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Tomasz Rewicz, Rémi Wattier, Thierry Rigaud, Michal Grabowski, Tomasz Mamos, et al.. The killer shrimp, *Dikerogammarus villosus*, invading European Alpine lakes: a single main source but independent founder events with an overall loss of genetic diversity.. *Freshwater Biology*, 2017, 62 (6), pp.1036-1051. <10.1111/fwb.12923>. <hal-01525665>

HAL Id: hal-01525665

<https://hal.science/hal-01525665v1>

Submitted on 28 Dec 2021

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

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Running title: Origin and introduction sources of *Dikerogammarus villosus* in Alpine Lakes

Key words: invasive species, dispersal, overland transport, demography, Ponto-Caspian

Abstract

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

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Introduction

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

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

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

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

The amphipod *Dikerogammarus villosus* (Sowinsky, 1894), aka the killer shrimp, is one of the most efficient invaders in aquatic ecosystems (DAISIE, 2009). Issued from the Ponto-Caspian area, where it lives in large rivers or deltas and limans, it has successfully colonized main lowland continental European waterways during the last thirty years. Large navigable waterways with intense commercial shipping traffic, connected through artificial channels, are recognized as primary colonization routes, often called “invasion highways”, for the killer shrimp in lowland continental Europe (Bij de Vaate *et al.*, 2002; Leuven *et al.*, 2009; Rewicz *et al.*, 2015a). *Dikerogammarus villosus* inhabits stony substrates and is often found within zebra mussel beds. Its feeding regime is scavenger/predatory at adult stage, while juveniles show a more diversified diet including algae (reviewed in Rewicz *et al.*, 2014). Its high trophic level resulting from predatory behaviour (van Riel *et al.*, 2006) as well as wide tolerance to temperature and conductivity fluctuations (Bruijs *et al.*, 2001; Wijnhoven, van Riel & van der Velde, 2003) combined with early sexual maturity and high fecundity (Grabowski, Bącela & Konopacka, 2007a; Pöckl, 2007; Pöckl, 2009) are favourable life-history traits in the initial phases of invasion. The presence of *D. villosus* induces negative effects on local communities, such as an increase of direct predation pressure on other invertebrates and fish spawn (Buric *et al.*, 2009; Platvoet *et al.*, 2009; van der Velde *et al.*, 2009), competition for shelters (MacNeil *et al.*, 2011) and modification of entire food webs (van Riel *et al.*, 2006).

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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).
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13 124 In addition to rivers and canals, at least twelve lakes at low altitudes in European Alps (here after
14 125 called ‘‘Alpine Lakes’’) have been invaded by *D. villosus* (Bącela-Spychalska *et al.*, 2013 and
15 126 references cited herein, Fig. 1b, Table 1). These lakes vary in size and intensity of tourist activities.
16 127 The hydrological connections between the lakes and to the ‘‘invasion highways’’ are also variable
17 128 (Leuven *et al.*, 2009) (Fig. 1b, Table 1). The western invasion route partly encircles the Alps from
18 129 the north and west and is directly connected to some Alpine Lakes, suggesting that the *D. villosus*
19 130 populations in the Alpine Lakes may originate from this source. Yet, the connections are scattered,
20 131 lowering the probability of a single massive introduction in this area. On the other side, a touristic
21 132 boat traffic from distant locations is known to occur in Alpine Lakes. Some boats are transported
22 133 overland from Northern and Central Europe, i.e. from countries along the eastern invasion route (de
23 134 Ventura *et al.*, 2016). *Dikerogammarus villosus* endures the overland transport: Martens & Grabow
24 135 (2008) reported that it can survive six days out of water within zebra mussels biofouling on a boat
25 136 propeller. Bącela-Spychalska *et al.* (2013) showed that *D. villosus* can survive two days in between
26 137 layers of scuba-diving suit. Such abilities may favour long-distance colonisation of the Alpine
27 138 Lakes, where sailing and diving is a very common touristic activity. Therefore, presence of founder
28 139 individuals from both the eastern and the western population of *D. villosus* are possible, opening the
29 140 chance for independent founder events in different parts of the Alps. De Ventura *et al.* (2016)
30 141 showed that boats are frequently transported overland between Swiss Alpine Lakes; some lakes
31 142 (e.g. lakes Bienne, Murten and Neuchâtel) being additionally interconnected by small waterways.
32 143 These features may result in secondary spread of *D. villosus* from lakes with already established
33 144 populations.
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35 145 The pattern of *D. villosus* invasion in Alpine Lakes is interesting to investigate because it may
36 146 strongly influence the pattern of its genetic diversity. The killer shrimp populations maintained their
37 147 genetic diversity along the highways of invasion (Rewicz *et al.*, 2015a). Yet, we cannot reliably

