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2 1 **The killer shrimp *Dikerogammarus villosus* invading the Alpine Lakes: A single main source**  
3 2 **but independent founder events with an overall loss of genetic diversity**  
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18 **Abstract**

- 19 1. The effects of biological invasions are generally more detrimental in isolated ecosystems  
20 than in the interconnected ones and freshwater lakes appear to be particularly fragile. The  
21 alien freshwater amphipod *Dikerogammarus villosus*, also known as the killer shrimp, is  
22 recognized as a major conservation problem. It has colonized most of the European main  
23 inland water bodies, including at least twelve lakes in the Alps – an area of high  
24 conservational priority and, at the same time, heavily affected by anthropogenic changes.  
25 Particularly, overland translocations of boats among touristic centres may have contributed  
26 to colonization of the Alpine Lakes.
- 27 2. We aimed to answer the following questions: Which of the two genetically differentiated  
28 populations invading continental lowland Europe has contributed to colonisation of the  
29 Alpine Lakes? Is it possible to detect independent founder events in different regions of the  
30 Alps and/or secondary spread among Alpine Lakes? Have the lacustrine populations  
31 suffered any reduction of genetic variation during colonization? Twelve populations from  
32 the Alpine Lakes were genotyped for a portion of mtDNA (COI and 16S) and for seven  
33 nuclear microsatellite loci. A wide range of methods was used to analyse the data, including  
34 haplotype network, Bayesian clustering and demography tests.
- 35 3. Our results show that the lowland western route (Danube-Rhine waterway) of the killer  
36 shrimp invasion was the source for introduction to the Alpine Lakes. Unlike invasive  
37 populations in the main lowland rivers, most of the Alpine populations suffered bottleneck  
38 and had significantly lower genetic diversity compared to their source population along the  
39 western route. We found clear patterns of genetic structure, revealing multiple independent  
40 lake colonisations followed by secondary spread between lakes. An overland transport of *D.*  
41 *villosus* with touristic boats is probably the major spreading vector to and among Alpine  
42 Lakes. Implementing safety programs such as the 'check, clean, and dry' procedures may  
43 stop or slow down spreading of *Dikerogammarus villosus* among the Alpine Lakes, and  
44 prevent additional long distance transport.

## 48 Introduction

49 Biological invasions are among the flagship symbols of globalization and a major threat to  
50 biodiversity at various scales (Simberloff *et al.*, 2013). Fast and uncontrolled spread of terrestrial  
51 and aquatic alien species may cause irreversible changes in local ecosystems. Alien invaders  
52 compete for shelters (Kinzler & Maier, 2006; van Kessel *et al.*, 2011) and food (Dick & Platvoet,  
53 2000; Kakareko *et al.*, 2013) with native biota, transmit new pathogens (Bącela-Spychalska *et al.*,  
54 2012; Peeler *et al.*, 2011; Poulin *et al.*, 2011) or even transform local habitats (Cuddington &  
55 Hastings, 2004; Rodríguez *et al.*, 2005; van Riel *et al.*, 2006).

56 Probability of successful establishment of an invasive population is, to some extent, associated with  
57 propagule pressure that combines the propagule size (i.e. the number of founding individuals) with  
58 the frequency of introduction events (Lockwood, Cassey & Blackburn, 2005; Von Holle &  
59 Simberloff, 2005). Small propagule pressure may result in genetic founder effect often causing  
60 negative consequences to genetic diversity, such as allelic loss and/or inbreeding (Deredec &  
61 Courchamp, 2007). Conversely, retention of genetic and phenotypic diversity associated with high  
62 propagule pressure may significantly increase further success of the founding population, especially  
63 if the new habitat is different from the source habitat (Dlugosch & Parker, 2008; Roman & Darling,  
64 2007). Populations of invasive species characterised by high level of genetic diversity may be less  
65 vulnerable to enemies (predators or diseases) (Zhu *et al.*, 2000) and respond faster and more  
66 efficiently to changing environmental conditions (Lavergne & Molofsky, 2007). There are now  
67 evidences that bottlenecks in invasive populations are not as common as thought before (Genton,  
68 Shykoff & Giraud, 2005; Kolbe *et al.*, 2004; Rewicz *et al.*, 2015a). High propagule pressure may  
69 originate either from massive single introduction or from multiple introductions from a single  
70 source (Forsman, 2014).

71 Inland waters are particularly vulnerable to biological invasions (Bij de Vaate *et al.*, 2002; Funnell  
72 *et al.*, 2009; Jazdzewski, 1980; Rewicz *et al.*, 2015a; Ricciardi & Rasmussen, 1998; Strayer, 2010).  
73 Various human activities favour the introduction or spread of alien species: (1) commercial  
74 shipping, through biofouling and ballast waters (Drake & Lodge, 2004), (2) recreational activities  
75 such as angling, boating, canoeing, diving, mainly through biofouling (Anderson *et al.*, 2014;  
76 Bącela-Spychalska *et al.*, 2013) and (3) intentional releases (Funnell *et al.*, 2009; Barbashova,  
77 Malavin & Kurashov, 2013). Introductions may result from overseas transport (Muirhead *et al.*,  
78 2015), from transport along waterways, notably through canals that connect previously isolated  
79 drainages (Bij de Vaate *et al.*, 2002; Jazdzewski, 1980), or even from overland transport (Anderson  
80 *et al.*, 2014; Bącela-Spychalska *et al.*, 2013).

1  
2 81 Inland lakes may be prone to invasion to a various extent. On one hand, some lakes, e.g. the  
3 82 Laurentian Great Lakes (LGL), are characterized by high commercial shipping allowing massive  
4 83 and recurrent introductions (Adebayo *et al.*, 2014; Holeck *et al.*, 2004). On the other hand, many  
5 84 lakes, especially those in the European Alps, may be at lower risk of alien species introduction due  
6 85 to their relative isolation from the main waterways. However, in many areas, lakes are  
7 86 geographically clustered. As a result, even if long distance introductions into a given lake are  
8 87 expected to be rare, once a given lake is colonized, the probability of secondary introduction to the  
9 88 neighbouring lakes is high. Even if such lakes are not connected by waterways, overland transport  
10 89 associated with recreational activities – such as angling, boating, canoeing or diving – creates such  
11 90 connexions ( Bączela-Spychalska, 2016; Bączela-Spychalska *et al.*, 2013; Johnson, Bossenbroek &  
12 91 Kraft, 2006). For example, small lakes in the vicinity of the LGL were shown to be susceptible to  
13 92 secondary spread of alien aquatic species by such overland transportation (Johnson, Bossenbroek &  
14 93 Kraft, 2006; Johnson, Ricciardi & Carlton, 2001; Kerfoot *et al.*, 2011). Small propagule size and  
15 94 bottlenecks associated with reduced genetic variation are expected in populations newly developed  
16 95 in such isolated minor lakes.

17  
18 96 The amphipod *Dikerogammarus villosus* (Sowinsky, 1894), aka the killer shrimp, is one of the most  
19 97 efficient invaders in aquatic ecosystems (DAISIE, 2009). Issued from the Ponto-Caspian area,  
20 98 where it lives in large rivers or deltas and limans, it has successfully colonized main lowland  
21 99 continental European waterways during the last thirty years. Large navigable waterways with  
22 100 intense commercial shipping traffic, connected through artificial channels, are recognized as  
23 101 primary colonization routes, often called “invasion highways”, for the killer shrimp in lowland  
24 102 continental Europe (Bij de Vaate *et al.*, 2002; Leuven *et al.*, 2009; Rewicz *et al.*, 2015a).

25 103 *Dikerogammarus villosus* inhabits stony substrates and is often found within zebra mussel beds. Its  
26 104 feeding regime is scavenger/predatory at adult stage, while juveniles show a more diversified diet  
27 105 including algae (reviewed in Rewicz *et al.*, 2014). Its high trophic level resulting from predatory  
28 106 behaviour (van Riel *et al.*, 2006) as well as wide tolerance to temperature and conductivity  
29 107 fluctuations (Bruijs *et al.*, 2001; Wijnhoven, van Riel & van der Velde, 2003) combined with early  
30 108 sexual maturity and high fecundity (Grabowski, Bączela & Konopacka, 2007a; Pöckl, 2007; Pöckl,  
31 109 2009) are favourable life-history traits in the initial phases of invasion. The presence of *D. villosus*  
32 110 induces negative effects on local communities, such as an increase of direct predation pressure on  
33 111 other invertebrates and fish spawn (Buric *et al.*, 2009; Platvoet *et al.*, 2009; van der Velde *et al.*,  
34 112 2009), competition for shelters (MacNeil *et al.*, 2011) and modification of entire food webs (van  
35 113 Riel *et al.*, 2006).

1  
2 114 Phylogeography and population genetics of *Dikerogammarus villosus* in its native and invaded  
3 115 ranges was studied by Wattier *et al.* (2007) and Rewicz *et al.* (2015a). Native populations of the  
4 116 Dnieper and the Danube deltas are genetically differentiated and provide sources for, respectively,  
5 117 eastern and western invasion routes of *D. villosus* invasion in continental lowland Europe (Fig. 1a).  
6 118 The eastern route is limited to the rivers or canals in Ukraine and in eastern and central Poland. The  
7 119 western route covers the rest of the vast colonized area in continental Europe, including the Danube  
8 120 with its tributaries, Rhine River, natural and artificial watercourses in France, Netherlands,  
9 121 Germany and western Poland (Fig. 1a). Neither loss of genetic diversity nor genetic differentiation  
10 122 were observed between native and invaded areas, even along the entire, ca. 4500 km long, western  
11 123 route (Wattier *et al.*, 2007; Rewicz *et al.*, 2015a).

