

Parasite communities and genetic structure of non-native pumpkinseed, *Lepomis gibbosus*, in different Black Sea drainages of Ukraine

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Abstract – In recent years, pumpkinseed *Lepomis gibbosus* (Actinopterygii: Centrarchidae) have spread intensively to many parts of Europe, including Ukraine. In this study, we (1) assess intra-population genetic variability in five widely-spaced Ukrainian pumpkinseed populations and compare their population genetic structure with other European populations and (2) provide a comprehensive survey of pumpkinseed parasites across the region. Discriminant analysis of principal components and F_{ST} analyses based on microsatellites indicated that all five populations formed discrete clusters. Within Europe, Ukrainian populations were most closely associated with populations from the River Danube, suggesting that the Danube is the main source of all Ukrainian pumpkinseed populations. The parasite fauna comprised 15 taxa, most of which (92%) were native to North America (monogenea *Onchocleidus similis* and *O. dispar*; myxozoan *Myxobolus dechtiari*). Parasites acquired in the species' new range occurred accidentally, with only *Trichodina* ciliates found relatively frequently and the eye flukes *Diplostomum pseudospathaceum* and *Tylodelphys clavata* occurring at higher prevalence. Absence of specific monogeneans in pumpkinseed from estuaries (Khadzhibey and Sukhyi Lymans) indicates low tolerance of *Onchocleidus* species to salinity.

Keywords: Invasive alien species (IAS) / parasite acquisition / enemy release / invasion corridors / microsatellites

1 Introduction

Despite the fact that fish represent an important aspect of the economy of many countries, risk management (*e.g.* quarantine control) for this group of animals is usually less rigid than that for other species (Copp *et al.*, 2005). Over recent decades, there has been a sharp increase in the number of alien freshwater fish introductions as a result of the socio-economic, ecological and evolutionary impacts of globalisation (Hulme, 2009; Vitule *et al.*, 2009; Gozlan *et al.*, 2010; Cucherousset and Olden, 2011). In many regions, such introductions have had a negative impact on local biodiversity through predation, food and spatial competition, hybridisation, spread of parasites and pathogens, modification of food chains or changes in biochemical cycles (Leunda, 2010; Cucherousset and Olden, 2011).

Alien species in invaded ecosystems may co-introduce new parasites species, which may be specific to the original host but

may also act as agents of new diseases for the local fauna (Taraschewski, 2006). According to Goedknecht *et al.* (2016), there are six main concepts of host/parasite interaction related to biological invasions, of which the most common are enemy release, parasite spillback and spillover, and parasite co-introduction. Indeed, one contributing factor to the invasion success of some species may be the temporary release of parasites and pathogens that are numerous in the native population but far less so in the new non-native population (enemy release; Torchin *et al.*, 2003; Prenter *et al.*, 2004). Likewise, escape from parasites may occur when parasites numerous at the beginning of invasion are lost due to local conditions, a process that forms part of the 'enemy release hypothesis' of Keane and Crawley (2002). Alternatively, the introduced hosts may be parasite-free at the start of invasion (*e.g.* when introduced as early life stages), which could help the invasive host adapt to its new environment and establish a stable population; however, local parasites may then quickly adapt to the new host (Tanaka *et al.*, 2007). Finally, when several alien species are introduced into a new environment at the same time,

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they may support the invasion success of each other, a process forming the basis of the ‘invasion meltdown hypothesis’ of Simberloff and von Holle (1999). In this way, a non-native host may help spread non-native parasites, even if they were introduced by some other vector (Taraschewski, 2006; Emde *et al.*, 2014; Hohenadler *et al.*, 2018).

The pumpkinseed, *Lepomis gibbosus* (L., 1758) (Actinopterygii: Centrarchidae), is native to fresh waters of eastern North America, from New Brunswick, Canada, to northeastern Georgia, USA (Scott and Crossman, 1973). The pumpkinseed was first transported from Canada to Europe (Paris) in 1877 as an ornamental species (Stansch, 1914). Shortly thereafter, the species was released into open waters, mainly in the southwest of France, where it established a stable population lasting to 1900 (Kunstler, 1908; Vivier, 1951). Since then, this invasive species has spread widely throughout Europe, with established populations now found from the Iberian Peninsula in the west to coastal rivers around the Sea of Azov in the east (Diripasko *et al.*, 2008; Ribeiro and Leunda, 2012). As a result, the species was included into the list of alien species of EU concern in 2019 (European Commission, 2019).

In Ukraine, the fish was first recorded in lakes of the lower Danube in 1918 (Pavlov and Bilko, 1962). In 1952, it was found in the Dniester river delta, approximately 150 km from the Danube delta, and in 1953, it was found in the Gulf of Odessa, in the northwestern Black Sea (Zambriborshch and Shumilo, 1953; Vinogradov, 1960). The fish is now found throughout the country, with populations in the lower and middle Dnieper, southern Bug and Tisza river drainages, various waterbodies in Crimea and several lagoons and estuaries along the Black Sea coast (Movchan, 2011). Its northernmost occurrence is believed to be the lakes of the Upper Dnieper basin near Kyiv, where it has been recorded since 2011 (Tsyba, 2011; Afanasyev *et al.*, 2017).

Analysis of genetic structure patterns across populations are now widely used in the study of biological invasions as they can provide useful information on the source of introduction, the number of introduction events, the level of propagule pressure (Roman and Darling, 2007; Zardi *et al.*, 2007) and help in reconstructing a species’ invasion history (Brown and Stepien, 2009, 2010). It may also help our understanding of the interrelationships between isolated non-native species populations over differing distances. Phylogeographic analyses of mitochondrial DNA (mtDNA; cytochrome *b* gene) variability have shown that all Ukrainian pumpkinseed populations along the lower Danube, Dniester and Dnieper exhibit a single haplotype, indicating a single source of introduction (Slynko *et al.*, 2014). Similarly, generally low genetic variation has been reported in a second mtDNA gene (NADH1) in a study analysing pumpkinseed populations over a wider area of Europe (Yavno *et al.*, 2020). Compared to mtDNA, microsatellite DNA markers provide better resolution for identifying population structure due to their high levels of variation and large number of alleles (O’Reilly and Wright, 1995). Accordingly, a detailed study of European pumpkinseed indicated the presence of three clear lineages, though there was relatively high variation within the lineage covering populations along the ‘southern invasion corridor’ (*sensu* Panov *et al.*, 2009), *i.e.* populations along the Rhine and Danube river basins (Ondračková *et al.*, 2021).

