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ORIGINAL ARTICLE

Daphnia diversity in water bodies of the Po River Basin

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ABSTRACT

Shallow water bodies dominate the areal extent of continental waters and host a proportion of biodiversity higher than the percentage of Earth's surface they cover. *Daphnia* is a key component of small aquatic ecosystems food webs. Here we present the result of a survey in 24 ponds located in the core of Po river Basin, to assess the actual spreading of *Daphnia* species in one of the most productive areas of the Northern hemisphere. By using diagnostic genetic markers (12S rRNA and ND5 genes) we identified five *Daphnia* species: *D. ambigua*, *D. curvirostris*, *D. longispina*, *D. obtusa* and *D. pulex* in fourteen ponds. Additional analyses of two nuclear genes (*LdhA* and *Rab4*) revealed that *D. pulex* in the study area is native European strain. In opposite, *D. ambigua* shared haplotype with the North-Eastern American lineage that was introduced to Europe by long-distance dispersal. In the Po river Basin we identified a highly divergent lineage of *D. longispina* group that formed a clade with individuals from northern European Russia and might represent a new *Daphnia* species. *Daphnia* species in the Cremona province have European origin, except for *D. ambigua* which is a North American species spreading across Europe. Future attention will require monitoring of invasive species, particularly *D. ambigua* and the North American invasive clone of *D. pulex* that is already present in Northern Italy.

Key words: Genetic diagnostic markers; invasive species; genetic differentiation; *D. pulex* complex; ponds.

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INTRODUCTION

Although freshwater ecosystems cover less than 1% of the earth's surface, they host almost 10% of the world's species that are directly supported through a wide range of habitats including running water in rivers, standing waters of lakes, ponds, pools and swamps and areas of ephemeral wetlands (Loh and Wackernagel, 2004; Feld *et al*., 2016). In recent years much attention has been paid to large lakes and rivers while small lakes and ponds that dominate the areal extent of continental waters have been completely ignored (Downing, 2010). However, small aquatic ecosystems play a major role in global cycles and in the Earth's most pressing environmental problems (Downing, 2010). In addition, permanent or temporary small, shallow water bodies offer a powerful potential for studies of ecology, evolutionary and conservation biology and provide high contribution to regional biodiversity (De Meester *et al.*, 2005). The organisms that colonise ponds and pools have evolved specific adaptations to deal with often extreme, disturbed, short hydro-period habitats, such as very effective spatial and temporal dispersal mechanisms (Ripley and Simovich, 2009). Furthermore, shallow water bodies are highly sensitive to climate warming and human activities due to substantial changes in thermal and hydrological regimes that play important role in freshwater biodiversity decline (Scheffer *et al.,* 2001; Dudgeon *et al*., 2006; Mitchell *et al.,* 2005; Meerhoff *et al.*, 2007; Feuchtmayr *et al.*, 2009; Landkildehus *et al.,* 2014). In Europe, up to 80% of the land is intensively used for settlements, infrastructure and production systems and aquatic biodiversity is probably impoverished accordingly (Feld *et al*., 2016).

Many of the cladoceran taxa recognized by traditional taxonomists are actually an admixture of genetically divergent species (Hebert and Wilson, 1994; Schwenk *et al.,* 2000). Now, the application of molecular tools is fundamental for the identification of new and cryptic species, for tracing distribution and dispersal of native and invasive species as well as for identification of hybrid taxa and their relatives (Pfenning and Schwenk, 2007). Furthering the study of freshwater zooplankton biodiversity by using techniques of molecular biology might help to reveal genetic diversity within and among species that could be otherwise hardly uncovered.

Cladocera, small aquatic crustaceans, are a key component of aquatic food webs. They inhabit a range of freshwater habitats throughout the world. Some genera, such as *Daphnia*, have been intensively studied over the past 250 years in different fields. Recently, the growing impact of climate change on inhabitants of the most vulnerable freshwater habitats, such small lakes and ponds, is undoubtedly

starting to be appreciated. Climate change is forcing *Daphnia* species to adjust their ways and may cause adverse effects on species diversity by means of elimination and/or replacement of sensitive species/strains by less sensitive ones, shifts in food-web interactions, acclimation of species/strains to stress, selection of tolerant genotypes, and outcome of predator–prey interactions (Belfiore and Anderson, 2001; Hunter and Pyle, 2004; Sakamoto *et al*., 2006; Riessen *et al.*, 2012; Vadadi-Fülöp *et al.*, 2012; Vadadi-Fülöp and Hufnagel, 2014). Additionally, environmental change creates ecological space for non-native species that can often replace native species. Recent invasion of the American *Daphnia* lineage to Africa was followed by rapid and complete continent-wide displacement of indigenous African *D. pulex* Leydig, 1860 throughout its native range (Mergeay *et al*., 2006) and was already recorded in Sardinia and Piedmont (Northern Italy) (Fadda *et al.,* 2011; Marková *et al.,* 2013).