12 124 In addition to rivers and canals, at least twelve lakes at low altitudes in European Alps (here after  
13 125 called ‘‘Alpine Lakes’’) have been invaded by *D. villosus* (Baćela-Spychalska *et al.*, 2013 and  
14 126 references cited herein, Fig. 1b, Table 1). These lakes vary in size and intensity of tourist activities.  
15 127 The hydrological connections between the lakes and to the ‘‘invasion highways’’ are also variable  
16 128 (Leuven *et al.*, 2009) (Fig. 1b, Table 1). The western invasion route partly encircles the Alps from  
17 129 the north and west and is directly connected to some Alpine Lakes, suggesting that the *D. villosus*  
18 130 populations in the Alpine Lakes may originate from this source. Yet, the connections are scattered,  
19 131 lowering the probability of a single massive introduction in this area. On the other side, a touristic  
20 132 boat traffic from distant locations is known to occur in Alpine Lakes. Some boats are transported  
21 133 overland from Northern and Central Europe, i.e. from countries along the eastern invasion route (de  
22 134 Ventura *et al.*, 2016). *Dikerogammarus villosus* endures the overland transport: Martens & Grabow  
23 135 (2008) reported that it can survive six days out of water within zebra mussels biofouling on a boat  
24 136 propeller. Baćela-Spychalska *et al.* (2013) showed that *D. villosus* can survive two days in between  
25 137 layers of scuba-diving suit. Such abilities may favour long-distance colonisation of the Alpine  
26 138 Lakes, where sailing and diving is a very common touristic activity. Therefore, presence of founder  
27 139 individuals from both the eastern and the western population of *D. villosus* are possible, opening the  
28 140 chance for independent founder events in different parts of the Alps. De Ventura *et al.* (2016)  
29 141 showed that boats are frequently transported overland between Swiss Alpine Lakes; some lakes  
30 142 (e.g. lakes Bienne, Murten and Neuchâtel) being additionally interconnected by small waterways.  
31 143 These features may result in secondary spread of *D. villosus* from lakes with already established  
32 144 populations.

33 145 The pattern of *D. villosus* invasion in Alpine Lakes is interesting to investigate because it may  
34 146 strongly influence the pattern of its genetic diversity. The killer shrimp populations maintained their  
35 147 genetic diversity along the highways of invasion (Rewicz *et al.*, 2015a). Yet, we cannot reliably

1  
2 148 predict if this diversity has been maintained after their introduction in the Alpine Lakes. Predictions  
3  
4 149 are ranging from low diversity in isolated lakes, associated with single introduction and strong  
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6 150 bottleneck, to high diversity in lakes well connected to invasion highways and colonized long time  
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8 151 ago with individuals arriving each year. In addition, it cannot be excluded that genetic diversity  
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10 152 (and consequently the invasive capacity) can increase in result of admixture between introductions  
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12 153 from multiple and previously isolated sources (here, the western and the eastern route) (Lombaert *et*  
13  
14 154 *al.*, 2011).

15  
16 155 In this study, populations of *Dikerogammarus villosus* from the twelve colonized Alpine Lakes  
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18 156 were genotyped for mtDNA (COI, 16S) and seven polymorphic nuclear microsatellite loci.  
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20 157 Molecular markers are known to be powerful tools in tracking invasion patterns and dynamics  
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22 158 (Ficetola, Bonin & Miaud, 2008). The markers used here were successfully applied in a large scale  
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24 159 study upon phylogeography and invasion dynamics of the killer shrimp in continental lowland  
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26 160 Europe (Rewicz *et al.*, 2015a). By revealing the population genetic structure in Alpine Lakes and  
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28 161 comparing it to results of this previous study, we addressed the following questions: (1) Is the  
29  
30 162 western route the only source for the populations established in the Alpine Lakes? 2) Is it possible  
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32 163 to detect cases of independent founder events in different areas of the Alps and cases of secondary  
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34 164 spreading between neighbouring lakes? (3) Have Alpine lacustrine populations suffered from a  
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36 165 reduction of genetic variation?

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## 166 167 **Materials and Methods**

### 168 *Sample collection and molecular marker data production*

169 *Dikerogammarus villosus* were collected from the littoral zone of twelve lakes in European Alps  
170 (Fig. 1b, Table 1) as described by Bączela-Spychalska *et al.* (2013) and conserved in 96% EtOH  
171 directly after sampling. Total DNA was extracted from 415 individuals with a standard phenol-  
172 chloroform method following Hillis, Moritz & Mable, (1996). Air-dried DNA pellets were eluted in  
173 100 µl of TE buffer, pH 8.00, stored at 4°C until amplification, and subsequently at -20°C long-  
174 term storage. Two mtDNA markers were amplified to a random subset of the initial 415 individuals  
175 used for microsatellite analyses, for a total of 128 specimens: 16S ribosomal RNA (16S rRNA; ca.  
176 320 bp fragment) with LR-J-GAM/LR-N-GAM primers (Müller, Schramm & Seitz, 2002) and  
177 reaction conditions following Grabowski *et al.* (2012) and cytochrome oxydase subunit 1 gene  
178 (COI; ca. 670 bp fragment) with LCOI490/HCO2198 primers (Folmer *et al.*, 1994) and reaction  
179 conditions following Hou, Fu & Li (2007). Sequences were obtained with BigDye sequencing

1  
2 180 protocol (Applied Biosystems 3730xl) by Macrogen Inc., Korea. The obtained sequences were  
3 181 edited, aligned and trimmed with CLUSTALW algorithm (Chenna *et al.*, 2003) using BIOEDIT©  
4 182 7.2.5, leading to 128 sequences of 16S (303 bp) and COI (654 bp) which were concatenated prior  
5 183 analyses. Haplotypes were retrieved using DNASP v5 both for individual markers and for the  
6 184 concatenated dataset (Librado & Rozas, 2009). Haplotypes for individual markers are presented in  
7 185 Table S1. One newly detected COI haplotype was deposited in GeneBank (KP814111). Other  
8 186 haplotypes were already identified after Rewicz *et al.* (2015a).

13  
14 187 Seven microsatellite loci (Msat) were used as nuclear co-dominant molecular markers: DikS, DikF  
15 188 (Wattier *et al.*, 2006), Dv11, Dv13, Dv17, Dv31, Dv33 (Rewicz *et al.*, 2015b). Altogether 415  
16 189 individuals were genotyped. The PCR conditions were described by Wattier *et al.* (2006) and by  
17 190 Rewicz *et al.* (2015b). It is to be noticed that DikF is not amplifying in the eastern source/route  
18 191 (Rewicz *et al.*, 2015b). Microsatellite alleles were visualized in 6.5% acrylamide, 25 cm long gels  
19 192 on a LICOR 4200 L automated sequencer and scored by eye. Reference individuals were included  
20 193 for inter-gel calibration. Absence of physical linkage disequilibrium was tested using the software  
21 194 GENEPOP (Rousset, 2008) and absence of recurrent genotyping errors and their possible sources  
22 195 e.g. short allele dominance (Wattier *et al.*, 1998) was checked with the software MICRO-  
23 196 CHECKER version 2.2.3. (van Oosterhout *et al.*, 2004).

24 197  
25 198 To compare the present data to the large-scale picture of *D. villosus* phylogeography in continental  
26 199 lowland Europe, 274 mtDNA sequences and microsatellite genotypes of 723 individuals analysed  
27 200 by Rewicz *et al.* (2015a) were also used in this paper.

#### 28 201 29 202 *Assessing the spatial distribution of diversity*

30 203 To visualize molecular divergence of mtDNA haplotypes and their spatial distribution, a Minimum  
31 204 Spanning Network was generated using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). Pairwise  
32 205 differentiation in haplotype and alleles frequencies was determined by two  $F_{ST}$  estimators:  $\Theta_{ST}$  with  
33 206 Tamura-Nei distance for mtDNA (Tamura & Nei, 1993) and  $\Theta$  for microsatellites (Weir &  
34 207 Cockerham, 1984), both implemented in ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010),  
35 208 statistical significance was measured using 10 000 permutations.

36 209 The population genetic structure was also analysed for Msat using individual-based Bayesian  
37 210 clustering method implemented in STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000). A  
38 211 first simulation was performed on a large dataset composed of populations from the Alpine Lakes,  
39 212 western route, eastern routes, and native area (Rewicz *et al.*, 2015a). This first simulation aimed to



1  
2 213 understand the source(s) of colonization of Alpine Lakes. A second simulation was restricted to the  
3 214 dataset including the 12 Alpine Lakes populations only (Table 1). This simulation aimed to  
4  
5 215 understand how these lakes clustered at the Msat level, completing the mtDNA analyses. Run for  
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7 216 each K value (1 to 8) was repeated 10 times. Each run included 750 000 iterations with a burn-in of  
8  
9 217 500 000 iterations. All simulations were performed using the admixture and correlated allele  
10  
11 218 frequencies models with no prior information. Selection of the most probable K value relied on the  
12  
13 219  $\Delta K$  method developed by Evanno, Regnaut & Goudet, (2005).

14 220 We also tested the hypothesis of a single introduction into one of the Alpine Lakes, followed by a  
15  
16 221 series of founder events according to a stepping-stone type of pattern of further expansion in other  
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18 222 lakes. Such a pattern would lead to increasing differentiation in allele frequencies relative to an  
19  
20 223 increasing the distance to the source. Based on the 12 lacustrine populations we therefore tested if  
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22 224 microsatellite differentiation increased positively with distance between sites (isolation-by-distance,  
23  
24 225 IBD). The geographic distances between lakes were estimated using Google Earth Pro7.1.5.1557.  
25  
26 226 The IBD was tested using Mantel's test between  $F_{ST} / (1 - F_{ST})$  and geographic distance as  
27  
28 227 recommended by Rousset (2008) for testing IBD in one-dimensional linear systems, with 100 000  
29  
30 228 permutations, using the GENEPOP on the Web 4.2 (Raymond & Rousset, 1995) and ISOLDE  
31  
32 229 software.