Considering the recent expansion of this fish in Ukraine, the aim of this work was to (1) evaluate the genetic structure of pumpkinseed populations in Ukraine and to assess relationships with other European populations using data previously published by Ondračková *et al.* (2021), and (2) to characterise their parasite communities. Despite the relatively long presence of pumpkinseed in Ukraine, data on the source of introduction and its parasites are somewhat lacking. Here, we included pumpkinseed populations of different ages. The oldest one is the population in Lake Kartal, which is situated in the area of Yalpuh-Kuhurlui lake system, where pumpkinseed were first documented in Ukraine (Berg, 1949). We, therefore, expect that this population might represent a source for further spread of the species in other Ukrainian areas. Furthermore, in accordance with other European populations, we expect generally low genetic diversity given the fish are predicted to originate from a single lineage (see Slynko *et al.*, 2014). The most comprehensive data to date on pumpkinseed parasites in Ukraine comes from the Danube Delta, where seven parasite species have been recorded, including the myxozoans *Myxobolus mülleri* and *M. exiguus*, the monogeneans *Onchocleidus similis* and *Gyrodactylus avalonia*, the digeneans *Nicolla skrjabini* and *Tylodelphys clavata*, and one nematode *Schulmanella petruschewskii* (Kulakovskaya and Koval, 1973; Kvach *et al.*, 2018). In addition, *Pomphorhynchus tereticollis* has been recorded in fish from Crimean waters (Stryukov and Moskvina, 2017) and *Onchocleidus dispar* from the Kakhovka reservoir and the lower Dnieper (Rubtsova, 2015). Therefore, we expect identifying parasites both native to pumpkinseed, *i.e.* co-introduced to Europe, and local species acquired in the new range, though at relatively low abundances in the latter ones, similar to other European regions (*e.g.* Ondračková *et al.*, 2019a).

2 Material and methods

2.1 Fish sampling and processing

Pumpkinseed were sampled at five localities in Ukraine (GPS coordinates in decimal degrees are provided), *i.e.* Lake Kartal (45.288477 N, 28.519946 E), Sukhyi Lyman (46.394228 N, 30.633834 E), Khadzhibey Lyman (46.593317 N, 30.661953 E), the lower Dnieper irrigation canal (46.272784 N, 32.734128 E) and Lake Almazne (50.504582 N, 30.627121 E) (Fig. 1). The salinity of the water at each site was assessed in the hydrochemical laboratory of the Institute of marine biology of the National Academy of Science, Odessa, Ukraine. Fish from Sukhyi Lyman, Khadzhibey Lyman and Lake Kartal were sampled using 5 m fyke-nets with a 10 mm mesh size, while the two sites in the Dnieper basin were sampled using a 6 mm mesh dipnet. After sampling, the fish were transported alive, in aerated water from the sampling locality, to the laboratories, where they were placed into an aquarium. Prior to dissection for parasite analysis, 10–12 scales from each fish were removed, cleaned of epithelial residue, and placed between two slides (Chugunova, 1959), after which they were examined for age determination using light microscopy. A sample of the caudal fin was also taken and preserved in 96% ethanol for further genetic analysis. In all, 97 individuals were used for genetic analysis and description of parasite communities.

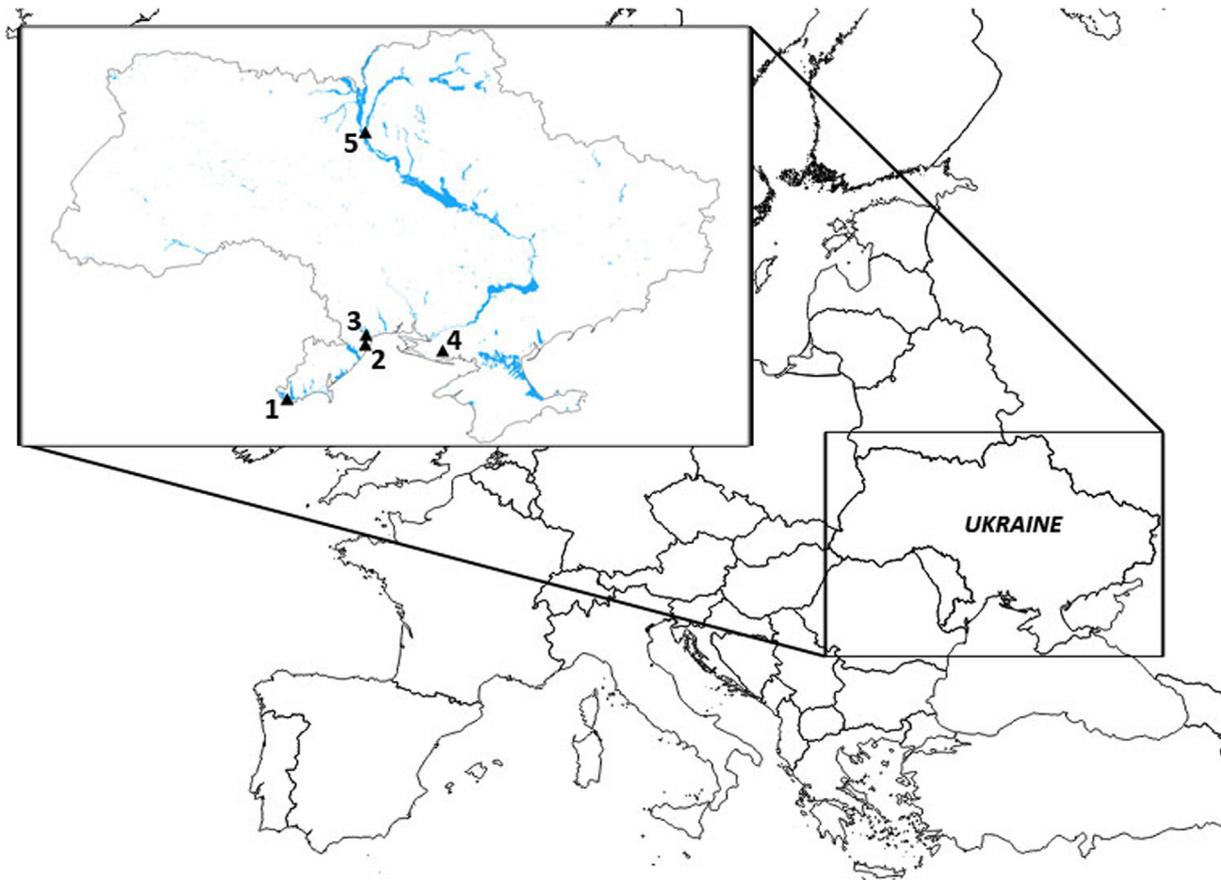


Fig. 1. Sampling localities in Ukraine: 1 = Lake Kartal, 2 = Sukhyi Lyman, 3 = Khadzhibey Lyman, 4 = Lower Dnieper irrigation canal, 5 = Lake Almazne.