In a previous paper, we reported the occurrence of the *D. pulex* group in the permanent pond named *bodrio del Pastore III*, in the Po River Basin (Northern Italy) where it co-exists with *D. longispina* O. F. Müller, 1776 (Rossi *et al.,* 2014). The presence of *D. pulex* in this area is important in the framework of conservation ecology. In fact, we have shown that it is the native European species instead of the invasive North American asexual clone (Rossi *et al*., 2015). Here we extend our research to 24 surrounding water bodies located in the Po river basin in the Cremona province that is one of the most productive areas of the Northern hemisphere (Bassanino *et al.*, 2011). Because of intensive farming, this area is threatened by diffuse pollution that, together with climatic changes, is a major driver of changes in freshwater ecosystems of Cremona province. Our main objectives are: 1) to assess the actual spreading and genetic diversity of native *D. pulex* and other *Daphnia* species in the area; 2) to verify morphological identification of different *Daphnia* species by using diagnostic mitochondrial gene (*12S rRNA*); and 3) to assess species identity and origin of *Daphnia* belonging to the *D. pulex* complex based on mitochondrial (the *ND5* gene) and nuclear phylogenies (*Rab4* and *LdhA* genes).

METHODS

The study place is located in the Cremona province where temporary pools and ponds, locally named *bodri,* originated by dramatic flooding events of the Po River (the largest Italian river with an annual mean discharge of 1500 $m^3 s^{-1}$) (Fig. 1). Erosive processes dig cone-shaped holes with depths up to 6-10 m and surface of some hundreds to some thousands m^2. At the end of flooding periods, these small ponds remain isolated from the main river course and start evolving as newly formed shallow aquatic environments. They display pronounced water level fluctuations, associated to the Po river hydrometric level, aquifer, pre-

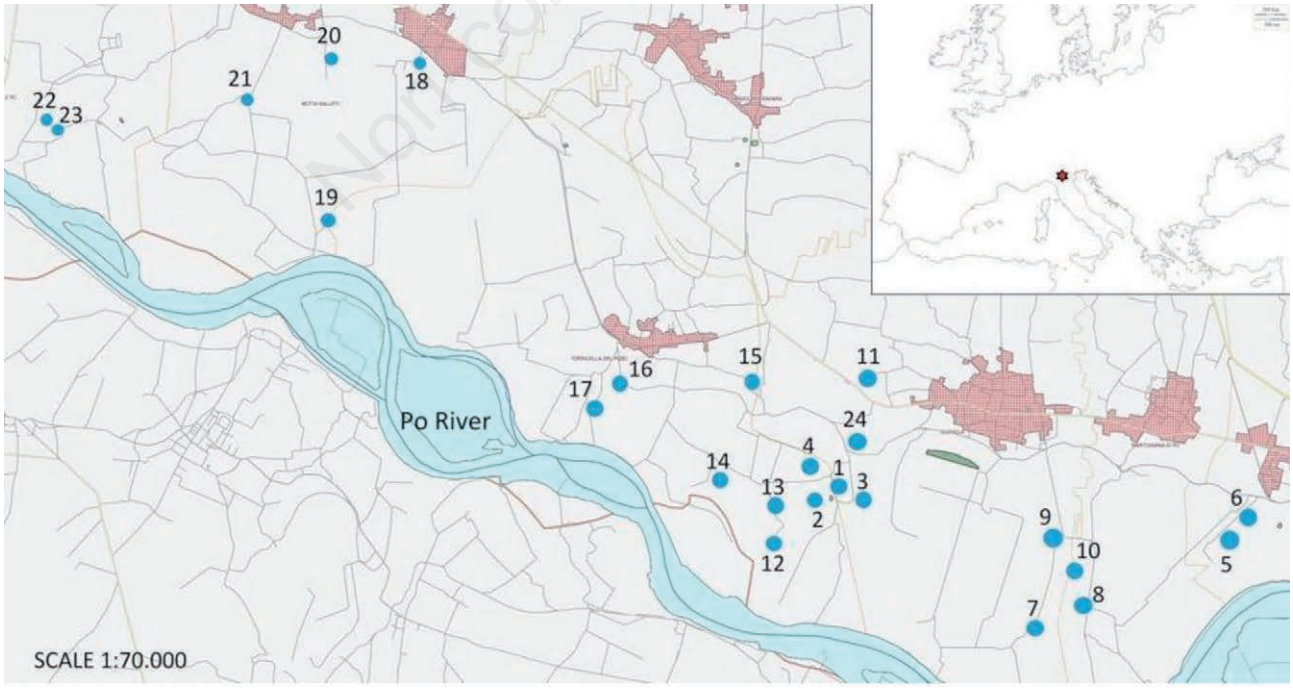

Fig. 1. Geographic location of the 24 sampling sites included in this study.

cipitations, runoff, groundwater vertical migration and summer evaporation. In 450 km^2 area, 61 water bodies were documented (AAVV, 1999). In this study, we focused on the occurrence of *Daphnia* species in 24 pools and ponds that were randomly selected in 200 km^2 area (Fig. 1). Many of the studied water bodies originated before 1723 [date of the first census of *bodri* (AAVV, 1999)]; however, some are more recent (for details see Tab. 1). Most of the time ponds and pools are separated from each other without possibility of seasonal stream connections, but some accidental link may occur during heavy flooding. Their size varied between 1529 to 7070 m^2; twelve of the 24 ponds contained fish and/or *Chaoborus* larvae [for details see Tab. 1; (AAVV, 1999)]. Each pond was sampled twice: first time between May and June 2014 and second time between June and July 2015.