33 230 As the result of the above analyses, we defined two groups of lakes where secondary spreads may  
34  
35 231 be suspected i.e. the Neuchâtel-group: Bienne (40L), Murten (39L), Neuchâtel (38L) and the  
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37 232 Zurich-group: Greifen (43L), Zurich (42L), Zug (41L); see results for details). The coalescence-  
38  
39 233 based program MIGRATE-N 3.6.8 (Beerli, 2006; Beerli, 2009) was used to estimate the intensity  
40  
41 234 and preferential direction of migration between pairs of locations within each of these groups. The  
42  
43 235 MIGRATE-N estimates the mutation-scaled migration rate M ( $M=m/\mu$ , where m is the immigration  
44  
45 236 rate per generation and  $\mu$  is the mutation rate) and population size  $\Theta$  ( $4N_e\mu$ , where  $N_e$  is effective  
46  
47 237 population size). All seven microsatellites loci were used in the analysis. The parameters were  
48  
49 238 inferred with Bayesian paradigm using the Brownian mutation model. The mutation rate among loci  
50  
51 239 was scaled so that the average change of the mutation rate was 1.0 and the relative mutation rates  
52  
53 240 were estimated for all loci. Initial M and  $\Theta$  were calculated from  $F_{ST}$  estimates. Two runs were  
54  
55 241 performed, each with one chain consisting of 1 000 000 recorded steps (increment 100) replicated  
56  
57 242 four times. Altogether 400 000 000 parameter values were sampled and 100 000 initial values per  
58  
59 243 chain were discarded as burn-in.

56 244

58 245 *Testing for loss of diversity and associated founder effect.*

1  
2 246 Genetic diversity was assessed for each lacustrine population, for both mtDNA and Msat markers  
3  
4 247 by calculating allelic richness ( $A_r$ ) corrected for a common sampling size using a rarefaction  
5  
6 248 approach (Leberg, 2002). Calculations were performed with HP-RARE 1.1 (Kalinowski, 2005). In  
7  
8 249 addition, expected heterozygosity ( $H_E$ ) was calculated for microsatellite markers using FSTAT  
9  
10 250 ( $A_r$  and  $H_E$ ) were compared to 11 geographically closest  
11  
12 251 populations (sites 14, 15, 17-19, 21, 22, 24-27) from the western route as defined by Rewicz *et al.*  
13  
14 252 (2015a), to track the potential loss of genetic diversity. This comparison was restricted to  
15  
16 253 populations from the western route on the basis of the analysis showing that this route was the only  
17  
18 254 source of the colonization of Alpine Lakes (see results). Differences in  $A_r$  and  $H_E$  were tested using  
19  
20 255 non-parametric Wilcoxon rank test in STATISTICA 10 (StatSoft Inc., 2011).

21  
22 256 A method implemented in the BOTTLENECK v1.2.02 software (Cornuet & Luikart, 1996; Piry,  
23  
24 257 Luikart & Cornuet, 1999), using summary statistics of the genetic diversity to assess significant  
25  
26 258 deviations from mutation/drift equilibrium, was applied. Heterozygosity excess was considered as  
27  
28 259 evidence of recent bottlenecks (or founder effect in the present situation) (Luikart & Cornuet,  
29  
30 260 1998). Presence of heterozygote excess was tested under the three following mutation models:  
31  
32 261 infinite alleles (IAM), two-phase (TPM), and the step-wise (SMM). For TPM we set  $ps = 0.95$  (the  
33  
34 262 frequency of single step mutations) and the variance of those mutations as 12. Significance for all  
35  
36 263 models was tested using Wilcoxon's signed rank tests. Altogether 100 000 simulations were run. To  
37  
38 264 account for multiple comparisons, the FDR procedure was applied (Benjamini & Yekutieli, 2001;  
39  
40 265 Narum, 2006).

41  
42 266 In addition, following Luikart *et al.* (1998) and as applied by Wattier *et al.* (2007), the number of  
43  
44 267 alleles over all microsatellite loci according to allele frequencies in native and invaded areas was  
45  
46 268 plotted. Bottlenecks are expected to reduce the number of rare alleles (frequency  $<0.1$  in the native  
47  
48 269 range), therefore shifting the mode of the allele frequency histogram towards alleles with higher  
49  
50 270 frequencies. We therefore compared the distribution of allele frequencies of the 12 Alpine lake  
51  
52 271 populations with those of the closest populations located on the Western route, on Danube or Rhine  
53  
54 272 or Rhône waterways, surrounding the Alpine arc (populations 15, 17, 19 and 27, see Fig. 1).  
55  
56 273 Comparisons were made using non parametric test of Dunn with Control for Joint Ranks. Since all  
57  
58 274 populations of the Western route showed the same allele distribution frequencies (Wilcoxon rank  
59  
60 275 test:  $\chi^2 = 6.75$ , 3 d.f.,  $P = 0.09$ ), we chose to illustrate this comparison with the closest from our  
276 Alpine lake data set: population 19 (Figure 2a).

277 In accordance with the results of Bączela-Spychalska *et al.* (2013) we may expect the overland  
278 transportation with leisure activity to be the most important vector of the killer shrimp to the Alpine  
279 Lakes. To test if the touristic pressure may be a proxy for the number and/or propagule size of

1  
2 280 introductions the allelic richness ( $A_r$ ) of the populations inhabiting the particular Alpine Lake was  
3  
4 281 correlated with boat number and boat density (number of boats/lake area). The boat number for  
5  
6 282 each lake was counted by eye on Google Earth picture (Table 1, for details see Baćela-Spychalska  
7  
8 283 *et al.*, 2013). A positive correlation between boat number/density and  $A_r$  was expected as increasing  
9  
10 284 probability of *D. villosus* translocation would allow rare alleles to be incorporated. In addition,  
11  
12 285 because recently-founded populations may show the weakest genetic diversity, we tested correlation  
13  
14 286 between  $A_r$  and the possible 'age' of the killer shrimp populations, i.e. years between the first record  
15  
16 287 in each lake and the year of sampling. It was tested by Spearman correlation test.

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18 288

## 19 289 **Results**

### 20 290 *Western route as the only source for populations established in the Alpine Lakes*

21  
22  
23 291 Based on the concatenated 16S and COI mtDNA sequences, six haplotypes (n°1, 2, 17, 18, 19 and  
24  
25 292 20) were identified out of 128 analysed individuals from all the twelve Alpine Lakes (Fig. 2a, Table  
26  
27 293 S1). The relatively abundant haplotype 13, specific for the eastern native area and route, was not  
28  
29 294 found in any of the lakes. Haplotype 19 observed in lakes: Zug (41L), Zurich (42L) and Greifen  
30  
31 295 (43L), and haplotype 20 found in Lake Geneva (44L) were not recorded by Rewicz *et al.* (2015a)  
32  
33 296 neither in native areas, nor in the eastern or western route of invasion. The Msat Bayesian clustering  
34  
35 297 from our first simulation clearly showed that all Alpine populations (34L-45L) cluster together with  
36  
37 298 those from the western invasion route (sites n° 12-31) and the associated native area in the Danube  
38  
39 299 delta (sites n° 6-7) (Fig. 3a). The analyses of both mtDNA and Msat data therefore revealed that the  
40  
41 300 eastern native area and the associated route can be excluded as a source for populations established  
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43 301 in the Alpine Lakes.

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### 46 303 *Independent founder events in different regions of the Alps and secondary spread between some* 47 304 *Alpine Lakes*

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49 305 Four main clusters were observed in Alpine Lakes based on the similarity of their mtDNA  
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51 306 haplotypes. The first cluster consisted of the ‘Zurich-group’ (Zug - 41L, Zurich - 42L and Greifen  
52  
53 307 - 43L), while the second one was composed of the ‘Neuchâtel -group’ (Neuchâtel - 38L, Murten -  
54  
55 308 39L and Bienne - 40L). These groups shared mtDNA haplotypes either rare or even absent  
56  
57 309 elsewhere (Fig. 2a). A third cluster consisted of the Geneva Lake (44L) and the Bourget Lake (45L)  
58  
59 310 (‘Geneva-group’), both sharing a rare haplotype present in the western route (Fig. 2). The last  
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1  
2 311 cluster consisted of the remaining lakes (34L, 35L, 36L and 37L) sharing only the most common  
3 312 haplotype 1. Thus, this last clustering was not really informative.

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6 313 The STRUCTURE analysis of Msat data restricted to Alpine Lakes provided more precise  
7 314 information on the above clustering. First, it confirmed the assignment of lakes clustered within the  
8 315 “Zurich-group”, the “Neuchâtel -group” and the “Geneva-group”. However, the remaining lakes  
9 316 were assigned to different genetic units (Fig. 3b). First, Lake Traun (34L) was recognised as  
10 317 belonging to the “Zurich-group” by STRUCTURE, but did not share the peculiar composition of  
11 318 mtDNA haplotypes characteristic of other lakes from this group (see above, Fig. 2). In addition, the  
12 319  $F_{ST}$  values between the Lake Traun and the other lakes from the Zurich group were relatively high  
13 320 ( $0.043 < F_{ST} < 0.054$ ). This pattern advocates for an independent colonization event in this lake  
14 321 compared to the Zurich group. Second, lakes 35, 36 and 37L, while sharing the same mtDNA  
15 322 haplotypes with 34L, were clearly assigned to a different Msat unit, and cannot be clearly assigned  
16 323 to the previously identified genetic units (these lakes were somehow between Geneva and  
17 324 Neuchâtel groups).

18  
19 325 Within the “Neuchâtel-group”, “Geneva-group” and “Zurich-group” (Lake Traun being excluded  
20 326 from the later, see above), the values of  $F_{ST}$  or  $\Phi_{st}$  between lakes were low and mostly not  
21 327 significant, while they were highly significant between lakes from the other groups (Table 2).  
22 328 Among the remaining lakes, the only non-significant  $F_{ST}$  were between Lake Garda (36L) and Iseo  
23 329 (37L), suggesting high gene flow between these two lakes only (Table 2).