2.1.1 Microsatellite analysis of fish

Six microsatellite loci were amplified for each fish using the method described in Ondračková *et al.* (2021). Analysis of population structure was performed using the discriminant analysis of principal components (DAPC) implemented in the R “adegenet” package v. 2.1.7 (Jombart, 2008), to differentiate pre-defined biological non-native pumpkinseed populations from this study (Ukraine) and nine European populations originally published by Ondračková *et al.* (2021), *i.e.* the lower Danube basin (Bulgaria), the middle Danube (Czech Republic and Austria), the Rhine river basin (Germany), the Girone basin (France) and the Sado and Tejo river basins (Portugal). The number of PCA axes retained was 25 and 20, explaining 96.1 and 81.6% of variance. The number of axes retained in the discriminant analysis was 4 and 13, respectively. The proportions of successful reassignment (based on the discriminant functions) of individuals to their original clusters were 0.979 and 0.895, for five Ukrainian populations and nine European, respectively. Scatterplot of DAPC was performed using the first two axes (LD1 and LD2) according to the discriminant analysis functions (DA) eigenvalues. Genetic divergence among five Ukrainian populations was estimated by pairwise F_{ST} (Weir and Cockerham, 1984), and the presence

of substructure (*i.e.*, F_{ST} significantly higher than zero) was tested by 1000 permutations in GENETIX 4.05 (Belkhir *et al.*, 1996–2004). The Hardy-Weinberg equilibrium (HWE) test was performed for each locus in all five populations using the Markov chain method (“Exact probability test”) in Genepop v.4.7 (Raymond and Rousset, 1995). Total (Total NA) the Shannon information index (I), expected heterozygosity (He), unbiased expected heterozygosity (UHe), observed heterozygosity (Ho), fixation index (F) and percentage of polymorphic loci (P) were all calculated in GenA1Ex v. 6.51 (Peakall and Smouse, 2006, 2012). Allelic richness (AR) corrected for sample size by the rarefaction method (*i.e.* estimated for a minimum sample size of 16 diploid individuals) were calculated for each of 5 populations in FSTAT 2.9.3.2 (Goudet, 2001). Excess heterozygosity with regard to that expected at mutation-drift equilibrium for the number of alleles present may suggest a genetic bottleneck (Cornuet and Luikart, 1996). We used BOTTLENECK 1.2.02 (Piry *et al.*, 1999) to estimate the deviation of gene diversity from mutation-drift equilibrium. This estimate was simulated using 1000 permutations in BOTTLENECK by choosing the two-phase mutation model (TPM), with a variance for TPM equal to 0.36 and a proportion of the stepwise mutation model in TPM equal to 0.7. The significance of deviation from the

Table 1. Size (mm) of pumpkinseed caught at five sites in Ukraine. n = number of fish, SL = standard length (mm), TL = total length (mm).

Parameters	Lake Kartal	Sukhyi Lyman	Khadzhibey Lyman	Lower Dnieper	Lake Almazne
n	19	16	20	21	21
SL					
m±sd	58.3±5.9	127.9±13.8	106.8±13.9	54.9±3.2	77.9±11.5
min–max	45–68	98–143	68–122	49–62	58–95
TL					
m±sd	71.9±6.8	150.4±15.0	126.0±16.5	68.1±4.2	77.0±11.5
min–max	55–84	119–170	80–144	60–76	63–107

Table 2. Mean (m) ± S.D. standard length (SL, mm) and weight (W, g) of pumpkinseed age groups at five Ukrainian localities. n = number of fish.

Parameters	Age	n	SL		W	
			m±sd	min–max	m±sd	min–max
Lake Kartal	1+	9	56.3±5.9	45.0–64.0	5.7±1.6	3.3–8.8
	2+	10	60.1±5.5	51.0–68.0	7.1±1.8	3.7–10.1
	Total	19	58.3±5.8	45.0–68.0	6.4±1.8	3.3–10.1
Sukhyi Lyman	4	3	125.7±5.1	120.0–130.0	67.4±10.7	56.6–77.9
	5	8	135.2±10.2	112.0–143.0	93.3±19.2	55.3–119.1
	Total	11	132.6±9.9	112.0–143.0	86.2±20.7	55.3–119.1
Khadzhibey Lyman	3	4	84.0±13.2	68.0–99.0	20.9±10.1	9.5–33.7
	4	9	108.3±4.2	103.0–115.0	46.5±6.3	39.3–56.5
	Total	20	106.7±13.9	68.0–122.0	45.8±15.4	9.5–65.2
Lower Dnieper	1	1	49.0	–	3.4	–
	2	7	52.2±1.9	50.0–55.0	3.9±0.4	3.3–4.3
	3	13	56.5±2.8	53.0–62.0	4.9±1.1	3.8–7.2
Lake Almazne	Total	21	54.9±3.5	49.0–62.0	4.6±1.1	3.3–7.2
	2	1	58.0	–	5.0	–
	3	18	68.1±4.8	58.0–78.8	11.0±2.4	6.3–17.3
Lake Almazne	4	2	86.5±6.4	82.0–91.0	21.5±4.7	18.2–24.8
	Total	21	69.4±7.7	58.0–91.0	11.7±4.3	5.0–24.8

expected heterozygosity under mutation-drift equilibrium was determined with a one-tailed Wilcoxon signed rank test (Luikart *et al.*, 1998).

2.2 Parasite collection and identification

All fish were dissected within three days of sampling to ensure maximum parasite recovery (Kvach *et al.*, 2016). Prior to dissection, each fish was measured for standard length (SL) and total length (TL) to the nearest 1 mm (Tabs. 1 and 2). The fins, skin, gills, muscles and internal organs were then examined for presence of parasites. Unicellular parasites were studied alive using light microscopy; monogeneans, myxozoans and mites were preserved in Glycerine-ammonium-picrate (GAP) and prepared as semi-permanent slides (Malmberg, 1970); and digeneans, cestodes and nematodes preserved in hot 4% formaldehyde and stained with iron acetic carmine, dehydrated in ethanol of increasing concentration and mounted in Canada balsam as permanent slides (Georgiev *et al.*, 1986; Cribb and Bray, 2010). Larval digeneans, which could not be determined by morphology, were identified using molecular methods following the procedure described in Georgieva *et al.* (2013). Briefly, the

DNA was extracted using the Invisorb® Spin Forensic Kit (STRATEC Molecular, Germany) following standard protocol. We used one fragment of nuclear ribosomal DNA (ITS1 rDNA) and one fragment of mitochondrial DNA (COI) were used for molecular determination, with the partial ITS1 rDNA gene amplified using primers BD1 (GTCGTAACAAGGT-TTCCGTA) (Luton *et al.*, 1992) and 4S (TCTAGATGCGTTC-GAARTGTCGATG) (Bowles *et al.*, 1993), Plat-diploCOX1F (CGTTTAAATTATACGGATCC) and Plat-diploCOX1R (AGCATAGTAATMGCAGCAGC) (Moszczynska *et al.*, 2009). Annealing temperatures were 57 °C and 50 °C for ITS1 and COI, respectively. PCR products were sequenced commercially at Eurofins Genomics Germany GmbH. All sequences were checked and aligned using Geneious v.9.0.5. software (<http://www.geneious.com>) and the newly generated sequences were compared with the NCBI database using BLASTn to assess sequence similarity. Glochidia and crustaceans were preserved in 4% formaldehyde and identified under light microscopy using Olympus SZX7 and SZX10 stereomicroscopes (Olympus Optical co., Hamburg, Germany). All parasites were identified to species level or to the lowest taxa when this was not possible.

Table 3. Total (Total NA) number of alleles per six microsatellite loci, Allelic Richness corrected for sample size (AR), Shannon Information index (I), expected heterozygosity (He), unbiased expected heterozygosity (UHe), observed heterozygosity (Ho), fixation index (F), percentage of polymorphic loci (P) and deviation from Hardy-Weinberg equilibrium (HWE) for different Ukrainian pumpkinseed populations. Bottleneck indicates the probability of mutation-drift equilibrium evaluated by Wilcoxon test in the program Bottleneck. Significance level = * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$.