Qualitative zooplankton samples were collected by 105 µm-mesh size plankton net. From 2 to 16 l of water were filtered for each sample according to the water volume and depth. All samples were preserved in 95% ethanol. All cladocerans present in the sample were sorted under a stereomicroscope and were identified to genus level. Then, all *Daphnia* species were morphologically identified according to Margaritora (1985). A contingency table analysis was performed to test whether the presence of *Daphnia* was negatively associated with the presence of fish or *Chaoborus* larvae (Tab. 1). The frequency of samples with and without *Daphnia* was compared in relationship with the presence or the absence of predators.

To verify morphological identification of *Daphnia* species, one to 11 collected specimens per each locality were used in molecular analyses according to the number of species per site. In the locality where species replacement was recorded, Pastore III (Rossi *et al*., 2015), up to 5 individuals per species were analysed. To verify *Daphnia* species identity, *12S rRNA* diagnostic marker was used. Because determining the species within the *D. pulex* complex based only on morphological characters is notoriously difficult (Colbourne *et al*., 1998) and *12S rRNA* gene is not a diagnostic marker for lineages within this complex, *D. pulex* complex, species from Po river Basin were analysed for an ~711 base-pair (bp) long fragment

Tab. 1. Collection sites of *Daphnia* included in this study. For each site Area and Depth refer to maximum value.

Code	Name	Latitude N	Longitude E	Area (m^2)	Depth (m)	Age	F	C
1	Pastore III	45°00’05”	10°19’26”	3130	6.3	1723-1870	1	1
2	Pastore I	45°00’00”	10°19’13”	2350	5.8	1935-1957	1	1
3	Pastore IV	45°00’01”	10°19’46”	3380	3.2	1723-1870	1	-
4	Temporary pond	45°00’12”	10°19’23”				-	-
5	Bosco Braca	44°59’41”	10°23’18”	2460	3.3	1935-1957	-	-
6	Cascina Pavarini	40°59’50”	10°23’29”	3763	5.6	1968	1	-
7	Bosco Valloni	44°59’06”	10°21’20”	1850	3.0	1723-1871	-	-
8	San Giorgio	44°59’15”	10°21’54”	2280	4.4	1723-1871	-	-
9	Forche	44°59’38”	10°21’47”	3140	3.2	1723-1870	-	-
10	Martignana	44°59’29”	10°21’47”	2230	5.3	1723-1871	-	-
11	S. Mariamaddalena	45°00’50”	10°19’44”	2280	1.8	B 1723	-	-
12	Bosco Bodini	44°59’42”	10°18’47”	1529	3.5	1957-1980	-	1
13	Cascina Mortara	44°59’59”	10°18’50”	2985	5.6	1723	1	1
14	Bosco Piazza	45°00’09”	10°18’18”	3390	4.0	1723-1870	-	1
15	Tavernelle	45°00’51”	10°18’37”	2420	4.5	B 1723	-	1
16	Vecchio	45°00’50”	10°17’18”	7070	3.7	B 1723	-	1
17	Bazzi	45°00’41”	10°17’04”	4350	7.5	1951	1	-
18	Motta	45°03’07”	10°15’23”	2230	4.0	1723-1869	1	1
19	Cascina Ronchetto	45°02’01”	10°14’27”	4150	4.8	1723-1871	-	-
20	Rita	45°03’10”	10°14’31”	5370	5.0	B 1723	-	-
21	Bicocca	45°02’52”	10°13’41”	1980	2.5	1723	-	-
22	Pescaroli Ovest	45°02’46”	10°11’41”	2640	5.9	1723-1871	1	-
23	Pescaroli Est	45°02’41”	10°11’49”	3090	5.5	1723-1871	1	1
24	Sabbie	45°00’25”	10°19’37”	2200	1.4	B 1723	-	-

F, presence (1) and absence (-) of fish in study sites; C, presence (1) and absence (-) of Chaoborus larvae in study sites, B 1723 in column Age mean that pond originated before year 1723.

of the gene coding for the NADH dehydrogenase subunit 5 (*ND5*). To assess the hybrid origin of specimens from *D. pulex* complex, we used two nuclear protein-coding loci: *Rab4* (subunit gene of the GTPase family) and *LdhA* (lactate dehydrogenase A).

Total genomic DNA was extracted from *Daphnia* stored in 95% ethanol using the QIAGEN DNeasy Tissue Kit (Valencia, CA). Parts of two mitochondrial genes, *12S rRNA* and *ND5,* were amplified and sequenced with published primers in accordance with the PCR conditions described by Taylor *et al.* (1996) and Dufresne *et al.* (2011), respectively. The entire *LdhA* gene and a part of the gene coding the small GTPase *Rab4* were amplified and sequenced according to Marková *et al*. (2013) and Omilian *et al*. (2008), respectively. All nucleotide sequence data from the present study have been deposited in the GenBank database under accession numbers KY196420 - KY196435 (12S rRNA), KY196436 - KY196439 (Rab4) and KY196440 (LdhA).

