Molecular data attest to the occurrence of autochthonous *Daphnia pulex* (Crustacea, Branchiopoda) populations in Sicily, Italy

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ABSTRACT

Biological invasions are known to be among the most important threats to the long-term conservation of native biota, and their effects might be even more difficult to contrast when they are cryptic, i.e., when the non-native invaders cannot be easily recognised based on morphology, and can thus be confused with native taxa. Such cryptic invasions are known to widely occur in the cladoceran genus *Daphnia* O.F. Müller, 1785, so that the actual distribution and status of most species and lineages need to be checked with a genetic approach. In the frame of this work, we investigated if the Sicilian populations of *D.* (*Daphnia*) *pulex* Leydig, 1860 belonged to the allochthonous North American lineage, which is known to occur in several regions of the Palearctic and Afrotropical biogeographical regions, or rather to the autochthonous European lineage of the species. The molecular results obtained, based on a fragment of the mitochondrial gene encoding for NADH subunit dehydrogenase 5 (ND5), allowed us to rule out the allochthonous status of the species, confirming the presence of autochthonous relictual lineages of *D. pulex* in Sicily. The native status of these populations is in agreement with their local distribution, limited to natural and poorly-impacted water bodies mostly located in wooded areas at medium and high altitudes. The current local distribution of *D. pulex* in Sicily is possibly linked to the end of the last glacial maximum and the onset of warmer climatic conditions in the early Holocene, which led the species to take refuge in colder microthermal refugia located at high altitudes, determining their current relictual distribution.

INTRODUCTION

Italian inland waters are known to be heavily affected by the occurrence of non-indigenous species (NIS) (Gherardi *et al.,* 2007). These biological invaders are one of the major threats to native freshwater fauna, altering the habitat structure and assemblage composition and ultimately leading to a significant loss of native biodiversity (Naselli-Flores and Marrone, 2019 and references therein).

Italy is characterized by a rich fauna of Cladocera, which includes 17 *Daphnia* O.F. Müller, 1785 species belonging to the subgenera *Daphnia* s.s. and *Ctenodaphnia*, plus several subspecies or hybrid taxa of dubious taxonomical value (Ruffo and Stoch, 2005; Marrone *et al*., 2007). Within the species belonging to the genus *Daphnia* s.s. occurring in Italy, two are allochthonous taxa of Nearctic origin, i.e., *D. ambigua* Scourfield, 1947 and *D. parvula* Fordyce, 1901 (Margaritora, 1985; Riccardi *et al.,* 2004). Furthermore, the presence of a non-native lineage of American origin within *D. pulex* Leydig, 1860 was recorded in Sardinia and Piedmont (Fadda *et al.,* 2011; Marková *et al.,* 2013).

To date, the only non-native cladocerans positively known to occur in Sicily are *Daphnia parvula* and *D. ambigua* (Marrone *et al.,* 2006; Marrone and Naselli-Flores, 2015), although also *D. galeata* G.O. Sars, 1864 and *D. cucullata* G.O. Sars, 1862 have a dubious status in the region (Marrone and Vecchioni, 2020). Moreover, no molecular data are to date available on the Sicilian populations of *D. pulex*, so that no information about the native or non-native status of Sicilian *D. pulex* is available. Furthermore, it must be taken into account that the taxonomy of the *Daphnia pulex* complex is rather difficult due to the absence of clear morphological features distinguishing among taxa listed under *Daphnia pulex* (Conde-Porcuna *et al.,* 2020 and reference therein), a binomen which is misleadingly used for distinct different evolutionary lineages belonging to different biogeographical regions (Mergeay *et al.,* 2008; Crease *et al.,* 2012; Ma *et al.,* 2019). Among them, the Nearctic lineage has been introduced in several countries, attaining an almost worldwide distribution with the sole exception of Antarctica (Crease *et al.,* 2012; Conde-Porcuna *et al.,* 2020).