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

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

Materials and Methods

Sample collection and molecular marker data production

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

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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©
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5 182 7.2.5, leading to 128 sequences of 16S (303 bp) and COI (654 bp) which were concatenated prior
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7 183 analyses. Haplotypes were retrieved using DNASP v5 both for individual markers and for the
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9 184 concatenated dataset (Librado & Rozas, 2009). Haplotypes for individual markers are presented in
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11 185 Table S1. One newly detected COI haplotype was deposited in GeneBank (KP814111). Other
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13 186 haplotypes were already identified after Rewicz *et al.* (2015a).

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15 187 Seven microsatellite loci (Msat) were used as nuclear co-dominant molecular markers: DikS, DikF
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17 188 (Wattier *et al.*, 2006), Dv11, Dv13, Dv17, Dv31, Dv33 (Rewicz *et al.*, 2015b). Altogether 415
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19 189 individuals were genotyped. The PCR conditions were described by Wattier *et al.* (2006) and by
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21 190 Rewicz *et al.* (2015b). It is to be noticed that DikF is not amplifying in the eastern source/route
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23 191 (Rewicz *et al.*, 2015b). Microsatellite alleles were visualized in 6.5% acrylamide, 25 cm long gels
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25 192 on a LICOR 4200 L automated sequencer and scored by eye. Reference individuals were included
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27 193 for inter-gel calibration. Absence of physical linkage disequilibrium was tested using the software
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29 194 GENEPOP (Rousset, 2008) and absence of recurrent genotyping errors and their possible sources
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31 195 e.g. short allele dominance (Wattier *et al.*, 1998) was checked with the software MICRO-
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33 196 CHECKER version 2.2.3. (van Oosterhout *et al.*, 2004).

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36 198 To compare the present data to the large-scale picture of *D. villosus* phylogeography in continental
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38 199 lowland Europe, 274 mtDNA sequences and microsatellite genotypes of 723 individuals analysed
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40 200 by Rewicz *et al.* (2015a) were also used in this paper.

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42 202 *Assessing the spatial distribution of diversity*

43 203 To visualize molecular divergence of mtDNA haplotypes and their spatial distribution, a Minimum
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45 204 Spanning Network was generated using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). Pairwise
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47 205 differentiation in haplotype and alleles frequencies was determined by two F_{ST} estimators: Θ_{ST} with
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49 206 Tamura-Nei distance for mtDNA (Tamura & Nei, 1993) and Θ for microsatellites (Weir &
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51 207 Cockerham, 1984), both implemented in ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010),
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53 208 statistical significance was measured using 10 000 permutations.

54 209 The population genetic structure was also analysed for Msat using individual-based Bayesian
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56 210 clustering method implemented in STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000). A
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58 211 first simulation was performed on a large dataset composed of populations from the Alpine Lakes,
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60 212 western route, eastern routes, and native area (Rewicz *et al.*, 2015a). This first simulation aimed to

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

We also tested the hypothesis of a single introduction into one of the Alpine Lakes, followed by a series of founder events according to a stepping-stone type of pattern of further expansion in other lakes. Such a pattern would lead to increasing differentiation in allele frequencies relative to an increasing the distance to the source. Based on the 12 lacustrine populations we therefore tested if microsatellite differentiation increased positively with distance between sites (isolation-by-distance, IBD). The geographic distances between lakes were estimated using Google Earth Pro 7.1.5.1557. The IBD was tested using Mantel's test between $F_{ST} / (1 - F_{ST})$ and geographic distance as recommended by Rousset (2008) for testing IBD in one-dimensional linear systems, with 100 000 permutations, using the GENEPOP on the Web 4.2 (Raymond & Rousset, 1995) and ISOLDE software.