Altogether, we analysed 54 individuals from fourteen localities in the Po river Basin for mitochondrial *12S rRNA* phylogeny and an additional 63 sequences from GenBank (Supplementary Tab. 1) were used to provide wider phylogenetic framework for our study. After we identified the specimens as *D. pulex* mtDNA type on the basis of *12S rRNA* phylogeny, we directly amplified and sequenced mitochondrial *ND5* gene of 14 individuals from 3 populations. Additionally, we analysed the sequences of two nuclear genes (the *Rab4*; the *LdhA*) of eight individuals from three populations possessing *D. pulex* mtDNA.

The *12S rRNA* sequences were used to estimate the nucleotide diversity Pi (Nei, 1987; Nei and Miller, 1990) within *Daphnia* groups (Tab. 2) by using DnaSP software (Librado and Rozas, 2009). Phylogenetic analyses using maximum-likelihood (ML) criterion were performed in MEGA software version 6 (Tamura *et al.*, 2013). Firstly, we reconstructed mitochondrial phylogenies of *12S rRNA* and *ND5* by employing T92+G+I (Tamura 92) and HKY+I (Hasegawa *et al*., 1985) substitution models selected according to the Bayesian information criterion (BIC), respectively. Then we applied ML criterion to reconstruct nuclear phylogeny of *Rab4* and *LdhA* by using T92 and T92 with gamma distribution substitution models, respectively. Bootstrap support estimated from 1000 replicates using NNI (nearest neighbour interchange) branch swapping algorithm was applied in all phylogenetic analyses.

RESULTS

Altogether, we identified five *Daphnia* species in fourteen of 24 analysed pools and ponds (Tab. 3). In 10 sites, no water fleas belonging to the genus *Daphnia* were found. The presence or the absence of *Daphnia* is not affected by the presence or the absence of vertebrate or invertebrate predators (Contingency coefficient=0.098,

Tab. 2. Genetic diversity within species.

	Population	Sample size	Number of haplotypes	Polymorphic sites	Haplotype diversity±SD	Nucleotide diversity±SD
D. ambiqua						
12 S rRNA	All	8	5	24	0.786±0.151	0.01491±0.00454
12 S rRNA	Po River Basin	1	1	NA	NA	NA
D. curvirostis						
12 S rRNA	All	17	6	6	0.647±0.120	0.00228±0.00068
12 S rRNA	Po River Basin	12	4	3	0.682±0.102	0.00180±0.00042
D. longispina						
12 S rRNA	All	26	13	17	0.855±0.056	0.00527±0.00090
12 S rRNA	Po River Basin	18	5	84	0.693±0.086	0.02077±0.01624
	Po River Basin without lineage V	17	4	4	0.654±0.089	0.00205±0.00059
12 S rRNA	Lineage V	3	2	20	0.667±0.09877	0.02874±0.01355
D. obtusa						
12 S rRNA	All	13	8	71	0.808±0.113	0.04561±0.01345
12 S rRNA	Po River Basin	8	3	2	0.464±0.200	0.00104±0.00049
D. pulex						
12 S rRNA	All	14	5	6	0.505±0.158	0.00370±0.00118
12 S rRNA	Po River Basin	10	1	NA	NA	NA
LdhA	All	30	13	19	0.989±0.031	0.00379±0.00042
LdhA	Po River Basin	16	1	NA	NA	NA
Rab4	All	22	8	7	0.805±0.069	0.00397±0.00049
Rab4	Po River Basin	16	4	6	0.708±0.091	0.00411±0.00062

χ^2=0.235, df=1, P=0.628). *Daphnia* species from each sampling site were firstly morphologically determined and then verified by molecular analyses. Among 54 analysed individuals, *D. ambigua* Scourfield, 1947, *D. curvirostris* Eylmann, 1887, *D. longispina*, *D. obtusa* Kurz, 1874 and *D. pulex* were determined (Tab. 3). The most frequent species, *D. curvirostris* and *D. longispina*, were found in six and five ponds, respectively. In contrast, *D. ambigua* was identified only in one pond (Pastore IV) while *D. obtusa* and *D. pulex* were detected in three ponds (Tab. 3). Three *Daphnia* species (*D. longispina*, *D. curvirostris* and *D. obtusa*) were identified in the pond Sabbie. Additionally, coexistence of *D. pulex* with *D. longispina* was found in three ponds (Pastore I, Pastore III and Bosco Valloni) and coexistence of *D. curvirostris* with *D. obtusa* in the pond Bosco Valloni (Tab. 3).

The *D. longispina* group was the most genetically variable group in the sampling area where we identified five unique *12S rRNA* haplotypes (DLONG A, B, C, D and E; Fig. 2 and Tab. 3). Nucleotide diversity of *D. longispina* from Cremona area was 0.02077 (Tab. 2). However, once we excluded the most divergent haplotype within this group (DLONG E), nucleotide diversity decreased to 0.00205. To reveal the phylogenetic origin of this haplotype, we included into the phylogenetic analyses also sequences of several divergent lineages of the *D. longispina* complex (Ishida *et al*., 2011; Petrusek *et al*., 2012; for details see Supplementary Tab. 1). Interestingly, the haplotype DLONG E clustered together with two individuals from the Pechora delta (northern European Russia) and formed the clade V of *D. longispina* complex (named by Petrusek *et al*., 2012).

