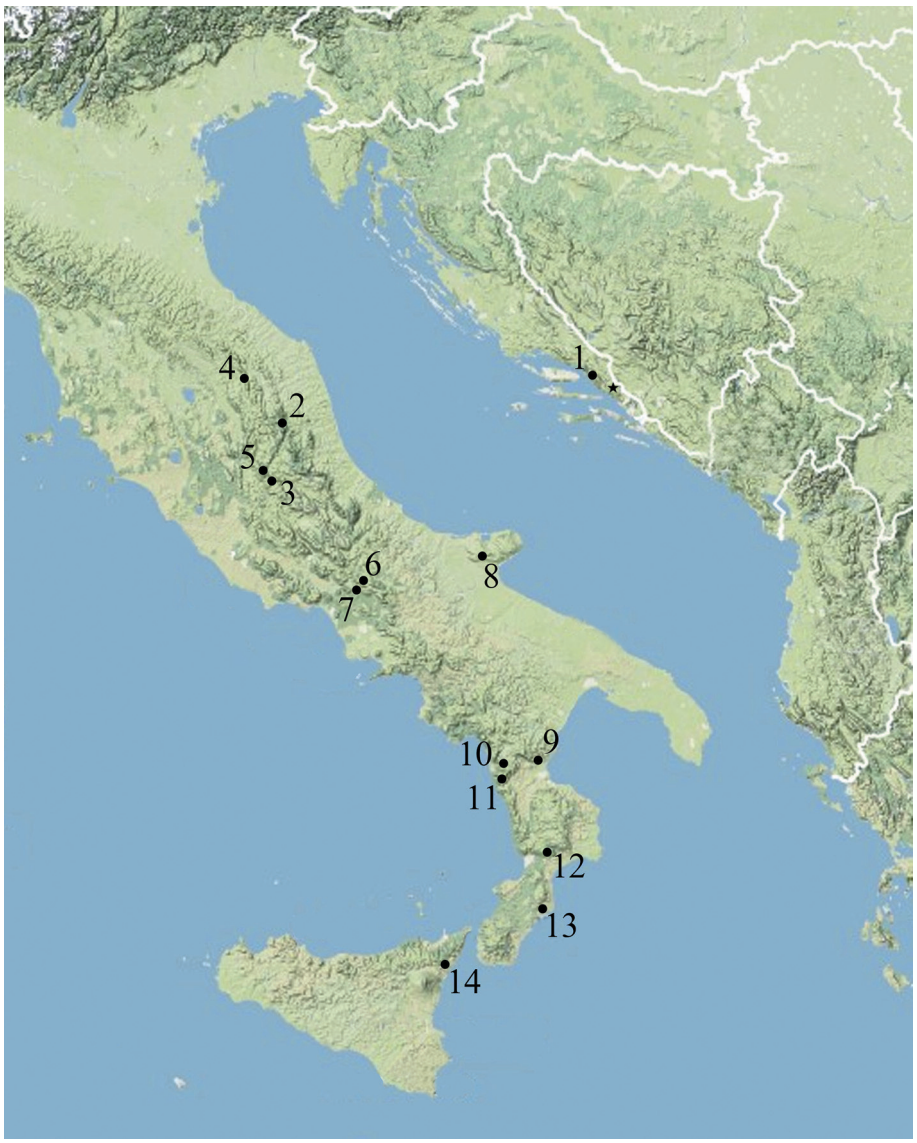

Figure 4. *M. dalmatina* (star: Vrgorac, Croatia) and *Medora* populations studied in this paper (see also Table 1). Dot 1) *M. macascarensis* (Makarska, Kotisina, Croatia). Dots 2-14 (Italy): 2) *M.* sp. 1 (Montefiorito, Rubbiano); 3) *M.* sp. 1 (Posta, Fiume Velino; 4) *M.* sp. 2 (Gualdo Tadino, La Rocchetta); 5) *M.* sp. 2 (Leonessa, Gola di Rio Fuggio); 6) *M. milettiana* (Campitello Matese, Monte Miletto); 7) *M. italiana* (Piedimonte Matese); 8) *M. garganensis* (San Marco in Lamis, Gargano); 9) *M. pollinensis* (Civita, Gole del Raganello); 10) *M. pollinensis* (Cosenza, Papasidero); 11) *M. pollinensis* (Cosenza, Orsomarso, Italy); 12) *M. punctulata punctulata* (Tiriolo, Monte Triolo); 13) *M. punctulata* ssp. (Stilo, Monte Consolino); 14) *M. punctulata peloritana* (Castelmola, Monte Veneretta).

different populations of italian *Medora* which cannot be ascribable to "*dalmatina*" and, one of the anatomical characters of *M. dalmatina*, the length of the zone of contact between the wall of the distal portion of the epiphallus and that of the proximal portion of the penis (DEP=PP) often differs among populations of *M. dalmatina* of the Balkan peninsula and *Medora* of Italy only by about 0.2 mm. Moreover, convergence phenomena between Italian and Balkan species have already been documented and confirmed with molecular evidence in invertebrate taxa which had differentiated along the Apennine chain (Sparacio, 2000; Audisio et al., 2009); and, in addition, the Pollino massif, where *M. pollinensis* occurs, is a geographic area of high biodiversity. Hence, although its affinity with *M. dalmatina* could be real, nevertheless waiting for further molecular evidence and considering that our data (Figs. 1-3) suggest a quite articulate differentiation among the three examined populations of *M. pollinensis*, at the moment we prefer to consider it as a distinct taxon rather than a subspecies of *M. dalmatina* of the Balkan peninsula.

Given that our results are to be further validated by (i) increasing the number of populations and of specimens per population, (ii) implementing the phylogenetic reconstruction using an additional mitochondrial gene, i.e. 12S rDNA, and (iii) performing also the Bayesian analysis, obtained results (although still preliminary) seem to indicate that:

- *M. i. garganensis* and *M. i. milettiana* considered (so far) subspecies of *M. italiana* can be elevated to the specific rank as *M. garganensis* and *M. milettiana* (for *M. garganensis* see also Nordsieck, 2012).

- *M. italiana* from Piedimonte Matese can be considered a distinct species, also distinct from *M. macascarensis*.

- The populations of Monte Tiriolo are a separate species, which we refer to as *M. punctulata* including the populations of Monte Consolino and Monte Veneretta.

From the above, it is clear that the genus *Medora* shows a much more complex and articulate differentiation than hitherto hypothesized by morphological surveys (Nordsieck, 1970; Giusti et al., 1986; Nordsieck, 2012) and that, in an attempt to clarify its organization and internal structure, at various taxonomic levels, a more detailed analysis including a higher number of molecular markers and additional *Medora* populations both from Italy (for example "*kobelti*") and Balkans (as *M. dalmatina*), is required.

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