In the light of the evidence of the presence of the allochthonous North American lineage of *Daphnia pulex* in Italian mainland and Sardinia (Fadda *et al.,* 2011; Marková *et al.,* 2013, 2017), the aim of this work was to identify the *Daphnia* lineages occurring in the Sicilian populations using a mitochondrial molecular marker, and thus verifying whether the evolutionary lineages occurring in Sicily belong to the allochthonous Nearctic or the autochthonous European groups. Accordingly, we carried out samplings in a selection of the known sites of occurrence of the species, and in an unpublished site where the species was found to occur.

METHODS

Based on the data available in the literature, *Daphnia pulex* is currently known for 19 sites in Sicily, which are limited to medium-high altitudes of the Nebrodi area, Madonie area, Etna and Bosco della Ficuzza (Marrone and Vecchioni, 2020). The populations from which samples of *Daphnia pulex* to be molecularly characterized

were collected were chosen based on the known occurrence sites, with the aim of including in the analyses at least a population from each of the four major distribution subareas of the species on the island. In addition, one unpublished site of occurrence was found in the context of this sampling campaign ("ME076" – 37.94158 N, 14.683676 E – 1559 m a.s.l.). A map of the known and sampled occurrence Sicilian sites of the species was made using QGIS software v. 3.18 (http://www.qgis.org).

Microcrustaceans were collected both in open waters and in the littoral areas of each water body through the use of hand- and\or towing-plankton nets with a mesh size of 125-200 µm, depending on size, depth and vegetation cover of the sampled sites. The collected samples were fixed *in situ* with 96% ethanol and microcrustaceans were sorted out in laboratory under a stereomicroscope. Cladocera were morphologically identified according to Margaritora (1985), Alonso (1996), and Benzie (2005). All the studied samples are currently stored in FM's branchiopod collection at the University of Palermo, Italy, and are available for loan on request.

A single *D. pulex* individual from each population was carefully cleaned of any impurities and soaked in double-distilled water for 5-10 minutes in order to eliminate the residual ethanol, and then processed for DNA extraction using the BIORON GmbH "Ron's Tissue DNA Mini Kit", following the manufacturer's instructions. The selective amplification of a fragment from the gene encoding NADH subunit dehydrogenase 5 (ND5), was carried out by polymerase chain reaction (PCR) using the primers ND5newF (5'-AAA CCT CTA AAB TTC YKA RCT- 3 ') and ND5newR (5'-CAT RTT YAT RTC RGG GGT TGT- 3'), described by Dufresne *et al.* (2011).

The PCR mix consisted of 18.9 µl of distilled water, 2.5 µl of Buffer 10X which includes 15 mM of $MgCl_2$, 0.4 µl of dNTPs (10 mM for each), 0.4 µl of each of the primers (10 µM), 0.4 µl of Taq polymerase (5 U / µl) and 2 µl of template DNA, for a total volume of 25 µl. The thermal cycle consisted of 35 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1 min), followed by five minutes at 72°C for the final extension step.

After PCR, 5 µl of each PCR product were used to perform electrophoresis on 2% agarose gel at 90 V for 20 min and then visualized with a UV transilluminator. When PCR products showed a clear single band, of the expected length, they were purified using the Exo-SAP-IT® kit (Affymetrix USB, Santa Clara, CA, USA). Sequencing was performed by Macrogen Inc. (Madrid, Spain; https://dna.macrogen.com/eng/) using an ABI 3130xL (Applied Biosystems, Waltham, MA, USA) sequencer. The same primers used previously for PCR were subsequently used for direct sequencing of the PCR products.