24  
25  
26 330 The analysis performed in MIGRATE-N detected high migration rates between lakes within  
27 331 Neuchâtel and Zurich groups. However, no asymmetry in gene flow pattern suggesting one peculiar  
28 332 lake to be a source was observed (Table S2).

29  
30 333 Finally, no significant correlation was detected between pairwise geographical distances and genetic  
31 334 differentiation among Alpine Lakes (Mantel test,  $R = -0.042$ ,  $p = 0.74$ ). A single introduction of *D.*  
32 335 *villosus* into one of the lakes followed by a series of founder events was therefore unlikely.

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34  
35 336 In summary, by combining mtDNA and microsatellite data we revealed that colonization of the  
36 337 Alpine Lakes was complex, with multiple independent population founding from the western route  
37 338 and secondary spread or admixture among some lakes. We may suggest that six independent  
38 339 founding event occurred: in the Geneva-group, the Neuchâtel-group, the Zurich-group, in Garda-  
39 340 Iseo, in the Lake Constance and in the Lake Traun.

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2 342 *An overall reduction of *D. villosus* genetic diversity in the Alpine Lakes*

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4 343 Allelic richness ( $A_r$ ) and the expected heterozygosity ( $H_E$ ) values of the Alpine Lakes populations  
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6 344 were significantly lower from those of the geographically closest populations from the western  
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8 345 invasion route ( $A_r$ :  $Z = 2.93$   $p = 0.003$ ;  $H_E$ :  $Z = 2.85$ ,  $p = 0.004$ ) (Figs 4a, 4b). The highest  $M_{sat}$   
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10 346 diversity was observed in the Lake Garda (36L) and Lake Constance (35L) ( $A_r = 3.5$  and 3.43,  
11  
12 347 respectively), while the lowest diversity was detected in the lakes: Bourget (45L), Traun (34L) and  
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14 348 Geneva (44L) ( $A_r = 2.35$ ; 2.39; 2.42).

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16 349 Genetic bottlenecks were evidenced in ten lakes: Traun (34L), Constance (35L), Garda (36L), Iseo  
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18 350 (37L), Neuchâtel (38L), Murten (39L), Bienne (40L), Zug (41L), Zurich (42L), Greifen (43L))  
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20 351 under IAM model, in four lakes (Constance (35L), Neuchâtel (38L), Bienne (40L), Zurich (42L))  
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22 352 under TPM model, and in two lakes: Bienne (40L) and Zurich (42L), under SMM model (Table 1).

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24 353 Cumulative distribution of allele frequencies showed mode shift for rare alleles (frequency  $< 0.1$ ) in  
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26 354 three to seven lakes, depending on the Western route population used as control (Table S3, Fig. 5).  
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28 355 Lakes of Geneva (44L), Traun (34L) and Bourget (45L) are those showing a significant difference  
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30 356 in allele distribution in all cases, while lakes Greifen (43L), Zurich (42L), Zug (41L) and Neuchatel  
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32 357 (38L) showed significant shifts for rare alleles depending on the population used as control (Table  
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34 358 S3).

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36 359 No correlation (Spearman correlation  $r = 0.39$ ,  $p = < 0.05$ ) was found between  $A_r$  and the possible  
37  
38 360 'age' of the killer shrimp populations, i.e. years between the first record in each lake and the year of  
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40 361 sampling. No correlation was found neither between  $A_r$  of populations inhabiting the Alpine Lakes  
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42 362 and the number of boats (Spearman correlation  $r = 0.33$ ,  $p = < 0.05$ ), nor between  $A_r$  and the boat  
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44 363 density (Spearman correlation  $r = -0.24$ ,  $p = < 0.05$ ).

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## 47 365 **Discussion**

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49 366 Many molecular studies help to reveal origins, founder events and secondary spreading of  
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51 367 freshwater alien species (Ficetola, Bonin & Miaud, 2008), e.g. gobiid fishes (Bronnenhuber *et al.*,  
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53 368 2011; Grabowski *et al.*, 2016), bivalves (Zhan *et al.*, 2012) or gammarids (Cristescu *et al.*, 2004).  
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55 369 However, such studies are very scarce not only for the Alpine Lakes (Schmidlin *et al.*, 2012) but  
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57 370 also for the Alpine terrestrial ecosystems (Henry *et al.*, 2009). Here, we evidenced that colonization  
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59 371 of the Alpine Lakes by *Dikerogammarus villosus* resulted from a process contrasting with the one  
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61 372 evidenced for rivers in continental Europe (Rewicz *et al.*, 2015a). This colonization in the Alps

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2 373 originated from several independent introductions but from a single route of invasion, followed by  
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4 374 secondary spreads among neighboring lakes. This pattern induced an overall decrease in genetic  
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6 375 diversity compared to the pattern observed in the invasion routes.  
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10 377 *The western route as the only source of invasion*

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12 378 Rewicz *et al.* (2015a) identified two invasion routes for *D. villosus* in continental lowland Europe  
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14 379 (see Fig. 1a). The comparison between native and invaded areas in each route revealed an absence  
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16 380 of loss of genetic diversity and, generally, the lack of genetic differentiation. High propagule  
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18 381 pressure associated with large propagule size and/or recurrent waves of migration may explain the  
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20 382 observed pattern. All the waterways associated with both invasion routes were already recognized  
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22 383 as effective invasion highways for Ponto-Caspian invasive species (Bij de Vaate *et al.*, 2002;  
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24 384 Jazdzewski, 1980; Leuven *et al.*, 2009; Panov *et al.*, 2009).

25 385 Our present results, combining mtDNA and Msat markers, showed clearly that the western route  
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27 386 was the only source for the colonization of the Alpine Lakes by the killer shrimp (Figs 2b, 3a). The  
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29 387 western route is geographically closer to the Alpine Lakes if compared to the eastern one, and there  
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31 388 are direct hydrological connections between the western route and some of the Alpine Lakes. For  
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33 389 example, Lake Geneva is directly connected to the Rhône River, and Lake Bourget is connected to  
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35 390 Rhône by the 4.1 km long navigable Canal de Savières. On the other hand, the two Italian lakes,  
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37 391 Lake Garda and Lake Iseo, located in the southern part of the Alps, are far away from the western  
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39 392 invasion route and without any direct aquatic connection. They were nevertheless colonized by the  
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41 393 killer shrimp (in 2003 and 2011, respectively). Here, instead of a direct colonization via  
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43 394 hydrological connection, overland transport with leisure boats is a more probable vector for its  
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45 395 introduction to these lakes. Other possible vectors of introduction to these isolated waterbodies may  
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47 396 be birds, e.g. waterfowls, as evidenced for several amphipod species (Rachalewski *et al.*, 2013 and  
48  
49 397 references cited therein). However, the occurrence of *D. villosus* in the Alpine Lakes is closely  
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51 398 associated with the intensity of recreational boating as shown by our previous studies (Bączela-  
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53 399 Szychalska *et al.*, 2013) and a high risk of spread with angling, sailing and diving equipment has  
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55 400 already been demonstrated (Anderson *et al.*, 2014). Touristic pressure is very high in the Alpine  
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57 401 region (Pechlaner & Tschurtschenthaler, 2003) and exchange of boats between various, sometimes  
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59 402 very distinct regions, happens quite often. Lakes such as Garda, Neuchâtel, Geneva and Constance,  
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61 403 are the biggest hubs for boat transport in the Alps (de Ventura *et al.*, 2016). An overland transport is  
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63 404 among the essential vectors for worldwide spread of other invasive species such as the *Corbicula*

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2 405 clams (Schmidlin *et al.*, 2012), zebra mussel *Dreissena polymorpha* (Pallas, 1771) (de Ventura *et*  
3 406 *al.*, 2016), or the aquatic plant *Cabomba caroliniana* (Gray 1837) (Bickel, 2015).

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6 407 Our results suggest that the eastern invasion route of *D. villosus* cannot be a source of introduction  
7  
8 408 to the Alpine Lakes. Since this route is not connected directly with Alpine Lakes investigated here,  
9  
10 409 the only potential way of colonization would have been an overland transport of recreational boats  
11 410 from central-eastern Europe. This cannot be excluded because *D. villosus* is known to resist adverse  
12 411 conditions associated with overland transport for a time compatible with long distance transport  
13 412 (Martens & Grabow, 2008). However, touristic boat translocations are rare from countries  
14 413 associated with the eastern invasion route of *D. villosus* (de Ventura *et al.*, 2016). Yet, the transport  
15 414 of *D. villosus* from the eastern route may perhaps be only a matter of time. It is to be noticed that  
16 415 the killer shrimp recently invaded the Masurian Lake District (MLD) in north eastern Poland (own  
17 416 unpublished data), which is directly connected to the eastern route. This district is highly  
18 417 fashionable for sailing and boating, with frequent relocation of boats both among lakes within the  
19 418 area and among MLD and foreign lakes, including the Alpine ones (personal communication, yacht  
20 419 transport company).

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#### 23 421 *Several independent founding events and cases of secondary spread or admixture*

24 422 Based on Msat data, several genetic clusters were defined, with low within- and high between-  
25 423 cluster genetic differentiations (Fig. 3b, Table 2). This result is reinforced by mtDNA data (Fig. 2b).  
26 424 Such a pattern may be explained by either a single introduction followed by a progressive  
27 425 colonization from this single source, by a massive colonization of this area followed by only small  
28 426 success of founding populations, or by recurrent and independent attempts for colonization with few  
29 427 founding populations, leading to independent colonization events. The absence of correlation  
30 428 between geographic distance and *Fst* suggest that a single introduction followed by a series of  
31 429 founder events (stepping-stone type pattern) is unlikely. A single massive colonization event is  
32 430 highly improbable as well. Indeed, even if the western route is the only source for invasion in the  
33 431 Alpine Lakes, only few lakes are directly connected to the main invasion route (Danube and low  
34 432 part of Rhine). In addition, only few lakes connected to the Danube drainage harbour *D. villosus*  
35 433 (Fig. 1b). Owing to the different genetic clusters deduced for both the mtDNA and Msat, it is  
36 434 probable that colonization attempts occurred recurrently, followed by very few settlements. These  
37 435 attempts were probably not simultaneous and could therefore initiate from various local populations  
38 436 along the western route. They would be independent introductions or at least independent founder  
39 437 events. The propagule size could be small, strengthening the founder effects, reshuffling allele

1  
2 438 frequencies and promoting the observed genetic differentiation. This result contrasts with the  
3  
4 439 absence of genetic structure along the western route which can, on the opposite, be associated with  
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6 440 recurrent invasions and/or large propagule size (Rewicz *et al.*, 2015a; Wattier *et al.*, 2007).