As the result of the above analyses, we defined two groups of lakes where secondary spreads may be suspected i.e. the Neuchâtel-group: Bienne (40L), Murten (39L), Neuchâtel (38L) and the Zurich-group: Greifen (43L), Zurich (42L), Zug (41L); see results for details). The coalescence-based program MIGRATE-N 3.6.8 (Beerli, 2006; Beerli, 2009) was used to estimate the intensity and preferential direction of migration between pairs of locations within each of these groups. The MIGRATE-N estimates the mutation-scaled migration rate M ($M=m/\mu$, where m is the immigration rate per generation and μ is the mutation rate) and population size Θ ($4N_e\mu$, where N_e is effective population size). All seven microsatellites loci were used in the analysis. The parameters were inferred with Bayesian paradigm using the Brownian mutation model. The mutation rate among loci was scaled so that the average change of the mutation rate was 1.0 and the relative mutation rates were estimated for all loci. Initial M and Θ were calculated from F_{ST} estimates. Two runs were performed, each with one chain consisting of 1 000 000 recorded steps (increment 100) replicated four times. Altogether 400 000 000 parameter values were sampled and 100 000 initial values per chain were discarded as burn-in.

Testing for loss of diversity and associated founder effect.

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2 246 Genetic diversity was assessed for each lacustrine population, for both mtDNA and Msat markers
3 247 by calculating allelic richness (A_r) corrected for a common sampling size using a rarefaction
4 248 approach (Leberg, 2002). Calculations were performed with HP-RARE 1.1 (Kalinowski, 2005). In
5 249 addition, expected heterozygosity (H_E) was calculated for microsatellite markers using FSTAT
6 250 (Goudet, 2001). Both parameters (A_r and H_E) were compared to 11 geographically closest
7 251 populations (sites 14, 15, 17-19, 21, 22, 24-27) from the western route as defined by Rewicz *et al.*
8 252 (2015a), to track the potential loss of genetic diversity. This comparison was restricted to
9 253 populations from the western route on the basis of the analysis showing that this route was the only
10 254 source of the colonization of Alpine Lakes (see results). Differences in A_r and H_E were tested using
11 255 non-parametric Wilcoxon rank test in STATISTICA 10 (StatSoft Inc., 2011).
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13 256 A method implemented in the BOTTLENECK v1.2.02 software (Cornuet & Luikart, 1996; Piry,
14 257 Luikart & Cornuet, 1999), using summary statistics of the genetic diversity to assess significant
15 258 deviations from mutation/drift equilibrium, was applied. Heterozygosity excess was considered as
16 259 evidence of recent bottlenecks (or founder effect in the present situation) (Luikart & Cornuet,
17 260 1998). Presence of heterozygote excess was tested under the three following mutation models:
18 261 infinite alleles (IAM), two-phase (TPM), and the step-wise (SMM). For TPM we set $ps = 0.95$ (the
19 262 frequency of single step mutations) and the variance of those mutations as 12. Significance for all
20 263 models was tested using Wilcoxon's signed rank tests. Altogether 100 000 simulations were run. To
21 264 account for multiple comparisons, the FDR procedure was applied (Benjamini & Yekutieli, 2001;
22 265 Narum, 2006).
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24 266 In addition, following Luikart *et al.* (1998) and as applied by Wattier *et al.* (2007), the number of
25 267 alleles over all microsatellite loci according to allele frequencies in native and invaded areas was
26 268 plotted. Bottlenecks are expected to reduce the number of rare alleles (frequency <0.1 in the native
27 269 range), therefore shifting the mode of the allele frequency histogram towards alleles with higher
28 270 frequencies. We therefore compared the distribution of allele frequencies of the 12 Alpine lake
29 271 populations with those of the closest populations located on the Western route, on Danube or Rhine
30 272 or Rhône waterways, surrounding the Alpine arc (populations 15, 17, 19 and 27, see Fig. 1).
31 273 Comparisons were made using non parametric test of Dunn with Control for Joint Ranks. Since all
32 274 populations of the Western route showed the same allele distribution frequencies (Wilconxon rank
33 275 test: $\chi^2 = 6.75$, 3 d.f., $P = 0.09$), we chose to illustrate this comparison with the closest from our
34 276 Alpine lake data set: population 19 (Figure 2a).
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36 277 In accordance with the results of Bączela-Spychalska *et al.* (2013) we may expect the overland
37 278 transportation with leisure activity to be the most important vector of the killer shrimp to the Alpine
38 279 Lakes. To test if the touristic pressure may be a proxy for the number and/or propagule size of
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introductions the allelic richness (A_r) of the populations inhabiting the particular Alpine Lake was correlated with boat number and boat density (number of boats/lake area). The boat number for each lake was counted by eye on Google Earth picture (Table 1, for details see Bączela-Spychalska *et al.*, 2013). A positive correlation between boat number/density and A_r was expected as increasing probability of *D. villosus* translocation would allow rare alleles to be incorporated. In addition, because recently-founded populations may show the weakest genetic diversity, we tested correlation between A_r and the possible 'age' of the killer shrimp populations, i.e. years between the first record in each lake and the year of sampling. It was tested by Spearman correlation test.