The quality of the obtained chromatograms was checked through the measurement of their "Phred score" (Richterich, 1998). Only those sequences that showed continuous high quality base readings (QV > 20) were used. Chromatograms were analysed and manually proofread using the software Chromas software v. 2.6.2 (Technelysium, Pty. Ltd., South Brisbane, Australia). Overall, six novel ND5 sequences of *Daphnia pulex* were produced. Moreover, in order to compare the new sequences with those publicly available, ten *Daphnia pulex* sequences, eight *D. pulicaria* Forbes, 1893 sequences, four *D. tenebrosa* Sars, 1898 sequences and one *D. magna* Straus, 1820 sequence (used as an outgroup) were downloaded from GenBank and included in the analyses (see Tab. 1 for their Accession Number, A.N.).

All sequences were aligned with MEGAX software (Kumar *et al.,* 2018) using the ClustalW method (Thompson *et al.,* 1997).

MrBayes software v. 3.2.6 (Ronquist *et al.,* 2012) and PhyML v. 3 (Guindon and Gascuel, 2003) were used for inferring the molecular identification and phylogenetic relationships between taxa, using Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. As support measures for the nodes, bootstrap values (Felsenstein, 1985) were calculated with 1000 replicates in ML trees, while the posterior probability values were reported in the BI tree. PartitionFinder v. 1.0.1 (Lanfear *et al.,* 2012) was used to choose the best evolutionary model following the "Akaike Information Criterion" (AIC; Akaike, 1974). A Hasegawa – Kishino – Yano evolutionary sequence model with a proportion of gamma and invariant sites (HKY+I+Γ; nst = 2) was used in the BI and ML analyses. In the BI analysis, two independent Markov Chain Monte Carlo analyses were performed with 1 million generations (temp.: 0.2; default priors). The trees and parameter values were sampled every 100 generations, resulting in 10,000 trees for each analysis. The convergence in the analysis was reached (Effective Sample Size (ESS) greater than 533.13 in all the analyses performed). The initial 25% of trees were discarded as "burn-in".

RESULTS

In addition to five of the published sites from the Madonie, Nebrodi and Bosco della Ficuzza areas, the species was collected in an novel site on the Nebrodi mountains (ME076), located close to the site "ME004", where the species was already known to occur (see Tab. 1 in Marrone and Vecchioni, 2020). In all the sampled populations, parthenogenetic females coexisted with males and ephippial females. Unfortunately, logistic constraints made not possible to collect fresh samples in the only known site for the species within the Etna area (CT011, see Tab. 1 in Marrone and Vecchioni, 2020), which was thus not included in the analyses.

Overall, six new sequences belonging to *Daphnia pulex* were produced from six different Sicilian water bodies (Fig. 1, Tab. 1). After having trimmed out the sequences, a properly aligned fragment of 624 bp long of the ND5 mtDNA gene was obtained. Novel sequences were deposited in GenBank (A.N., MZ489122-MZ489127).

The BI and ML trees based on the mitochondrial ND5 mtDNA fragment and rooted on *D. magna* showed a congruent and well supported topology highlighting how the analysed sequences of *Daphnia pulex* s.l. create two paraphyletic clades: the first including North American *D. pulex* populations and the allochthonous North American *D. pulex* populations occurring in Europe ("NAPX"); the second one includes the autochthonous European *D. pulex* populations ("EPX") including the analysed Sicilian populations (i.e., PA074, PA079, PA081, ME004, ME013, ME076; Tab. 1). All the Sicilian sequences clustered together in a monophyletic clade showing only two slightly different haplotypes (i.e., there is only a single base of difference between the sequence that belongs to PA079 and the remaining ones).

Similarly to what was observed in *D. pulex*, also *D. pulicaria* shows a marked paraphyly (Fig. 2) that separates the North American populations ("NAPC") from the European ones ("EPC").

DISCUSSION

Based on the molecular evidence here reported, the Sicilian populations of *Daphnia pulex* unequivocally belong to the native European lineage.

Taking into account the distribution of the species in Sicily, this result is not surprising. In fact, Sicilian *D. pulex* populations seem to be linked to natural, poorly-

Tab. 1. Origin and GenBank accession numbers (A.N.) for the analysed *Daphnia* specimens. Geographic coordinates are expressed as decimal degrees (Map Datum: WGS84).