Sampling locality	N fish	Total NA	AR	I	He	UHe	Ho	F	HWE	Bottleneck
Lake Kartal	17	23	3.79	1.02	0.58	0.60	0.75	-0.28	<0.001	0.039
Sukhyi Lyman	16	26	4.33	1.19	0.64	0.66	0.61	0.02	0.054	0.039
Khadzhibey Lyman	20	35	5.38	1.25	0.64	0.66	0.67	-0.03	0.696	0.719
Lower Dnieper	21	28	4.39	1.05	0.55	0.56	0.53	0.04	0.179	0.500
Lake Almazne	21	18	2.92	0.74	0.42	0.43	0.49	-0.17	0.650	0.219

2.3 Community description and statistical analyses

The student's *t*-test was used to assess differences in fish size between different localities following the Shapiro–Wilks normality test. Indices of prevalence (P, %), intensity range (IR), mean intensity (MI) and mean abundance (A) were calculated for each parasite species (Bush *et al.*, 1997). The ‘importance’ of different parasite taxa was assessed using the adapted core-/satellite concept based on abundance, where values >2 = core species, $0.6–2$ = secondary species, $0.2–0.6$ = satellite species and <0.2 = rare species (Zander *et al.*, 2000). The ‘infracommunity’ is used in accordance to Bush *et al.* (1997) as a parasite community in a single host individual. A parasite's tendency to join the infracommunity was evaluated according to the infracommunity index (ICI; Zander, 2004), according to the formula:

$$ICI = M_{ij} / (N_j + I_j)$$

where M_{ij} is the ratio (number) of multiple infected hosts j with parasite i and other parasites, N_j is the ratio (number) of infected hosts j , and I_j is the mean number of parasite species in host j (= mean infracommunity). ICI levels >0.30 indicate a high tendency to join the infracommunity (Zander, 2004). Pumpkinseed parasite communities in different localities were compared by qualitative similarity (%), calculated using the index of Czekanowski–Sørensen (ICS) (Sørensen, 1948).

Generalised linear models (GLM) were used to compare parasite abundance (negative binomial distribution of errors) and infracommunity species richness (poisson distribution of errors) between the five sites. As the number of parasites and their species are expected to increase with fish age and size, both these parameters were considered as covariates in the models. As age and size (SL) were strongly correlated with each other (Pearson correlation moment = 0.88), we included size only as the covariate as models using size had a lower Akaike information criterion (AIC) than those using age (species richness models: AIC = 205.53 and 209.35; abundance models: AIC = 412.71 and 414.23 for size and age, respectively). The Tukey HSD approach was used to control for type II errors in multiple post-hoc pairwise comparisons in all models (using functions *glht* and *mcp* from the *multcomp*

package; Hothorn *et al.*, 2008). All statistical analyses were undertaken using R v.4.1.1 (R Core Team, 2015).

3 Results

3.1 Characterisation of fish host populations

Three localities (lakes Almazne and Kartal, lower Dnieper irrigation canal) were confirmed as freshwater and two as having brackish-water, with Sukhyi Lyman having a salinity of 2.2‰ and Khadzhibey Lyman 5.0‰.

Fish size (SL) differed significantly between all sampling sites ($p \leq 0.05$), with the largest fish found in the two brackish-water localities (Sukhyi and Khadzhibey Lyman; Tab. 1). While all populations comprised fish of 1–5-years, the overall age structure differed between localities (Tab. 2).

In all pumpkinseed populations, the six microsatellite loci amplified proved to be polymorphic, with allelic richness ranging from two to 19 alleles per locus. The population from the lower Dnieper irrigation canal was characterised by a higher *F*-index than the other populations, indicating the possible occurrence of inbreeding (Tab. 3). The range of AR was 2.92–5.38 (rarefaction estimate for the lowest sample size $N = 16$). While the majority of populations were in HWE, the Lake Kartal population showed a significant deviation ($p < 0.001$), attributable to an excess of heterozygotes (Tab. 3). This population is likely to represent recent founder effect, supported by the results of Bottleneck tests, where two populations showed a significant heterozygosity excess (Sukhyi Lyman and Lake Kartal), indicating recent decrease in effective population size (Tab. 3). DAPC and F_{ST} (Tab. 4) confirmed that all five Ukrainian populations formed highly structured clusters, in European view similar to each other, with Kartal being the most distanced (Fig. 2A). Aside from the Kartal population, Ukrainian populations are most closely associated with populations from the lower Danube (Bulgaria) and middle Danube (Czech Republic) basins (Fig. 2B).

3.2 Parasite community

A total of 15 parasite species were recorded in the five Ukrainian pumpkinseed populations (Tab. 5), including two microparasites (a ciliate and a myxozoan), three monogeneans,

Table 4. Population pairwise *F*_{st} values for five Ukrainian pumpkinseed populations.

	Sukhyi Lyman	Khadzhibey Lyman	Lower Dnieper	Lake Almazne
Lake Kartal	0.18208	0.11426	0.23570	0.22794
Sukhyi Lyman		0.07790	0.12787	0.21248
Khadzhibey Lyman			0.08229	0.13847
Lower Dnieper				0.21871