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8 441 Many lakes in the Alps are geographically aggregated. Assuming that a given lake is exposed to an  
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10 442 introduction as a consequence of touristic pressure, the probability of secondary introduction to  
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12 443 neighbouring and connected lakes is therefore predicted to be high. Our results suggest such  
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14 444 independent founding events and secondary spread or admixture in two groups of lakes: i) the  
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16 445 Neuchâtel-group: Neuchâtel (38L), Murten (39L), Bienne (40L) and ii) the Zurich-group: Zug  
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18 446 (41L), Zurich (42L), Greifen (43L). Each group combined the signature of an absence of within-  
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20 447 group genetic differentiation while being differentiated from other lakes.

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22 448 This within-group lack of genetic differentiation indicates that, after a colonization event within  
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24 449 each group, there was gene flow between lakes belonging to the group. The lakes of the Neuchâtel  
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26 450 group are interconnected by a system of canals: Lake Murten is connected to Lake Neuchâtel by the  
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28 451 Broye Canal and Lake Neuchâtel is connected to Lake Bienne by the Thielle canal. The latter lake  
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30 452 drains to the River Aare which ends in the River Rhine. These three lakes form a kind of system of  
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32 453 reservoirs to control water level. Thus, occasionally, the water from Lake Bienne flows backwards  
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34 454 through the Thielle and Broye canals in order to retain the overflow from the river Aare (Cioc,  
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36 455 2009). Whatever the scenario of *D. villosus* introduction to these lakes, either these direct  
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38 456 connexions or the high touristic pressure and boat traffic on the Lake Neuchâtel (de Ventura *et al.*,  
39  
40 457 2016) may explain intense genetic flow within this group of lakes. The observed bottleneck  
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42 458 supports a scenario with a small initial population, probably a single settlement in one of these  
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44 459 lakes. However, the results of the migration pattern analysis did not allow to identify any particular  
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46 460 lake as being the one colonized first. In the 'Zurich group' of lakes, the lack of differentiation and  
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48 461 symmetrical migration pattern also suggests very large or/and multiple transfers of the killer shrimp  
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50 462 among these lakes and also prevents, based on the current data, identification which of the lakes  
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52 463 was colonised first. Even if there is no direct hydrological connection between these lakes, their  
53  
54 464 geographic proximity suggest that the secondary spreads may have occurred through overland  
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56 465 transport. Considering the Neuchâtel group, the similarity in haplotype or allele frequencies among  
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58 466 lakes also suggests an intense and continuous gene flow among these lakes since their first  
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60 467 colonization.

56  
57 469 *Overall reduction in genetic diversity*



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2 470 The overall genetic diversity of *D. villosus* populations in the Alpine Lakes was significantly lower  
3 471 in comparison to populations from the western route. The bottleneck effect was detected in 25 % to  
4 472 75 % of the twelve investigated lakes. The proportion of lakes where bottlenecks were detected was  
5 473 highly dependent on the method used for detection. The relatively low number of Msat available for  
6 474 *D. villosus* is probably the cause for the variation observed in the proportion of bottleneck detection.  
7  
8 475 No correlation was found between allelic richness of populations inhabiting Alpine Lakes and the  
9 476 age since colonization. For example, Lake Traun, sampled eight years after its colonisation, still had  
10 477 very low genetic diversity. Additionally, loss of genetic diversity does not seem to be the only result  
11 478 of lake isolation from the western route. For example, populations from the isolated Lake Garda and  
12 479 Lake Iseo did not suffer large reduction of genetic diversity. Lake Garda has the highest level of  
13 480 allelic richness among the studied lakes. No correlation was found in the Alpine Lakes between  
14 481 allelic richness and (i) the number of boats, (ii) the boat density, which could have explain higher  
15 482 propagule size at the time of introduction. The diverse patterns of genetic diversity found in our  
16 483 study illustrates the complex colonization pattern of the Alpine Lakes compared to the invasion  
17 484 highways.

18  
19 485 The short term ecological success of the killer shrimp was not impeded by reduced genetic  
20 486 diversity. This case is not an isolated one. Populations of species such as the gammarid  
21 487 *Echinogammarus ischnus* (Stebbing, 1899) (Cristescu *et al.*, 2004), cladoceran *Cercopagis pengoi*  
22 488 (Ostroumov, 1891) (Cristescu *et al.*, 2001), mysid *Hemimysis anomala* G. O. Sars, 1907  
23 489 (Audzijonyte, Wittmann & Vainola, 2008) or guppy fish *Poecilia reticulata* W. K. H. Peters, 1859  
24 490 (Lindholm *et al.*, 2005) experience founder effects and bottlenecks without any negative  
25 491 consequences. A mechanism explaining such patterns could be a purge of recessive deleterious  
26 492 alleles, which fuelled further population expansion, as evidenced in the ladybird *Harmonia axyridis*  
27 493 (Pallas, 1773) (Facon *et al.*, 2011). Reduction of genetic diversity was also observed in populations  
28 494 of *D. villosus* after the colonization of waterbodies in the UK (Arundell *et al.*, 2015; Rewicz *et al.*,  
29 495 2015a). Since the first initial population was discovered in 2010, the killer shrimp was detected in  
30 496 further four localities (MacNeil *et al.*, 2010). Its surprisingly slow spread in UK compared to the  
31 497 Alpine Lakes, can be explained by the effective implementation of 'check, clean and dry' procedure  
32 498 (Arundell *et al.*, 2015).

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#### 53 54 500 *Conservation implications*

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56 501 Among the Ponto-Caspian invaders, *D. villosus* is considered as one of the most deleterious for  
57 502 local fauna (Bij de Vaate *et al.*, 2002; Rewicz *et al.*, 2014). In the Alpine Lakes, negative impact of

1  
2 503 *D. villosus* on the native ecosystems in already known. It includes displacing the native amphipod  
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4 504 *Echinogammarus stammeri* (S. Karaman, 1931) in lake Garda (Casellato, Visentin & La Piana,  
5  
6 505 2007), *Gammarus pulex* (Linnaeus, 1758) and *Gammarus fossarum* Koch, 1836 in the Lake Zurich  
7  
8 506 (Steinmann, 2008) and *Gammarus roeselii* Gervais, 1835 in Lake Constance (Gergs, Schlag &  
9  
10 507 Rothhaupt, 2013). Decrease of the whitefish *Coregonus lavaretus* (Linnaeus, 1758) population in  
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12 508 the Lake Garda also followed invasion of the killer shrimp (Casellato, Visentin & La Piana, 2007).  
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14 509 Currently, at least twelve lakes are invaded by the killer shrimp in the Alpine region (Baçela-  
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16 510 Spsychalska *et al.*, 2013). High touristic pressure and intense overland transport of boats and water  
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18 511 sport gear may promote its further spread (Baçela-Spsychalska *et al.*, 2013; de Ventura *et al.*, 2016).  
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20 512 As clearly evidenced for the Zurich and Neuchâtel lakes groups, such secondary spread may be a  
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22 513 fast process.  
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24 514 Eradication of invasive aquatic macroinvertebrates such as *D. villosus* without heavy damage to the  
25  
26 515 entire ecosystem is extremely difficult, expensive, or even impossible (Genovesi, 2005). Only few  
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28 516 successful examples of eradication of aquatic invertebrates are known e.g. rusty crayfish  
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30 517 (*Orconectes rusticus* (Girard, 1852)) from the Sparkling Lake, Wisconsin (LTER, 2014). Thus,  
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32 518 some measures should be implemented to avoid further expansion. Some countries like United  
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34 519 Kingdom, New Zealand, Canada and the United States of America have implemented programs  
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36 520 which may prevent or reduce the chance of transferring invasive species with relocation of yachts,  
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38 521 and other sport gear (Anderson *et al.*, 2015). These programs are generally called 'check, clean and  
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40 522 dry' and they are addressed to tourists, anglers etc., to aware them about the threats from invasive  
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42 523 species (Aldridge, Ho & Froufe, 2014; StateDept, 2015). These procedures are easy to implement  
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44 524 and should be forced immediately in the Alpine marinas to prevent spreading of the killer shrimp  
45  
46 525 and other invasive species. Stopping the expansion of invasive species in lacustrine ecosystems  
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48 526 should be a primary objective due to their detrimental impact on local biota.  
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50 527

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534

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25 868 **Table 1.** Sampling and diversity of *D. villosus* in twelve Alpine Lakes. Acro. = Acronym. Lat. =  
26 869 Latitude. Long. = Longitude. Alt. = Altitude. Nb. Boats = Number of boats that could be scored on  
27 870 an aerial/satellite picture. Dates = year of first record / year of sampling. Msat = microsatellites (7  
28 871 loci). mtDNA = mitochondrial DNA (2 targets COI and 16S). N = number of individual analysed.  
29 872 Na = total number of alleles or haplotypes.  $H_E$  average expected heterozygosity.  $A_r$  = average allelic  
30 873 richness estimated with correction for sample size through rarefaction. Bottleneck = results for the  
31 874 Wilcoxon rank test computed in BOTTLENECK under IAM, TPM and SMM microsatellite  
32 875 mutation models. Significant deviation from mutation/drift equilibrium ( $P \leq 0.05$ ) and deviation  
33 876 after the application of false discovery rate procedure (FDR=0.016) are in bold (Benjamini and  
34 877 Yekutieli 2001).