Taxon	Country	Location	Latitude (N)	Longitude (E)	A.N.	Clade	Reference
D. pulicaria	Albania	Ohrid	40.95	20.71	KC536551	EPC	Marková *et al.*, 2013
	Switzerland	Alps	46.67	8.04	KC536536	EPC	Marková *et al.*, 2013
	Czech Republic	Bohdaneč	49.78	15.23	KC536543	EPC	Marková *et al.*, 2013
	Italy	Dolomites	46.48	11.66	KC536525	EPC	Marková *et al.*, 2013
	Greenland	Nuuk	64.15	-51.31	KC536586	EPC	Marková *et al.*, 2013
	Canada	Winnipeg, Manitoba	52.12	-97.25	KC536611	NAPC	Marková *et al.*, 2013
	USA	Indiana	39.9	-85.43	KC536600	NAPC	Marková *et al.*, 2013
	Iceland	Reykjavik	64.13	-21.94	KC536621	NAPC	Marková *et al.*, 2013
D. pulex	Czech Republic	Blatná	49.42	13.78	KC536544	EPX	Marková *et al.*, 2013
	Germany	Regensburg	49	12.15	KC536593	EPX	Marková *et al.*, 2013
	UK	Streetly End	52.14	0.35	KC536555	EPX	Marková *et al.*, 2013
	Lithuania	Vilnius	54.75	25.29	KC536560	EPX	Marková *et al.*, 2013
	Canada	Listowel pond, Ontario	43.73	-80.95	KC536602	NAPX	Marková *et al.*, 2013
	Canada	Disputed Road, Ontario	42.22	-83.03	HQ434640	NAPX	Vergilino *et al.*, 2011
	Canada	Res. Duchesnier, Quebec	48.13	-68.63	KC536603	NAPX	Marková *et al.*, 2013
	Italy	PA079	37.889944	13.394575	MZ489122	EPX	Present work
	Italy	PA074	37.823410	14.127241	MZ489123	EPX	Present work
	Italy	PA081	37.901131	13.408438	MZ489124	EPX	Present work
	Italy	ME013	37.951944	14.698331	MZ489125	EPX	Present work
	Italy	ME076	37.941580	14.683670	MZ489126	EPX	Present work
	Italy	ME004	37.939456	14.682634	MZ489127	EPX	Present work
	Italy	Avigliana	45.070000	7.390000	KC536565a	NAPX	Marková *et al.*, 2013
	Italy	Sardinia	40.560000	9.320000	KC536565b	NAPX	Marková *et al.*, 2013
	Italy	Bodrio del Pastore III	45.001389	10.323889	KR233296	EPX	Marková *et al.*, 2017
	Spain	Lake Borreguil, Sierra Nevada	37.052	-03.30	MW883468	NAPX	Conde-Porcuna *et al.*, 2021
	Sweden	-	-	-	HQ434644	EPX	Vergilino *et al.*, 2011
	Kenya	Lake Naivasha	-0.771667	36.361667	DQ235240	NAPX	Mergeay *et al.*, 2006
	South Africa	Cape Flats	-33.98	18.656667	DQ235233	NAPX	Mergeay *et al.*, 2006
	Kenya	Lake Baringo	0.535764	36.063759	DQ235242	NAPX	Mergeay *et al.*, 2006
	Japan	Kosugi, Toyama	36.68	137.09	JX532913	NAPX	Crease *et al.*, 2012
	Japan	Higashi Hiroshima, Hiroshima	34.35	132.8	JX532907	NAPX	Crease *et al.*, 2012
	China	Lake Wusutu	40.86	111.55	MH632088	NAPX	Ma *et al.*, 2019
D. tenebrosa	Canada	Churchill, Manitoba	58.77	-94.17	KC536605	-	Marková *et al.*, 2013
	Russia	Petchora Delta	68.06	53.58	KC536564	-	Marková *et al.*, 2013
	Svalbard	Storvatnet, Ny-Alesund	78.92	11.88	KC536580	-	Marková *et al.*, 2013
	Russia	Tsvetkov cape, Taimyr	74.92	112.62	KC536608	-	Marková *et al.*, 2013
D. magna	-	-	-	-	MT199637	-	Lee (Unpublished)