Results

Western route as the only source for populations established in the Alpine Lakes

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

Independent founder events in different regions of the Alps and secondary spread between some Alpine Lakes

Four main clusters were observed in Alpine Lakes based on the similarity of their mtDNA haplotypes. The first cluster consisted of the "Zurich-group" (Zug - 41L, Zurich - 42L and Greifen - 43L), while the second one was composed of the "Neuchâtel -group" (Neuchâtel - 38L, Murten - 39L and Bienne - 40L). These groups shared mtDNA haplotypes either rare or even absent elsewhere (Fig. 2a). A third cluster consisted of the Geneva Lake (44L) and the Bourget Lake (45L) ("Geneva-group"), both sharing a rare haplotype present in the western route (Fig. 2). The last

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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).
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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 Φ_{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).
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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).
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31 333 Finally, no significant correlation was detected between pairwise geographical distances and genetic
32 334 differentiation among Alpine Lakes (Mantel test, $R = -0.042$, $p = 0.74$). A single introduction of *D.*
33 335 *villosus* into one of the lakes followed by a series of founder events was therefore unlikely.
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36 336 In summary, by combining mtDNA and microsatellite data we revealed that colonization of the
37 337 Alpine Lakes was complex, with multiple independent population founding from the western route
38 338 and secondary spread or admixture among some lakes. We may suggest that six independent
39 339 founding event occurred: in the Geneva-group, the Neuchâtel-group, the Zurich-group, in Garda-
40 340 Iseo, in the Lake Constance and in the Lake Traun.
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342 *An overall reduction of *D. villosus* genetic diversity in the Alpine Lakes*

343 Allelic richness (A_r) and the expected heterozygosity (H_E) values of the Alpine Lakes populations
 344 were significantly lower from those of the geographically closest populations from the western
 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}
 346 diversity was observed in the Lake Garda (36L) and Lake Constance (35L) ($A_r = 3.5$ and 3.43 ,
 347 respectively), while the lowest diversity was detected in the lakes: Bourget (45L), Traun (34L) and
 348 Geneva (44L) ($A_r = 2.35$; 2.39 ; 2.42).