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Name	Acro.	Lat	Long	Riv. Bas.	Alt (m)	Surface (km <sup>2</sup> )	Nb. boats	Dates	Msat				mtDNA			Bottleneck		
									N	Na	H <sub>E</sub>	A <sub>r</sub>	N	Na	A <sub>r</sub>	IAM	TPM	SMM
Traun	34L	47.901	13.768	Danube	425	25.6	810	03/11 <sup>a</sup>	32	17	0.43	2.39	12	1	1.00	<b>0.012</b>	0.027	0.027
Constance	35L	47.748	9.137	Rhine	395	536.0	16744	03/07 <sup>b</sup>	32	25	0.53	3.43	6	2	1.00	<b>0.004</b>	<b>0.012</b>	0.020
Garda	36L	45.874	10.867	Po	66	370.0	5234	03/11 <sup>c</sup>	32	26	0.54	3.50	12	1	1.00	<b>0.008</b>	0.055	0.234
Iseo	37L	45.666	10.063	Po	200	61.8	1467	11/11 <sup>d</sup>	32	23	0.49	3.12	12	1	1.00	<b>0.016</b>	0.055	0.055
Neuchâtel	38L	46.924	6.953	Rhine*	440	218.3	7360	03/11 <sup>e</sup>	31	23	0.50	3.12	12	2	2.00	<b>0.008</b>	<b>0.016</b>	0.039
Murten	39L	46.924	7.037	Rhine*	450	22.8	1535	06/11 <sup>f</sup>	32	22	0.56	3.09	11	2	2.00	<b>0.008</b>	0.020	0.027
Bienne	40L	47.107	7.218	Rhine*	440	39.3	2749	05/11 <sup>e</sup>	32	24	0.55	3.34	12	2	1.95	<b>0.008</b>	<b>0.008</b>	<b>0.016</b>
Zug	41L	47.180	8.467	Rhine	420	38.3	723	10/11 <sup>g</sup>	32	23	0.50	3.06	11	2	2.00	<b>0.008</b>	0.023	0.039
Zurich	42L	47.251	8.627	Rhine	420	88.7	7506	06/11 <sup>h</sup>	32	21	0.47	2.93	10	2	1.99	<b>0.008</b>	<b>0.016</b>	<b>0.016</b>
Greifen	43L	47.360	8.654	Rhine	425	8.5	557	08/11 <sup>i</sup>	32	21	0.49	2.87	12	2	1.99	<b>0.012</b>	0.148	0.148
Geneva	44L	46.366	6.303	Rhone	372	582.4	14996	02/02 <sup>j</sup>	32	17	0.35	2.42	12	3	2.52	0.055	0.578	0.578
Bourget	45L	45.800	5.845	Rhone	238	44.5	2738	07/07 <sup>k</sup>	32	17	0.33	2.35	6	2	2.00	0.047	0.594	0.594

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4 \* these lakes are also interconnected through small navigable canals. a = (Holdich & Pöckl, 2007);  
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6 b = (Mürle, Becker & Seitz, 2003); c = (Casellato *et al.*, 2006); d = (Bącela-Spychalska *et al.*,  
7  
8 2013); e = (Lods-Crozet & Reymond, 2006); f = (Lubini *et al.*, 2006); g = (Steinmann, 2010); h =  
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10 (Steinmann, Mürle & Ortlepp, 2006); i = (Steinmann, 2008); j = (Bollache, 2004); k = (Grabowski,  
11 Bącela & Wattier, 2007b)  
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**Table 2** Genetic pairwise differentiation between twelve Alpine Lakes *D. villosus* populations estimated for microsatellite ( $F_{ST}$ , below diagonal) and mitochondrial DNA data ( $\Theta_{ST}$ , above the diagonal). See Table 1 for acronym definition.

$F_{ST}/\Theta_{ST}$	34L	35L	36L	37L	38L	39L	40L	41L	42L	43L	44L	45L
34L	-	0.000 ns	0.000 ns	0.000 ns	0.364 ns	<b>0.613</b> **	0.091 ns	<b>0.576</b> **	<b>0.670</b> **	<b>0.590</b> **	0.000 ns	0.124 ns
35L	<b>0.174</b> **	-	0.000 ns	0.000 ns	0.260 ns	0.516 ns	0.008 ns	<b>0.475</b> **	<b>0.574</b> **	<b>0.496</b> **	- 0.069 ns	0.000 ns
36L	<b>0.129</b> **	<b>0.037</b> **	-	0.000 ns	0.364 ns	<b>0.613</b> **	0.091 ns	<b>0.576</b> **	<b>0.670</b> **	<b>0.590</b> **	0.000 ns	0.124 ns
37L	<b>0.176</b> **	<b>0.057</b> **	0.010 ns	-	0.364 ns	<b>0.613</b> **	0.091 ns	<b>0.576</b> **	<b>0.670</b> **	<b>0.590</b> **	0.000 ns	0.124 ns
38L	<b>0.153</b> **	<b>0.106</b> **	<b>0.030</b> **	<b>0.031*</b> *	-	0.006 ns	0.063 ns	0.471 ns	<b>0.511</b> **	<b>0.488</b> **	0.303 ns	0.246 ns
39L	<b>0.175</b> **	<b>0.120</b> **	<b>0.047</b> **	<b>0.056*</b> *	<b>0.023</b> **	-	0.316 ns	0.584 ns	<b>0.617</b> **	<b>0.597</b> **	<b>0.538</b> **	0.483 ns
40L	<b>0.082</b> **	<b>0.073</b> **	0.020 ns	<b>0.042*</b> *	0.009 ns	<b>0.029</b> **	-	0.448 ns	<b>0.508</b> **	<b>0.466</b> **	0.061 ns	0.034 ns
41L	<b>0.043</b> **	<b>0.094</b> **	<b>0.060</b> **	<b>0.108*</b> *	<b>0.095</b> **	<b>0.122</b> **	<b>0.036</b> **	-	- 0.090 ns	0.312 ns	<b>0.489</b> **	<b>0.438</b> **
42L	<b>0.054</b> **	<b>0.130</b> **	<b>0.067</b> **	<b>0.123*</b> *	<b>0.100</b> **	<b>0.144</b> **	<b>0.052</b> **	0.007 ns	-	0.410 ns	<b>0.562</b> **	<b>0.522</b> **
43L	<b>0.045</b> **	<b>0.138</b> **	<b>0.088</b> **	<b>0.147*</b> *	<b>0.145</b> **	<b>0.152</b> **	<b>0.074</b> **	0.021 ns	0.020 ns	-	<b>0.506</b> **	<b>0.460</b> **
44L	<b>0.198</b> **	<b>0.092</b> **	<b>0.077</b> **	<b>0.120*</b> *	<b>0.148</b> **	<b>0.190</b> **	<b>0.107</b> **	<b>0.101</b> **	<b>0.129</b> **	<b>0.144</b> **	-	- 0.091 ns
45L	<b>0.229</b> **	<b>0.126</b> **	<b>0.089</b> **	<b>0.133*</b> *	<b>0.160</b> **	<b>0.187</b> **	<b>0.127</b> **	<b>0.130</b> **	<b>0.146</b> **	<b>0.155</b> **	- 0.003 ns	-

ns, not significant; bold \*\* $P \leq 0.01$

## Supplementary Table

Supplementary Table 1. GenBank accession numbers of COI and 16S haplotypes

Supplementary Table 2. Migration rates between source and receiver lakes within two groups of lakes.

Supplementary Table 3. Comparisons of allele distributions between control populations (populations on the Western Route of invasion), and Alpine lake populations

## Figure Captions:

Fig. 1. (a) The dynamics of *Dikerogammarus villosus* invasion in main water bodies of Europe: dates of first records along the invasion highways (e.g. the Western route = WR and the Eastern route = ER) are indicated, shadowed area - the Alpine Arch. (b) Small black dots represent the positions of the 48 lakes sampled during the field survey along the Alpine Arch, where *D. villosus* was absent (for all lake names see Bączela-Spychalska et al 2013a). Big black dots indicating lakes with *D. villosus* are supplemented by locality acronyms (details in Table 1) and date of first record of *D. villosus*. Main rivers and their affluent are indicated by colours: Rhone - yellow, Rhine - red, Po - blue, Danube - green.

Fig. 2. Haplotype distribution of *D. villosus* in continental Europe. (a) Native area, western route and eastern route of invasion in continental lowlands (from Rewicz et al. (2015a)). (b) Alpine Lakes region. Black dots represent lakes with *D. villosus*. (c) Minimum spanning network based on data from Rewicz et al. (2015a) and from present study. \* - new haplotypes associated with the present study.

Fig. 3. Bayesian clustering of *D. villosus* based on six microsatellite loci from: (a) 36 populations collected in Alpine Lakes (L), native (N) area, western route (WR) and eastern route (ER) of invasion for  $K = 3$ . Acronyms refer to sites as detailed in Rewicz et al. (2015a) and in Table 1. (b) Bayesian clustering of *D. villosus* based on seven microsatellite loci from 12 populations from the Alpine Lakes with  $K = 3$ . Each individual is represented by a thin vertical line, with proportional assignment to different clusters indicated by colours. Black vertical lines separate sampling sites, identified below the plot.