Altogether, four *12S rRNA* haplotypes of *D. curvirostris* group were identified in the study area (DCUR A, B, C and D; Fig. 2 and Tab. 3). Interestingly, in the temporary pond located close to the pond Pastore III, different *12S rRNA* haplotypes were found in 2014 and 2015. In 2015, the dominant haplotype DCUR C was replaced there by a new haplotype DCUR D. This is the sole case of shift in genetic variability between years within *D. curvirostis* group. In ponds Bosco Valloni and Sabbie, where *D. curvirostris* coexists with *D. obtusa* and with *D. longispina* and *D. obtusa*, respectively, only the most common haplotype DCUR C was identified. Even though the nucleotide diversity of *D. curvirostris* from the study area was low (0.00180), overall nucleotide diversity of *D. curvirostris* group was just a little bit higher (0.00228; Tab. 3).

D. obtusa inhabited four pounds in the study area and within the *D. obtusa* group three *12S rRNA* haplotypes were identified (DOBT A, B, and C; Fig. 2 and Tab. 3). The nucleotide diversity of *D. obtusa* from the study area was the lowest one (0.00104) within all analysed *Daphnia* groups, while total nucleotide diversity of the *D. obtusa* group was relatively high (0.04651; Tab. 2).

The pond Pastore IV was the only locality in the study area where we found *D. ambigua* in two years sampling period (2014 and 2015). The *D. ambigua* we identified shared *12S rRNA* haplotype (DAMB A) with individuals from France and USA (Fig. 2).

D. pulex was found in three ponds (Pastore III, Pastore I and Forche) during our sampling in 2014 and 2015 (Tab. 3). Altogether 12 individuals were analysed and genetically determined as European *D. pulex* (Fig. 2, Supplementary Figs. 1 and 2). All individuals carried unique haplotype (DPX A) at both mitochondrial genes (*12S rRNA* and *ND5*) and clustered into the European *D. pulex* clade. Additional analyses of two nuclear genes (*LdhA* and *Rab4*) revealed that *D. pulex* from the study area carried only European *D. pulex* haplotypes at both analysed nuclear markers (Supplementary Figs. 1 and 2). All eight

Tab. 3. Distribution of haplotype diversity within sampling sites included in this study.

Species	Haplotype	GenBank Accession N.	Sampling site
D. ambigua	DAMB A	KY196426	Pastore IV
D. curvirostris	DCUR A	KY196435	Isola Pescaroli East
	DCUR B	KY196428	San Giorgio, Bicocca
	DCUR C	KY196425	T. Pond, Bosco Valloni, Sabbie
	DCUR D	KY196432	T. Pond
D. longispina	DLONG A	KY196434	Vecchio
	DLONG B	KY196431	Sabbie
	DLONG C	KY196423	Pastore III
	DLONG D	KY196424	Pastore I, Bosco Piazza, Sabbie
	DLONG E	KY196433	Bosco Piazza
D. obtusa	DOBT A	KY196427	Bosco Valloni, Martignana
	DOBT B	KY196430	Sabbie
	DOBT C	KY196429	Isola Pescaroli West
D. pulex	DPX A	KY196422	Pastore I, Pastore III, Forche

T., temporary.

European *D. pulex* were homozygote at the *LdhA* gene and shared the *LdhA* haplotype DPX A (Supplementary Fig. 2). Higher genetic variability was found at a second nuclear gene the *Rab4* (Supplementary Fig. 2). Altogether, we identified four *Rab 4* haplotypes (haplotypes DPX A, DPX B, DPX C and DPX D) among individuals from the study area with nucleotide diversity equal to 0.00411. While two *Rab4* haplotypes (DPX B and DPX C; Supplementary Fig. 2) were determined as unique for the Cremona region, two other *Rab4* haplotypes (DPX A and DPX D) were shared by individuals from Czech Republic, Sweden, Germany, Lithuania and United Kingdom (Supplementary Fig. 2).

DISCUSSION

In this study, we have sampled 24 pools and ponds that covered 200 km^2 area of the Po river Basin in Cremona province. By using diagnostic genetic markers, we assessed