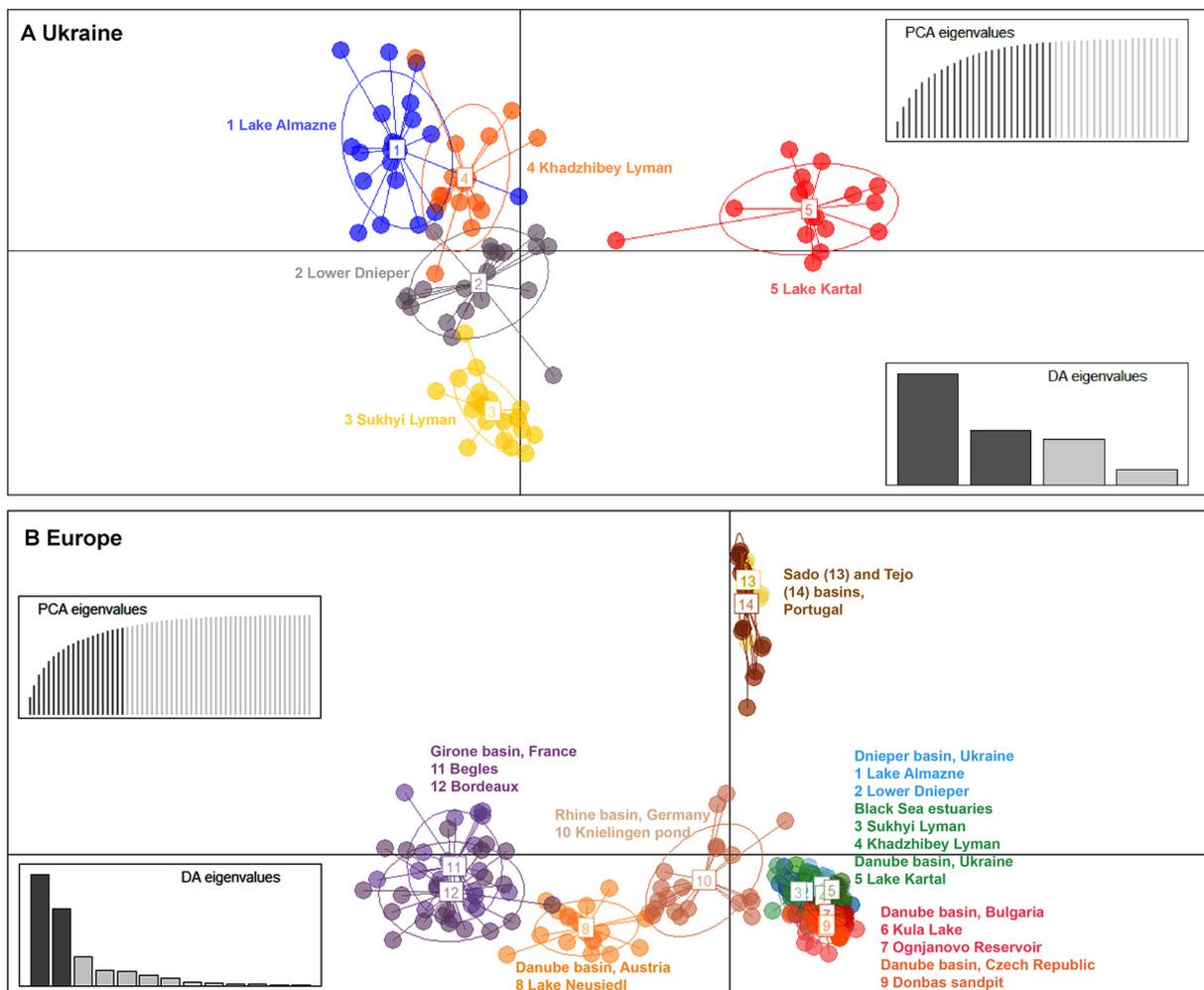


Fig. 2. Scatterplot of discriminant analysis of principal components (DAPC) of population structure of (A) five Ukrainian populations, and (B) European populations, including river basins in Ukraine, basins in the lower and middle Danube; the Rhine; Gironne and the Rivers Tejo & Sado. The axes represent the first two linear discriminants (LD1 and LD2) according to the discriminant analysis functions (DA) eigenvalues. Each dot represents an individual, and each colored circle represents a group identified by DAPC analysis. Legend: 1=Lake Kartal, Lower Danube; 2=Sukhyi Lyman; 3=Khadzhibey Lyman; 4=Lower Dnieper basin; 5=Lake Almazne, Dnieper basin, Ukraine; 6=Kula and 7=Ognyanovo Reservoirs, Danube basin, Bulgaria; 8=Lake Neusiedl, Danube basin, Austria; 9=Donbas sandpit, Morava/Danube basin, Czech Republic; 10=Kniefingen pond, Rhine basin, Germany; 11=Bègles Plage; 12=Bordeaux Lac, Gironne basin, France; 13=Landeira Reservoir, Sado River basin and 14=Coruche Reservoir, Tejo River basin, Portugal.

Table 5. Parasite infection parameters for different Ukrainian pumpkinseed populations. P= prevalence index (%), MI = mean intensity \pm sd, IR = intensity range, A = mean abundance. met – metacercaria, gl – glochidia.

Parasite species		Lake Kartal	Sukhyi Lyman	Khadzhibey Lyman	Lower Dnieper	Lake Almazne
CILIATA						
	P	5.3	25.0	30.0	9.5	
<i>Trichodina</i> spp.	MI	10.0	5.5 \pm 5.2	1.5 \pm 0.8	5.5 \pm 6.4	
fins	IR	10–10	1–10	1–3	1–10	
	A	0.5	1.4	0.5	0.5	
MYXOZOA						
	P	10.5		10.0		
<i>Myxobolus dechtiari</i>	MI \pm sd	50.5 \pm 70.0		15.5 \pm 20.5		
gills	IR	1–100		1–30		
	A	5.3		1.6		
MONOGENEA						
	P			5.0		
<i>Gyrodactylus sprostonae</i>	MI \pm sd			3.0		
gills	IR			3		
	A			0.2		
	P	15.8			100.0	
<i>Onchocleidus dispar</i>	MI \pm sd	2.3 \pm 0.6			39.9 \pm 20.9	
gills	IR	2–3			5–77	
	A	0.4			39.9	
	P, %	21.1				
<i>Onchocleidus similis</i>	MI \pm sd	9.5 \pm 11.0				
gills	IR	1–24				
	A	2.0				
DIGENEA						
	P, %		12.5			4.8
<i>Bucephalus polymorphus</i> met	MI \pm sd		1.0 \pm 0.0			1.0
fins	IR		1			1
	A		0.1			0.05
	P, %	10.5				23.8
<i>Diplostomum pseudospathaceum</i> met	MI \pm sd	1.5 \pm 0.7				2.6 \pm 1.9
eyes	IR	1–2				1–6
	A	0.2				0.6
	P, %		6.3			33.3
<i>Tylodelphys clavata</i> met	MI \pm sd		2.0			2.7 \pm 2.1
eyes	IR		2			1–7
	A		0.1			0.9
	P, %		6.3			
<i>Posthodiplostomum braevicaudatum</i>	MI \pm sd		2.0			
met eyes	IR		2			
	A		0.1			
NEMATODA						
	P, %	5.3	18.8			
<i>Raphidascaris acus</i> L3	MI \pm sd	2.0	1.0 \pm 0.0			
mesentery	IR	2	1			
	A	0.1	0.2			
	P, %	5.3				
<i>Spiroxys contortus</i> L3	MI \pm sd	1.0				
mesentery	IR	1				
	A	0.1				
CRUSTACEA						
	P, %				9.5	
<i>Neoergasilus japonicus</i>	MI \pm sd				2.0 \pm 1.4	
gills	IR				1–3	
	A				0.2	
	P, %				4.8	

Table 5. (continued).

Parasite species		Lake Kartal	Sukhyi Lyman	Khadzhibey Lyman	Lower Dnieper	Lake Almazne
<i>Lernaea cyprinacea</i> fins	MI±sd				1.0	
	IR				1	
	A				0.05	
ARACHNIDA						
<i>Porohalacarus hydrachnoides</i> gills	P, %		12.5			
	MI±sd		1.5±0.7			
	IR		1–2			
BIVALVIA	A		0.2			
	P, %	5.3				
	MI±sd	1.0				
<i>Sinanodonta woodiana</i> gl	IR	1				
	A	0.1				
	Parasite richness	8	6	3	4	3