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2  
3 Fig. 4. Comparison of (a) allelic richness and (b) expected heterozygosity between eleven  
4 populations from the western route of invasion and the populations from the Alpine Lakes.  
5 The boxes represent 25th–75th percentiles, upper and lower whiskers indicate minimum and  
6 maximum values. Square inside each box indicates median value. Non-parametric Wilcoxon  
7 rank test ( $A_r$ :  $Z = 2.93$   $p = 0.003$ ;  $H_E$ :  $Z = 2.85$ ,  $p = 0,004$ )  
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11 Fig. 5. Allele frequency distribution in populations of *D. villosus* from the 12 Alpine Lakes  
12 (black bars), compared with population WR19 (grey bars - same population in each diagram)  
13 that has not experienced a bottleneck and. Bottlenecks are expected to shift the mode by  
14 reducing the number of rare alleles. The population of WR19 is representative of other  
15 populations surrounding the Alpine Arch (not showed). It was chosen to illustrate the allele  
16 distribution in populations outside the Alps as it is the geographically closest population to the  
17 Alpine Lakes.  
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Illustrations

Fig. 1

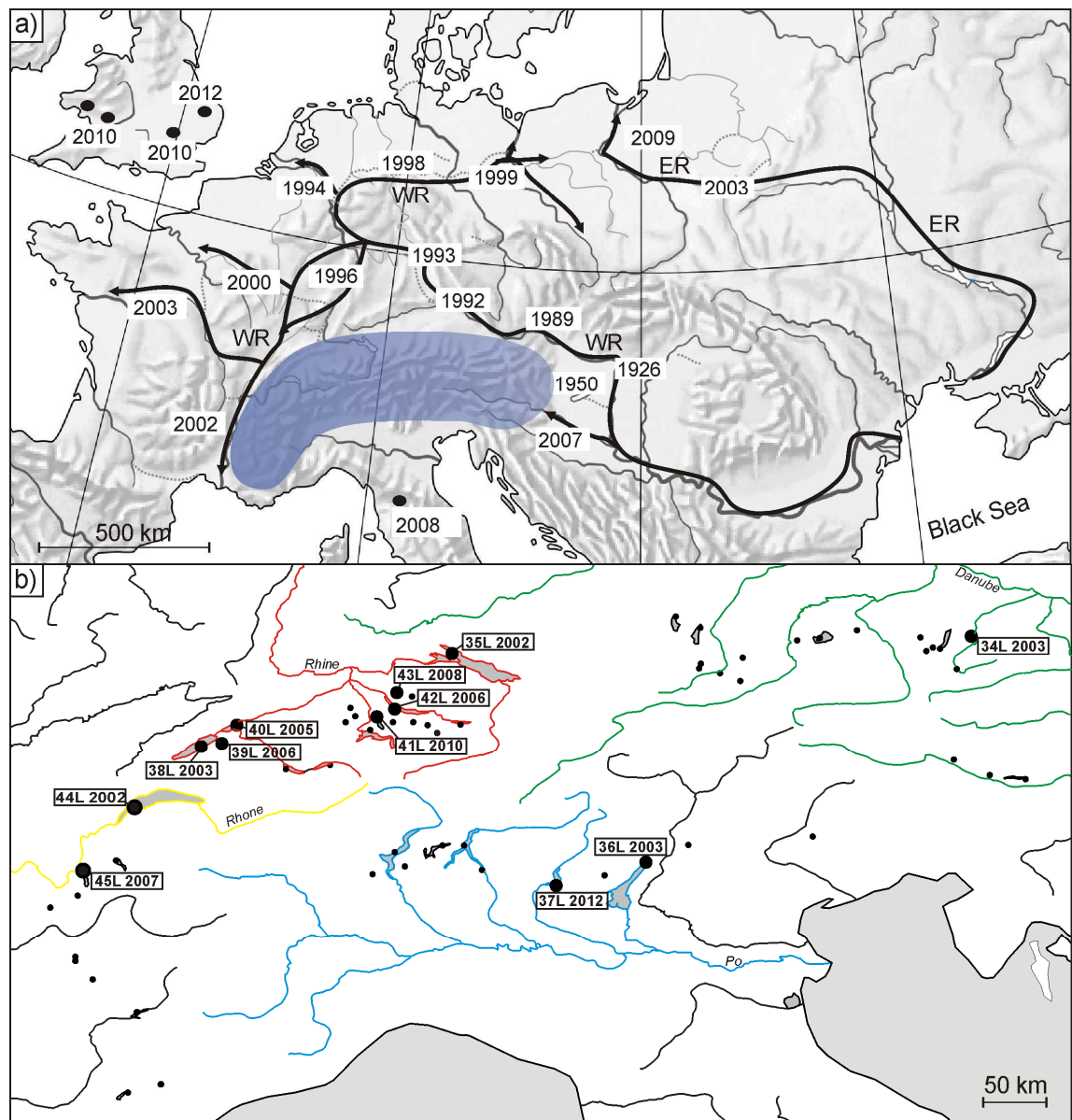


Fig. 2

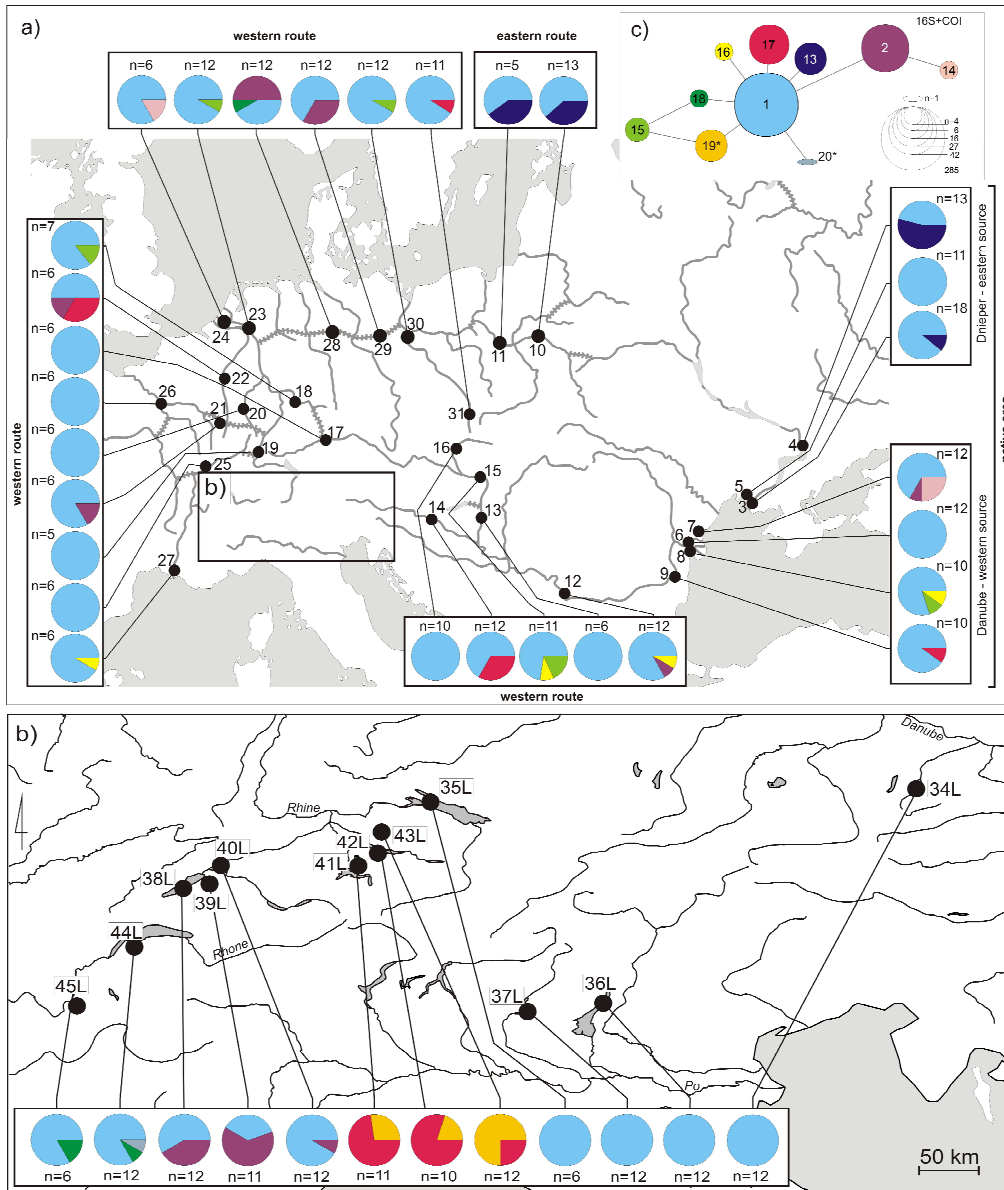




Fig. 3

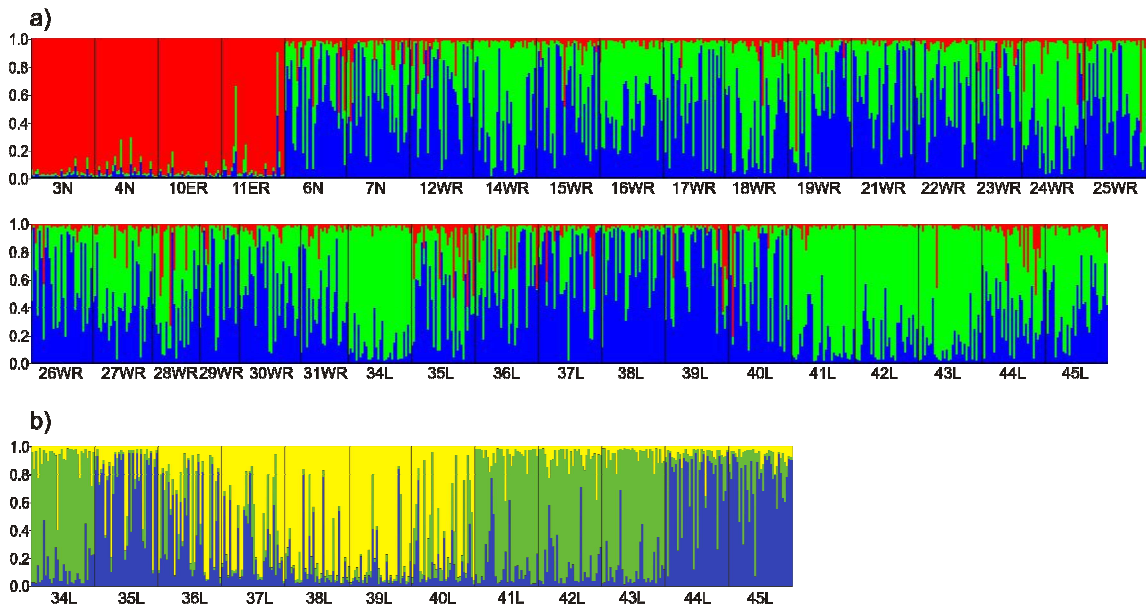


Fig. 4

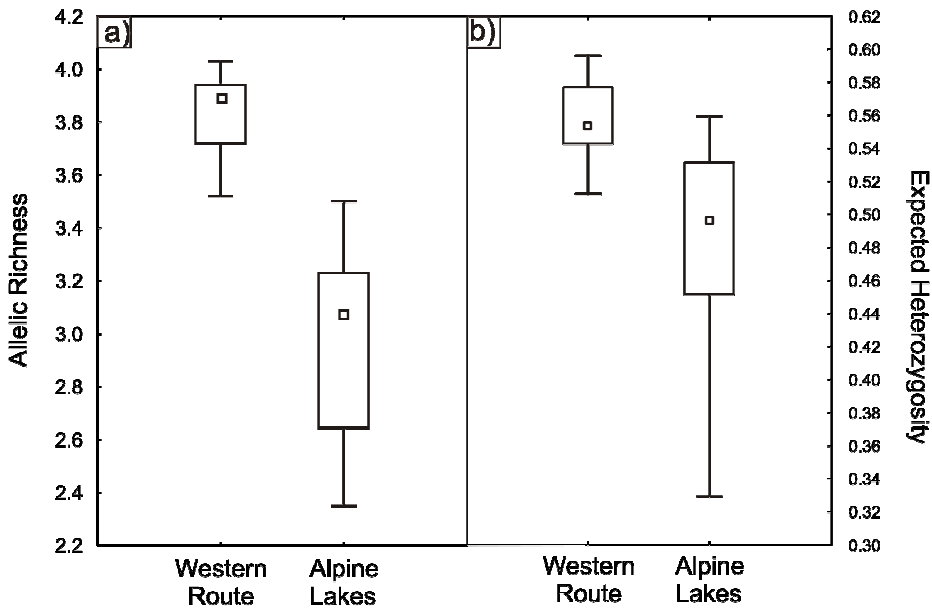


Fig. 5

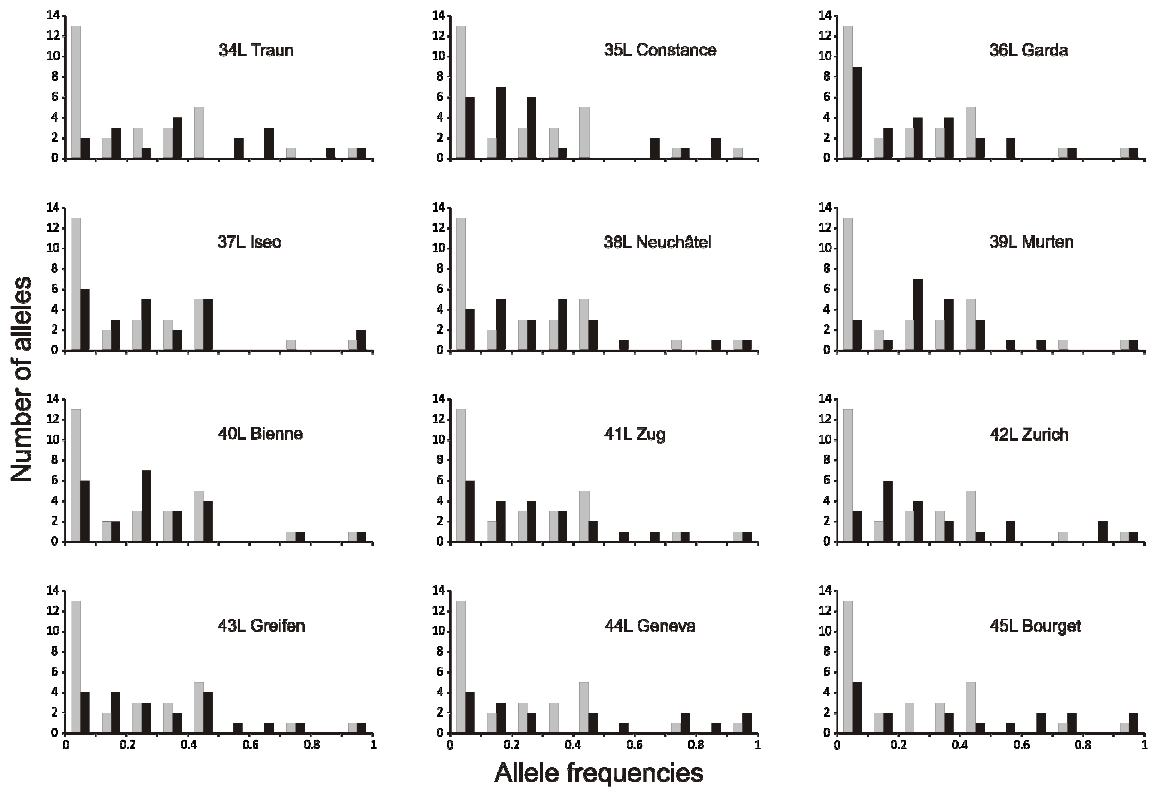


Table S1 Assignment of the individuals to the COI and 16S haplotypes as well as concatenated ones (16S+COI) with GeneBank accession numbers. Code for the individual consist of the lake number as in Figure 1 and number of the individual.

16S+COI	16S	COI	Individuals
hap 1	hap 1 KM208876	hap 13 KM208874	L34/1; L34/10; L34/11; L34/12; L34/15; L34/16; L34/17; L34/19; L34/24; L34/6; L34/8; L34/9; L35/10; L35/5; L35/6; L35/7; L35/8; L35/9; L36/1; L36/10; L36/14; L36/15; L36/16; L36/19; L36/2; L36/20; L36/3; L36/4; L36/8; L36/9; L37/10; L37/11; L37/12; L37/14; L37/15; L37/3; L37/4; L37/5; L37/6; L37/7; L37/8; L37/9; L38/14; L38/15; L38/16; L38/17; L38/5; L38/6; L38/7; L39/10; L39/13; L39/5; L39/8; L40/10; L40/11; L40/12; L40/13; L40/4; L40/5; L40/6; L40/7; L40/8; L40/9; L44/11; L44/12; L44/16; L44/18; L44/19; L44/2; L44/3; L44/4; L44/7; L44/9; L45/1; L45/2; L45/3; L45/5; L45/6