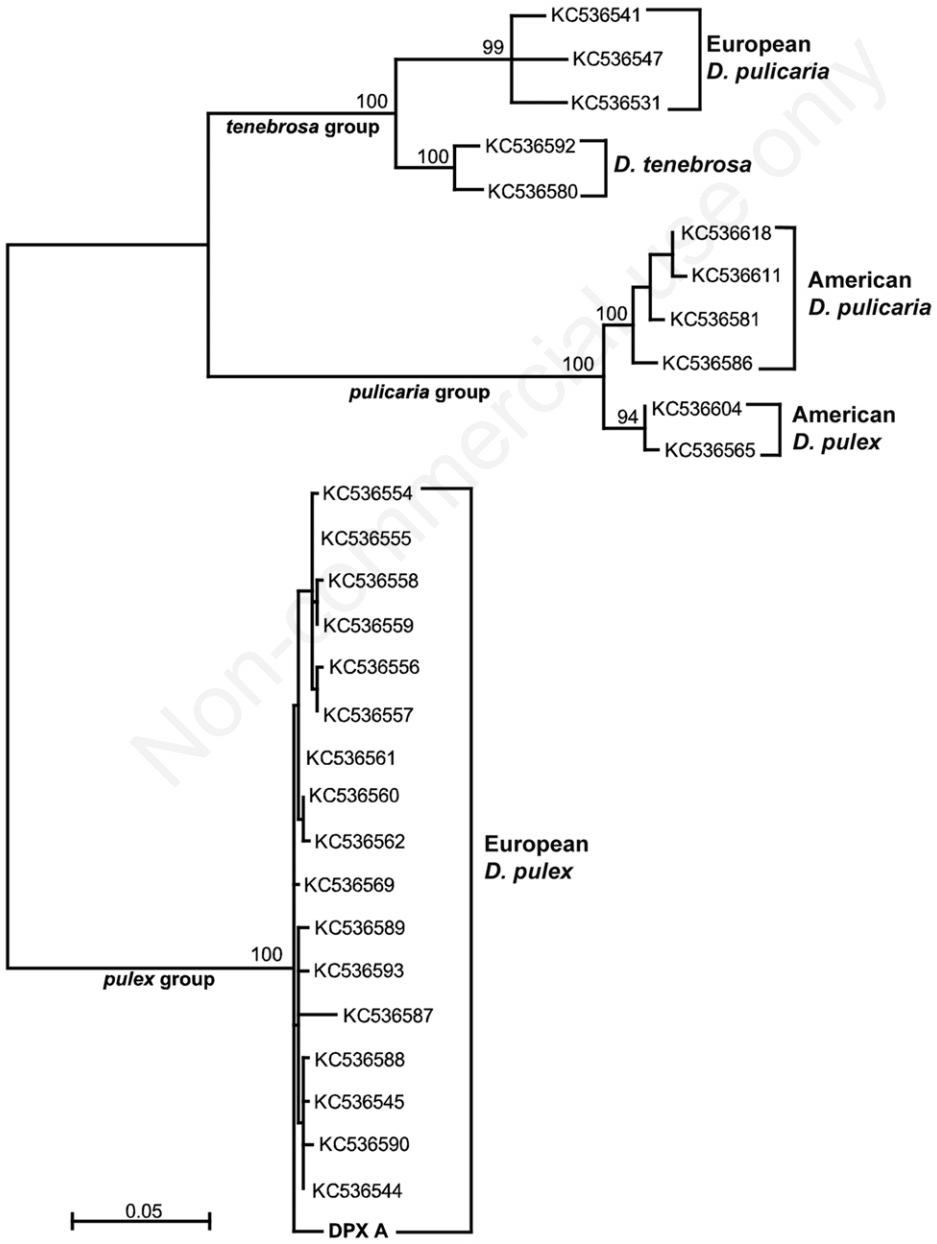

Fig. 2. Maximum likelihood mitochondrial phylogeny based on *ND5* haplotypes. Numbers along branches indicate the percent bootstrap frequencies for bipartitions with greater than 70% support.

the presence of five *Daphnia* species (*D. ambigua*, *D. curvirostris*, *D. longispina*, *D. obtusa* and *D. pulex*) in a relatively small area of the Po river Basin intensively devoted to agricultural activities and vulnerable to diffuse pollution. We discuss these results in wider phylogenetic context.

In the 70's, *D. pulex* was identified in the Po river Basin (Caorso and Ozzano dell'Emilia, about 50 km west and 100 km east from our study area, respectively) by Margaritora (personal communication), however no information about its origins was reported. Firstly, we recorded *D. pulex* in the pond Pastore III in 2011 (Rossi *et al*., 2014) and later we extended our sampling and focused on phylogenetic origin of those *Daphnia* species that could pose a threat for local populations. A well-known invasive asexual clone of *D. pulex* has spread from North America and replaced the native one throughout Africa, Spain (Mergeay *et al*., 2006) and recently has been also recorded in Sardinian reservoir (Sardinia Island; Fadda *et al*., 2011) and in Avigliana Lake (Piedmont Region, Northern Italy; Vergilino *et al*., 2011; Crease *et al*., 2012; Marková *et al.*, 2013) that is only about 200 km west from our study area. Because of that, the chance that this highly invasive clone could be present also in the Po river Basin was high. Phylogenetic analyses of several individuals from the Po river Basin, morphologically determined as *D. pulex*, revealed that *D. pulex* from the study area is true European *D. pulex* (Fig. 3; Supplementary Figs. 1 and 2). While mitochondrial haplotypes of *12S rRNA* and *ND5* genes were carried only by individuals from the Po river Basin, nuclear *LdhA* and *Rab4* haplotypes were shared also by *D. pulex* from the Czech Republic, Sweden, Germany, Lithuania and United Kingdom with exception of two unique *Rab4* haplotypes for the Po river Basin (DPX B and C; Supplementary Fig. 2). Even though the low genetic variability was identified at most of the analysed loci, the presence of adult males, ephippial females and free ephippia indicate that the population of *D. pulex* from the Po river Basin reproduce by cyclical parthenogenesis, and so clearly differ also by reproductive strategy from invasive asexual clone inhabited Sardinian reservoirs and Lake Avigliana (Rossi *et al*., 2014). Additional analyses of tens of *D. pulex* from the study area could reveal some genetic diversity also at the mitochondrial genes.