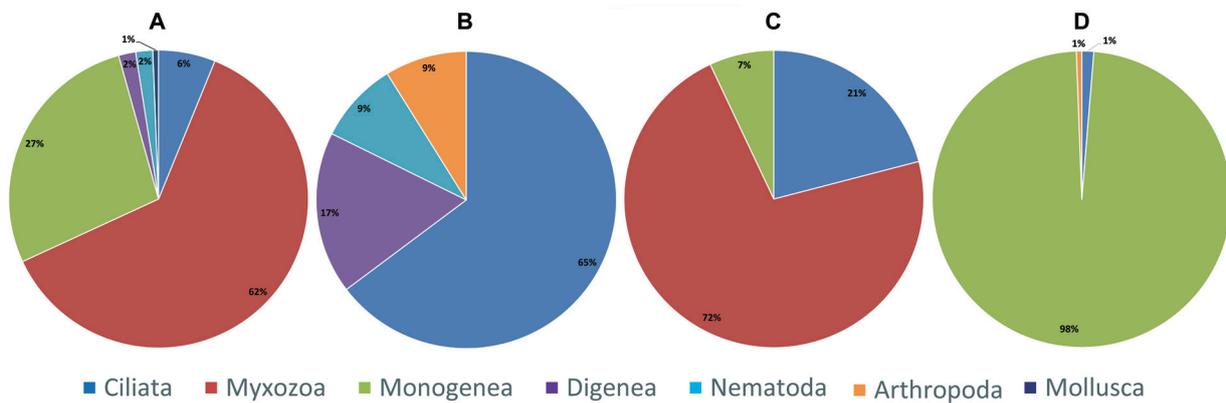


Fig. 3. Pumpkinseed parasite species composition at four Ukrainian localities. A = Lake Kartal, B = Sukhyi Lyman, C = Khadzhibey Lyman, D = Lower Dnieper.

four digeneans, two nematodes and crustaceans, one mite and one bivalve (Fig. 3). Highest parasite richness was recorded in Lake Kartal (eight spp.) and Sukhyi Lyman (six spp.; Tab. 5), while lowest richness was observed in the lower Dnieper (four spp.), Khadzhibey Lyman and Lake Almazne (three spp.).

The parasite communities included representatives of native European, non-native North-American and non-native Asian parasite species. In Lake Almazne, the parasite fauna was solely composed of native European parasites, while the other localities had communities comprised of both native and non-native (co-introduced) parasites. Three North-American species were recorded, the monogeneans *O. similis* and *O. dispar* and the myxozoan *Myxobolus dechtiari*, which together represented 92% of all parasite species found (Tab. 4). The two non-native Asian species recorded, the copepod *Neoergasilus japonicus* and the bivalve *Sinanodonta woodiana*, occurred only rarely.

Only two parasites were characterised as core species, *i.e.* *M. dechtiari* in Lake Kartal and *O. dispar* in the lower Dnieper. Five parasite taxa were classed as secondary species, *i.e.* *O. similis* in Lake Kartal, *Trichodina* spp. in Sukhyi Lyman, *M. dechtiari* in Khadzhibey Lyman and the eye flukes *Diplostomum pseudospathaceum* and *T. clavata* in Lake

Almazne, while two taxa were registered as satellite species, *i.e.* *Trichodina* spp. in Lake Kartal, Kadzhibey Lyman and the lower Dnieper, and *O. dispar* in Lake Kartal. All other species were classed as rare (Tab. 5).

Parasite community similarity between the five localities was relatively low, with ICS values of <50% in all cases (Tab. 6). The Lake Almazne community differed most from that at the other localities, having no parasites in common with Khadzhibey Lyman and the lower Dnieper. In just three cases were similarity >30%, *i.e.* between Sukhyi Lyman and Lake Almazne (44.4%), Khadzhibey Lyman and Lake Kartal (36.4%) and the lower Dnieper and Lake Kartal (33.3%). In all other cases, similarity levels were <30%.

Parasite fauna species composition differed between all sites (Fig. 3). In terms of parasite abundance, myxozoans dominated in Lake Kartal and Khadzhibey Lyman, followed by monogeneans in Lake Kartal and ciliates in Khadzhibey. At Sukhyi Lyman, ciliates dominated, followed by larval trematodes (metacercariae). In the lower Dnieper, monogeneans represented 97% of all parasites recorded, while the parasite fauna at Lake Almazne (not illustrated in Fig. 3) was solely represented by metacercariae (*Bucephalus polymorphus*, *D. pseudospathaceum* and *T. clavata*), all other taxa

Table 6. Czekanowski-Sørensen index matrix for parasite communities of Ukrainian pumpkinseed populations.

	Lake Kartal	Sukhyi Lyman	Khadzhibey Lyman	Lower Dnieper	Lake Almazne
Lake Kartal	100.0				
Sukhyi Lyman	28.6	100.0			
Khadzhibey Lyman	36.4	22.2	100.0		
Lower Dnieper	33.3	20.0	28.6	100.0	
Lake Almazne	18.2	44.4	0.0	0.0	100.0

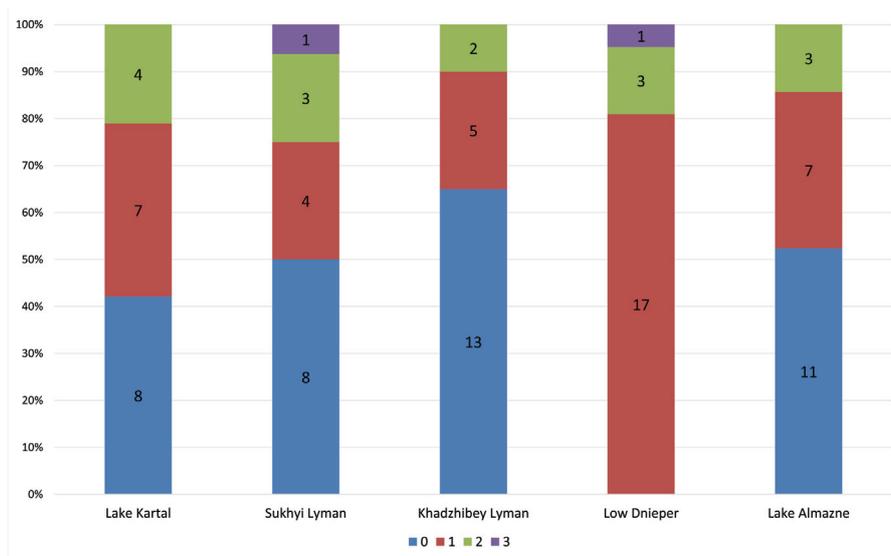


Fig. 4. Pumpkinseed (*Lepomis gibbosus*) parasite infracommunity richness in different Ukrainian water bodies.

being absent. Fish size was not a significant predictor of parasite abundance and was thus removed from the final model (likelihood ratio test, $df=1,6$, $\chi^2=1.77$, $P=0.183$; AIC of model with and without fish size=412.7 and 412.4), which revealed significant differences between the sites (GLM, $df=4, 87$, $P < 0.001$; for details see Supplementary Table S2). Parasite abundance in fish from the lower Dnieper was significantly higher than that at all other sites (GLM, post-hoc comparisons, all $P < 0.001$), with the abundance in fish from Khadzhibey being significantly lower than all other sites except Sukhyi Lyman (GLM, post-hoc comparisons, $P=0.006$, <0.001 , 0.001 and 0.074 for Almazne, Dnieper, Kartal and Sukhyi Lyman, respectively).

Fish free of parasites were recorded at all localities except the lower Dnieper. Parasite infracommunities comprised zero to two species at Khadzhibey Lyman, Lake Almazne and Lake Kartal, and zero to three species at Sukhyi Lyman and the lower Dnieper (Fig. 4). *Trichodina* sp. at Khadzhibey and Sukhyi Lyman showed a tendency to join the infracommunity, along with *O. dispar* in the lower Dnieper and *D. pseudospathaceum* and *T. clavata* in Lake Almazne (Tab. 7). Fish size was a significant predictor of parasite infracommunity richness (likelihood ratio test, $df=1,6$, $\chi^2=4.91$, $P=0.027$; AIC of model with and without fish size=205.5 and 208.4) and, as such, remained in the final model which determined significant differences in parasite richness between sites (GLM, $df=4,86$, $P=0.002$).