mineralized ponds located within wooded areas at medium and high altitudes (Marrone and Vecchioni, 2020), where phenomena of biological invasions are less frequent than in disturbed water bodies located in highly-anthropized areas. In fact, in Italy, the non-native *D. pulex* lineage was to date found in artificial or highly-anthropized water bodies both in Sardinia and Piedmont (Fadda *et al.,* 2011; Marková *et al.,* 2017). In light of these results, the hypothesis by Marrone *et al.* (2009) that Sicilian *D. pulex* populations are relictual elements that colonised Sicily during the late Pleistocene glacial events, coming from the Balkan peninsula or northern Italy, and which later found a "cool" refuge at higher altitude with the onset of warmer and low-moisture climatic conditions in the early Holocene (Curry *et al*., 2016), seems to be supported.

Interestingly, based on the currently available data, it seems that native European *D. pulex* populations preferentially inhabit small, natural water bodies, whereas the populations of the non-native American lineage are mostly occurring in artificial or highly-disturbed habitats (Schwenk *et al.,* 2000; Fadda *et al.,* 2011; Vergilino *et al.,* 2011) at medium and low altitude with a single high-altitude exception reported for a Spanish lake located in the Sierra Nevada (Conde-Porcuna *et al.,* 2020). Native and non-native *D. pulex* lineages are known to coexist sympatrically, but not syntopically, in the Po River basin, with the native populations occurring in the "*bodri"* (i.e., temporary natural ponds) (Marková *et al.,* 2017), and the non-native one found in the highly-anthropized Avigliana lakes (Virgilino *et al.,* 2011; Marková *et al.,* 2013). Conversely, for Sardinian populations molecular data are available only from a single site, i.e., an artificial reservoir built in Sos Canales (Fadda *et al.,* 2011). However, *D. pulex* is also occurring in smaller, natural astatic and temporary ponds located in the southern part of the island (Margaritora *et al.,* 2021; Marrone and Stoch, *unpublished data*), and it should be checked whether the *D. pulex* populations inhabiting these marginal, natural habitats belong to the alien or the native lineage.