D. ambigua was the only invasive *Daphnia* species in the Po river Basin, and belongs to the North-Eastern American strain that was introduced by long-distance dispersal and is spreading across Europe (Hebert *et al*., 2003). We determined *D. ambigua* only in one of 24 studied ponds (Pastore IV) which is located in the core of our sampling area. However, this *Daphnia* species is very successful in spreading because of small body size, which makes it preadapted to eutrophied ponds that are over-stocked with fish (De Meester *et al.,* 2002). In Belgium (Lake Donk, Eastern Flanders), *D. ambigua* was recorded for the first time in the 60's by Dumont (1974). By 2002 it was already widespread in Flanders (De Meester *et al*., 2002). Even though *D. ambigua* was recorded in England (Johnson, 1952), France (Amoros, 1980), Germany (Krause-Dellin, 1992; Maier and Buchholz, 1996) and Czech Republic (Hamrová and Černý, 2007), the only available European *12S rRNA* sequences in GenBank database are from France (Schwenk *et al*., 2000; Hebert *et al*., 2003). We showed that *D. ambigua* haplotype from the Po river Basin (DAMB A) is identical with haplotypes from France (AF277283, AF523738) and New York (USA; AF523735; Fig. 3). The rapid colonization of Europe by *D. ambigua* and the occupancy of large blocks of territory by a single haplotype support little genetic diversity in the early phases after colonization of a new continent (De Meester *et al.,* 2002). Our record of invasive *D. ambigua* supports well known high dispersal capacity of freshwater cladocerans inhabiting island-like aquatic habitats (Slusarczyk and Pietrzak, 2008). In fact, *D. ambigua* has been already recorded in Northern Italy (Margaritora, 1985; Manca *et al*., 2005) and in Sicily (Marrone *et al*., 2005) but no information about its origin was reported. Manca *et al.* (2005) recorded *D. ambigua* only in the Lago di Mantova superiore that is less than 50 km east from our study area. *D. ambigua* from Lago di Endine (Northern Italy) was not cyclomorphic and showed some morphological characters that differentiated it from other European populations (Margaritora, 1985). *D. ambigua* from the pond Pastore IV seems cyclomorphic, showing a small pointed helmet, and does not have the ocello (Rossi; personal observation). Dispersal potential of *D. ambigua* is enormous and will require monitoring, because this small invasive species could pose a threat to native *Daphnia* populations in the Po river Basin where eutrophication and fish presence made local water bodies suitable for it.

In the Po river Basin, we found a high genetic diversity within the *D. longispina* complex (five haplotypes with Pi 0.02077). Between 2011 and 2014, *D. pulex* dominated in the pond Pastore III while in 2015 it was partly replaced by *D. longispina* (Rossi *et al*., 2015; Rossi *et al*., in preparation). Due to high population fluctuation and spatial/temporal dispersal by resting eggs, *D. longispina* could have colonized the pond Pastore III from surrounding ponds. An alternative scenario is that *D. longispina* have been present in the pond Pastore III already before, but in low hardly detectable density and/or in ephippial eggs bank, or could colonized this pond recently by long distance dispersal from other sources (*e.g.* some introductions by huntsmen, by fishers, by vehicles, by migrant birds). Interestingly the haplotype DLONG E, carried by *Daphnia* from the pond Bosco Piazza, clustered into the highly divergent clade V of *D. longispina* complex (named by Petrusek *et al*., 2012) together with two individuals from Pechora Delta (northern European Russia). However, *12S rRNA* haplo-

types (JX069356, JX069357) carried by individuals from Pechora Delta are distinct from the Po river Basin one (nucleotide diversity 0.02874) that negotiates a scenario of recent invasion. Instead of that, our phylogenetic data suggested that the clade V of *D. longispina* complex is not restricted only to northern European Russia but has a much wider area of distribution. *Daphnia* from the pond Bosco Piazza (DLONG E, Fig. 3) likely represents a distinct biological species. Because this putative new species co-exists in the pond Bosco Piazza with *D. longispina*, sequencing of additional marker(s) with focus on hybridization will be required.

An additional two *Daphnia* species (*D. curvirostris* and *D. obtusa*) with low nucleotide diversity (0.00180 and