Pumpkinseed from Sukhyi Lyman displayed significantly higher infracommunity species richness than Almazne and Khadzhibey, but not than Kartal and the lower Dnieper (GLM, post-hoc comparisons, $P=0.033$, 0.014 , 0.063 and 0.166 for Almazne, Khadzhibey, Kartal and lower Dnieper, respectively).

4 Discussion

Microsatellite analysis indicated that all pumpkinseed populations in Ukraine are highly structured, but genetically similar within the European comparison, potentially originating from the River Danube. This is in accordance with the results of Slynko *et al.* (2014), who suggested a single introduction source using mtDNA analysis. Based on such results, the lower Danube would appear to be the most likely source of introduction owing to its accessibility and distance. Pumpkinseed were first reported in the lower Danube basin along the Romanian stretch in 1918 (Cărăușu, 1952). Further reports from the Dniester delta and Gulf of Odessa in the 1950s (Zambriborshch and Shumilo, 1953; Vinogradov, 1960) suggest that the fish were introduced from the lower Danube, though actual historical data are lacking. Despite the pumpkinseeds relatively recent occurrence in the Dnieper and its adjacent lakes (e.g. Lake Almazne in Kyiv), our results suggest that they may have been sourced from other

Table 7. Infracommunity index for taxa in the parasite communities of Ukrainian pumpkinseed populations. Numbers in bold indicate a strong tendency to join the infracommunity.

Parasite species	Lake Kartal	Sukhyi Lyman	Khadzhibey Lyman	Lower Dnieper	Lake Almazne
<i>Trichodina</i> spp.	0.07	0.31	0.67	0.08	
<i>Myxobolus dechtiari</i>	0.13		0.22		
<i>Gyrodactylus sprostonae</i>			0.11		
<i>Onchocleidus dispar</i>	0.20			0.81	
<i>Onchocleidus similis</i>	0.27				
<i>Bucephalus polymorphus</i>		0.15			0.08
<i>Diplostomum pseudospathaceum</i>	0.13				0.38
<i>Tylodelphys clavata</i>		0.08			0.54
<i>Posthodiplostomum braevicaudatum</i>		0.08			
<i>Raphidascaris acus</i>	0.07	0.23			
<i>Spiroxys contortus</i>	0.07				
<i>Neoergasilus japonicus</i>				0.08	
<i>Lernaea cyprinacea</i>				0.04	
<i>Porohalacarus hydrachnoides</i>		0.15			
<i>Sinanodonta woodiana</i>	0.07				

Ukrainian populations, and ultimately the River Danube (see Fig. 2B).

Low intra-population genetic variability in pumpkinseed from Lake Kartal (Danube basin) indicates that this population has probably undergone a bottleneck (following the founder effect) at some point. Likewise, the Lake Kartal cluster was most isolated in DAPC plots (Fig. 2A), with heterozygote excess and deviation from HWE (Tab. 3) and mutation-drift equilibrium also suggestive of a population bottleneck, respectively founder effect, representing typical pattern in biological invasions. Lake Kartal, inhabited by the oldest pumpkinseed population in this study, is located in the Danube delta and connected by wetlands and artificial canals with both the River Danube and the Yalpuh-Kuhurlui lake system, the Ukrainian locality where pumpkinseed were first documented (Berg, 1949). Indeed, it is possible that the pumpkinseed population at Lake Kartal is the same age as that at Lake Yalpuh. Based on historical data and genetic similarity of pumpkinseed populations in Ukraine related to the Danube River (Fig. 2B), we assumed that Lake Kartal as the oldest population represents a source of other Ukrainian populations. Nevertheless, our data indicate that this is not the case and that other areas may have been colonized from other sources.

Compared to Lake Kartal, the high intra-population variability at Khadzhibey Lyman suggests that this population may have been formed by repeated introductions from several sources, or that the population began with a large number of individuals. Khadzhibey Lyman is an estuary of the Malyi Kuyalnyk river, an isolated river drainage artificially separated from the Black Sea. The estuary is actively used for fisheries purposes and is regular stocked with freshwater fish (mainly cyprinids) (Shekk, 2015). As at other localities, the source of pumpkinseed introduction is unknown, but it may have been accidentally introduced as a ‘hitchhiker’ with commercially important fishes. Thus, both possibilities, *i.e.* repeated unintentional introductions with commercial fish stock and introduction of a large number of juveniles, may be responsible

for the high allelic richness and heterozygosity observed at Khadzhibey Lyman.

Pumpkinseed parasite species richness in Ukraine was strongly dependent on fish length and/or age, while parasite abundance was primarily associated with locality and type of water body. While fish from brackish waters, for example, showed faster growth (measured as size range in each age cohort), three-year-old fish from the lower Dnieper were characterised by a very low SL, this cohort being almost the same size as two-year-old fish (see Tab. 2). The lower Dnieper is an artificial irrigation canal with managed water levels, and each year the water level is allowed to drop considerably, meaning that fish only tend to survive at high densities close to the pumping-stations, limiting their growth in the summer season. This lower Dnieper population was also characterised by an absence of large-sized individuals, which cannot survive in such conditions (Demchenko, unpublished data).

This study provides the first comprehensive data on non-native pumpkinseed parasites in both fresh and brackish Ukrainian waters. The pumpkinseed were infected with 15 taxa of both protozoan and metazoan parasites, with the vast majority represented by species native to North America, *i.e.* co-introduced with the pumpkinseed into Europe. The first Ukrainian studies on pumpkinseed parasites were conducted in the 1960s on the Danube delta, when Kulakovskaya and Koval (1973) found six species, of which just one, *O. similis*, was co-introduced with the fish host from North America. A few years later, Pashkevichute (1971) found two American parasites, *O. dispar* and *O. similis*, in the Ukrainian part of the lower Danube, and a further American species, the monogenean *G. avalonia*, has been registered in the Danube delta just recently, possibly having switched from sympatric stickleback hosts (Kvach *et al.*, 2018). Though the diversity of North American monogeneans infecting pumpkinseed is higher in other European populations, reaching up to seven species on the River Durance in France (Havlátová *et al.*, 2015), populations inhabiting the lower Danube basin and those derived from the

River Danube, e.g. the rivers Struma (see Ondračková *et al.*, 2021) or Dnieper (Rubtsova, 2015; present study), appear to be limited to just two *Onchocleidus* species, i.e. *O. similis* and *O. dispar* (Ondračková *et al.*, 2011; 2021).

In addition to monogeneans, a North American myxozoan species, *M. dechtiari*, was also found in fish from Khadzhibey Lyman and Lake Kartal. This parasite has recently been confirmed in Hungary and Portugal as a species co-introduced with pumpkinseed to Europe from its native North American range (Goswami *et al.*, 2021). Representatives of the genus *Myxobolus* require oligochaete and vertebrate hosts to complete their life cycles (Lom and Dyková, 2006; Yokoyama *et al.*, 2012), and Khadzhibey Lyman is an organically polluted water body where oligochaetes are a common component of the benthic fauna (Zaitsev *et al.*, 2006).