hap 2	hap 1 KM208876	hap 2 KM208863	L38/10; L38/11; L38/12; L38/2; L38/9; L39/1; L39/11; L39/12; L39/14; L39/3; L39/6; L39/9; L40/1; L40/2
hap 17	hap 1 KM208876	hap 14 KM208875	L41/12; L41/2; L41/3; L41/4; L41/5; L41/6; L41/7; L41/8; L42/10; L42/12; L42/17; L42/2; L42/3; L42/7; L42/8; L42/9; L43/1; L43/11; L43/5
hap 18	hap 1 KM208876	hap 13 KM208874	L44/20; L45/9
hap 19	hap 4 KM208879	hap 1 KM208862	L41/10; L41/11; L41/13; L42/11; L42/5; L43/10; L43/12; L43/13; L43/3; L43/4; L43/6; L43/7; L43/8; L43/9
hap 20	hap 1 KM208876	hap 15 KP814111	L44/13

Table S2. Migration rates between Source and Receiver lakes within two groups of lakes. Median values for two runs (1 and 2) are given for mutation-scaled migration rate  $M$  ( $M = m/\mu$ , where  $m$  is the immigration rate per generation and  $\mu$  is the mutation rate) estimated using Bayesian inference in program MIGRATE-N.

Group	Source	Receiver	run 1	run 2	
Neuchâtel -group	Murten	39L Neuchâtel	38L	21.1	17.5
	Bienne	40L Neuchâtel	38L	16.1	19.5
	Neuchâtel	38L Murten	39L	14.5	12.7
	Bienne	40L Murten	39L	21.1	21.1
	Neuchâtel	38L Bienne	40L	23.9	28.7
	Murten	39L Bienne	40L	20.5	23.7
Zurich-group	Zurich	42L Zug	41L	13.9	30.7
	Greifen	43L Zug	41L	17.9	21.9
	Zug	41L Zurich	42L	20.9	11.5
	Greifen	43L Zurich	42L	20.1	19.7
	Zug	41L Greifen	43L	13.9	15.5
	Zurich	42L Greifen	43L	18.1	18.7

Table S3: Comparisons of allele distributions between control populations (populations on the Western Route of invasion, the closest from the Alpine arc), and Alpine Lake populations, using non parametric test of Dunn with Control for Joint Ranks. Only significant comparisons are provided.

<b>Control population</b>	<b>Lake population</b>	<b>Z</b>	<b>P-value</b>
WR15	Geneva	4.82	< 0.0001
WR15	Traun	4.58	< 0.0001
WR15	Bourget	4.47	< 0.0001
WR15	Greifen	3.98	0.0008
WR15	Zurich	-3.29	0.01
WR15	Zug	-3.07	0.02
WR15	Neuchatel	2.85	0.05
WR17	Geneva	3.94	0.001
WR17	Traun	3.71	0.002
WR17	Bourget	3.56	0.004
WR17	Greifen	3.04	0.03
WR19	Geneva	4.35	0.0002
WR19	Traun	4.11	0.0005
WR19	Bourget	3.97	0.0008
WR19	Greifen	3.50	0.005
WR27	Geneva	3.67	0.003
WR27	Traun	3.45	0.007
WR27	Bourget	3.23	0.01