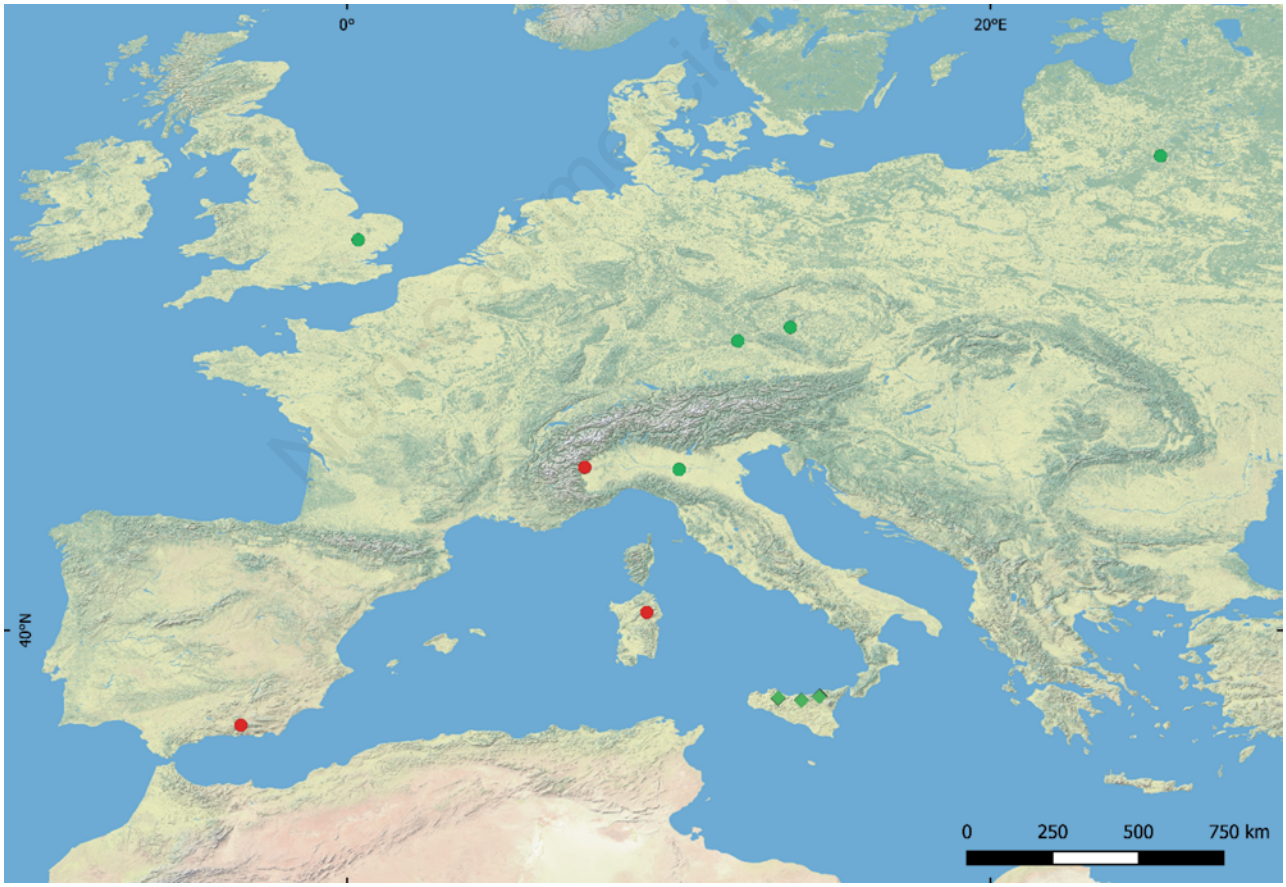

Fig. 1. Geographic location of *Daphnia pulex* sampling sites for which molecular data are available. Red circles indicate sites where the North American lineages, "NAPX", of *Daphnia pulex* occur. Green circles and diamonds indicate where the European lineages, "EPX", of the species occur. Green diamonds indicate the novel sampled sites. Due to the scale of the map some of the novel sites overlap and thus are not displayed. See Tab. 1 for the coordinates of the sampling sites and for more information on the collected species.