While most of the parasite species found in Ukrainian pumpkinseed were represented predominantly by common native species acquired in their new range along the lower Danube basin (see Kulakovskaya and Koval, 1973; Kvach *et al.*, 2020), most of these were only found accidentally, with low prevalence and abundance (Tab. 4), with just two native eye flukes, *D. pseudospathaceum* and *T. clavate*, found more commonly. Both species are generalists infecting a wide range of fish intermediate hosts in Europe (WoRMS, 2022a,b). Among the more prevalent parasites, trichodinid ciliates were found at four of the five localities, confirming that pumpkinseed is highly susceptible to this group of parasites (Yurshynets *et al.*, 2019). Unfortunately, these parasites could not be determined to species level, hence we cannot draw any conclusions as to whether they were co-introduced or acquired. In addition to native species, we also recorded non-native Asian parasites parasitising this North American fish, though always with low infection parameters. Occurrence of the copepod *N. japonicus* represents the first report of this parasite in Ukraine, and confirms the high susceptibility of pumpkinseed to this copepod, as recently reported for other European regions by Ondračková *et al.* (2019b, 2021) and Alekseev *et al.* (2021), whose results suggest that abundant pumpkinseed populations may potentially support this copepod species in invaded areas, such as the lower Dnieper.

Diversity and composition of pumpkinseed parasite communities varied greatly between localities in Ukraine, despite their relatively high degree of genetic relatedness in comparison to other non-native European populations, reflecting the generally low parasite community similarity between pumpkinseed populations. In species introduced into a new range, parasite community richness often reflects the age of the host population (Gendron *et al.*, 2012; Ondračková *et al.*, 2015). It is, therefore, no surprise that the richest parasite fauna among Ukrainian sites is found at Lake Kartal, the oldest pumpkinseed population in this study (Tab. 5; Fig. 3). A relatively high diversity was also recorded at Sukhyi Lyman, a landscape formation made up of the estuary of two small rivers, Akkarzhanka in the south and Dalnyk to the north. In the 1950s, a navigation canal was built in the southern part of the estuary, transforming the system into three parts, i.e. a marine section (the sea bay), a central brackish section and a northern oligohaline section (Murkalov *et al.*, 2018). Both the source and age of the pumpkinseed population in this water body is unknown; however, the species was first reported at

Sukhyi Lyman in 2001, having been reported along the adjacent sea coast in 1953 (Vinogradov, 1960; Vinogradov and Khutornoy, 2013). It is probable, therefore, that the species penetrated into the estuary in the 1950s, which may explain the relatively high parasite diversity in this population. On the other hand, the pumpkinseed is a new component of the fish fauna at Khadzhibey Lyman, having first been recorded in 2016 (Snigirov and Finogenov, 2017), possibly as an unintentional introduction with commercial fish stocks. Parasite species richness at Khadzhibey Lyman was much lower than that at Lake Kartal and Sukhyi Lyman, with just three species. Of these, only North American myxozoans were relatively numerous, the other two species only occurring sporadically. The accidental occurrence of *Gyrodactylus sprostonae*, which is considered by some authors to be non-native to Europe (Hanel *et al.*, 2011), is more usually a parasite of carps, and their presence is probably the result of intensive commercial carp stocking in the water body. The generally low parasite abundance at Khadzhibey Lyman may be explained by the young age of the pumpkinseed population, or alternatively may be a general trend affecting parasite supracommunities caused by the unstable salinity regime (Kvach, 2004, 2010).

In the Dnieper basin, pumpkinseed have been recorded since the 1970s (Yelesovich and Kozlova, 1974); nevertheless, its occurrence near Kyiv was only confirmed in 2011 (Tsyba, 2011; Afanasyev *et al.*, 2017). The pumpkinseed population in Lake Almazne, a recently established lake in Kyiv, was characterised by the lowest genetic variability and lowest parasite diversity recorded, being represented by just three widely distributed digeneans. Parasite diversity in fish from the irrigation canal of the lower Dnieper was also very low, with the North American monogenean *O. dispar* forming 98% of the overall parasite fauna (Fig. 3). The River Dnieper forms part of the “central invasion corridor” (Panov *et al.*, 2009), and is separated from the “southern invasion corridor” by the brackish waters of the Black Sea. The introduction route from the Danube delta to the Dnieper and Dniester along the Black Sea coast has been discussed previously (Zambriborshch and Shumilo, 1953; Afanasyev *et al.*, 2017). In these cases, North American monogeneans (*O. dispar* in this case), which are specific for centrarchids, would have to survive spreading upstream through salt/brackish waters. However, our data from brackish localities (Sukhyi and Khadzhibey Lyman) indicated that these dactylogyrid parasites exhibit low tolerance to salinity, suggesting that the finding of *O. dispar* in the lower Dnieper may be the result of artificial introduction from another freshwater source. This may be one case where the presence of specific parasites represents a useful tool for determining the host source population (Ondračková *et al.*, 2021).

5 Conclusions

This study provides the first comprehensive insight into the parasite community structure and population genetics of non-native pumpkinseed in Ukraine. Despite having been introduced into the country over a hundred years ago, the spread of pumpkinseed has become especially noticeable in recent years.

Our data show that all Ukrainian populations are highly genetically structured and, in accordance with our hypothesis, are most closely related to populations in the Danube basin. However, the oldest Ukrainian pumpkinseed population, situated in Lake Kartal in the Danube delta, does not appear to be the source from which the other Ukrainian populations were derived. Further investigations will be needed to reveal the species' full invasion history in the Black Sea drainage. This will also help identify the main introduction routes for non-native fish and, subsequently, support management efforts to prevent further species invasions.

Parasite community data showed that the pumpkinseed has co-introduced several parasite species into its new Ukrainian range. Nevertheless, species richness and abundance native pumpkinseed parasites were much lower than in other European populations (e.g. Ondračková *et al.*, 2021), particularly in those regions where the species was first introduced in the 19th century such as France and Germany. These parasites are highly specific and apparently do not represent a significant threat to local fish species. Pumpkinseed have also been determined as hosts for a range of local parasite species, though generally at low prevalence and abundance. Interestingly, this non-native fish species appears to be highly susceptible to the non-native Asian crustacean *N. japonicus*, a recent invader in Europe and North America (Marshall *et al.*, 2019; Ondračková *et al.*, 2019b; Alekseev *et al.*, 2021; Kvach *et al.*, 2021). Further spread of pumpkinseed along the Dnieper River, where *N. japonicus* is known to occur, may subsequently support the spread of the parasite as a “hitchhiker”. Our results, therefore, highlight the importance of non-native species as not only hosts of co-introduced parasites, potentially threatening native fish species, but also as hosts or vectors of invasive parasites introduced from other regions.

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Supplementary Material

Figure S1. Percentage of parasites by taxon according to fish age and sampling site.

Table S1. Standard length (SL, mm) and weight (W, g) of pumpkinseed by age group and sex.

Table S2. Table of post-hoc pairwise comparisons of infracommunity species richness and abundance of parasites between five localities.

The Supplementary Material is available at <https://www.kmae-journal.org/10.1051/kmae/2022023/olm>.

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