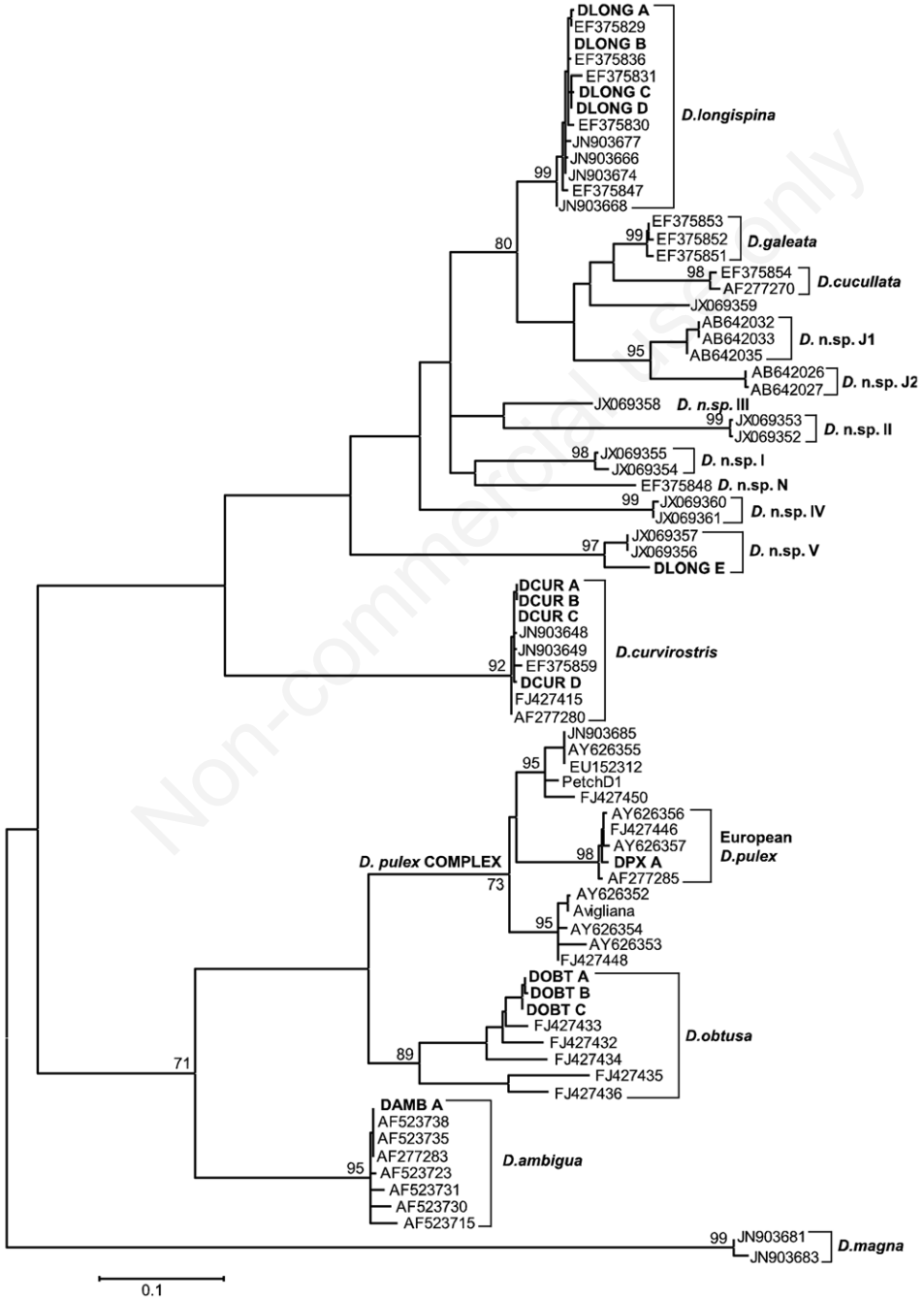

Fig. 3. Maximum likelihood mitochondrial phylogeny based on *12S rRNA* haplotypes. Numbers along branches indicate the percent bootstrap frequencies for bipartitions with greater than 70% support.

0.00104) inhabited six and four ponds in the study area, respectively. Generally, low genetic variability within whole *D. curivirostris* group (nucleotide diversity 0.00228) containing *D. curvirostris* from Europe (JN903648, JN903649) and from Canada (FJ427415, AF277280; Fig. 3) was detected. The *D. obtusa* clade involved two groups, one formed by *D. obtusa* from Europe including study area and the other by *D. obtusa* from North America. Phylogenetic analyses showed that both *Daphnia* species from the Po river Basin have European origin.

The finding of five *Daphnia* species in a relatively small area (20,000 ha) of the Po river Basin is in accordance with previous studies at the regional scale and from different parts of Europe. The analysis of Cladocera community including 29 lakes in the Po River watershed during the period 1998-1999 revealed the presence of five *Daphnia* species (*D. longispina*, *D. ambigua*, *D. hyalina*, *D. galeata* and *D. cucullata*) and four relative hybrids (Manca *et al*., 2005). Between November 1987 and May 1989, during a study focused on distribution and genetic variability of *D. obtusa,* in 97 ponds and pools sampled in the Po river catchment (Parma province), Bachiorri found four *Daphnia* species (*D. obtusa*, *D. longispina*, *D. ambigua* and *D. hyalina*) in 18 sampling sites (Bachiorri *et al*., 1991 and unpublished data). In 25 temporary ponds located in a wetland zone spread over an area of 180,000 ha in Doñana (South-west Spain) 3 species of *Daphnia* (*D. longispina*, *D. magna* and *D. pulex*) were found (Frish *et al*., 2006). In 33 interconnected shallow ponds in a small area (200 ha) in Belgium, 3 species, *D. pulex*, *D. ambigua* and *D. galeata* were recorded (Cottenie *et al*., 2001).

CONCLUSIONS

Here we identified phylogenetic origin of five *Daphnia* species from Cremona province and showed that four of them are native European species. Until now, only the *D. ambigua* invasive species in the Po River Basin was found. In addition, we identified, within the *D. longispina* complex, a highly divergent haplotype that clustered together with individuals from northern European Russia and might represent a distinct biological species. This finding has important implication for biodiversity conservation in agricultural areas. Future attention will require monitoring of the dispersal of North American *D. pulex* which presents a high risk for natural populations in the Po River Basin.

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