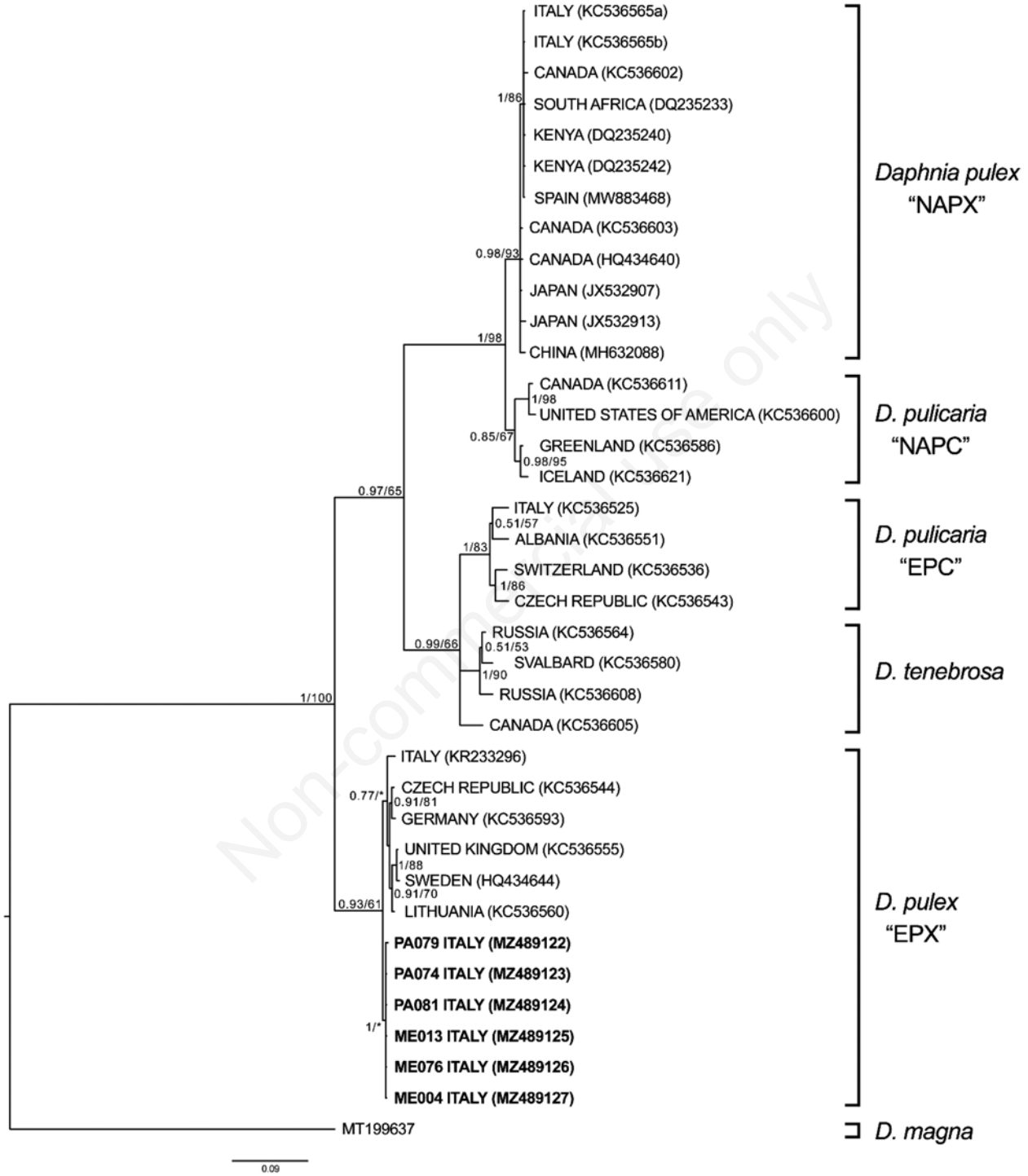

Fig. 2. Bayesian phylogram of *Daphnia* spp. based on the 624 bp fragment of the mtDNA ND5. *D. magna* was used as outgroup. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (Maximum Likelihood, ML). *, Nodal statistical supports <0.50. Square brackets group the samples according to the current taxonomy of the genus. Novel sequences are reported in bold. NAPX, North American Pulex; NAPC, North American Pulicaria; EPX, European Pulex; EPC, European Pulicaria. The analysed specimens are reported using the codes listed in Tab. 1.

CONCLUSIONS

This study provides the first molecular data about the Sicilian populations of the water flea *Daphnia pulex*. All the sampled populations proved to belong to the autochthonous European lineage of the species, in accordance with their previously hypothesised native and relictual status (Marrone *et al.,* 2009; Marrone and Vecchioni, 2020). Sicilian *D. pulex* populations are thus important management units, to be attentively managed and preserved. Considering the threats to native taxa linked with the occurrence and spreading of invasive, alien species (Marrone and Naselli-Flores, 2015), and the importance of timely acting to have a chance to effectively manage the biological invasions, the molecular characterization of the *D. pulex* populations occurring in southern Sardinia, peninsular Italy, and Malta (Margaritora *et al.,* 2021) is urgent and desirable.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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