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

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

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

365 **Discussion**

366 Many molecular studies help to reveal origins, founder events and secondary spreading of
 367 freshwater alien species (Ficetola, Bonin & Miaud, 2008), e.g. gobiid fishes (Bronnenhuber *et al.*,
 368 2011; Grabowski *et al.*, 2016), bivalves (Zhan *et al.*, 2012) or gammarids (Cristescu *et al.*, 2004).
 369 However, such studies are very scarce not only for the Alpine Lakes (Schmidlin *et al.*, 2012) but
 370 also for the Alpine terrestrial ecosystems (Henry *et al.*, 2009). Here, we evidenced that colonization
 371 of the Alpine Lakes by *Dikerogammarus villosus* resulted from a process contrasting with the one
 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
3 374 secondary spreads among neighboring lakes. This pattern induced an overall decrease in genetic
4 375 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
13 379 (see Fig. 1a). The comparison between native and invaded areas in each route revealed an absence
14 380 of loss of genetic diversity and, generally, the lack of genetic differentiation. High propagule
15 381 pressure associated with large propagule size and/or recurrent waves of migration may explain the
16 382 observed pattern. All the waterways associated with both invasion routes were already recognized
17 383 as effective invasion highways for Ponto-Caspian invasive species (Bij de Vaate *et al.*, 2002;
18 384 Jazdzewski, 1980; Leuven *et al.*, 2009; Panov *et al.*, 2009).
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21 385 Our present results, combining mtDNA and Msat markers, showed clearly that the western route
22 386 was the only source for the colonization of the Alpine Lakes by the killer shrimp (Figs 2b, 3a). The
23 387 western route is geographically closer to the Alpine Lakes if compared to the eastern one, and there
24 388 are direct hydrological connections between the western route and some of the Alpine Lakes. For
25 389 example, Lake Geneva is directly connected to the Rhône River, and Lake Bourget is connected to
26 390 Rhône by the 4.1 km long navigable Canal de Savières. On the other hand, the two Italian lakes,
27 391 Lake Garda and Lake Iseo, located in the southern part of the Alps, are far away from the western
28 392 invasion route and without any direct aquatic connection. They were nevertheless colonized by the
29 393 killer shrimp (in 2003 and 2011, respectively). Here, instead of a direct colonization via
30 394 hydrological connection, overland transport with leisure boats is a more probable vector for its
31 395 introduction to these lakes. Other possible vectors of introduction to these isolated waterbodies may
32 396 be birds, e.g. waterfowls, as evidenced for several amphipod species (Rachalewski *et al.*, 2013 and
33 397 references cited therein). However, the occurrence of *D. villosus* in the Alpine Lakes is closely
34 398 associated with the intensity of recreational boating as shown by our previous studies (Bącela-
35 399 Spychalska *et al.*, 2013) and a high risk of spread with angling, sailing and diving equipment has
36 400 already been demonstrated (Anderson *et al.*, 2014). Touristic pressure is very high in the Alpine
37 401 region (Pechlaner & Tschurtschenthaler, 2003) and exchange of boats between various, sometimes
38 402 very distinct regions, happens quite often. Lakes such as Garda, Neuchâtel, Geneva and Constance,
39 403 are the biggest hubs for boat transport in the Alps (de Ventura *et al.*, 2016). An overland transport is
40 404 among the essential vectors for worldwide spread of other invasive species such as the *Corbicula*

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

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

Several independent founding events and cases of secondary spread or admixture

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

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2 438 frequencies and promoting the observed genetic differentiation. This result contrasts with the
3 439 absence of genetic structure along the western route which can, on the opposite, be associated with
4 440 recurrent invasions and/or large propagule size (Rewicz *et al.*, 2015a; Wattier *et al.*, 2007).
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7 441 Many lakes in the Alps are geographically aggregated. Assuming that a given lake is exposed to an
8 442 introduction as a consequence of touristic pressure, the probability of secondary introduction to
9 443 neighbouring and connected lakes is therefore predicted to be high. Our results suggest such
10 444 independent founding events and secondary spread or admixture in two groups of lakes: i) the
11 445 Neuchâtel-group: Neuchâtel (38L), Murten (39L), Bienne (40L) and ii) the Zurich-group: Zug
12 446 (41L), Zurich (42L), Greifen (43L). Each group combined the signature of an absence of within-
13 447 group genetic differentiation while being differentiated from other lakes.
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16 448 This within-group lack of genetic differentiation indicates that, after a colonization event within
17 449 each group, there was gene flow between lakes belonging to the group. The lakes of the Neuchâtel
18 450 group are interconnected by a system of canals: Lake Murten is connected to Lake Neuchâtel by the
19 451 Broye Canal and Lake Neuchâtel is connected to Lake Bienne by the Thielle canal. The latter lake
20 452 drains to the River Aare which ends in the River Rhine. These three lakes form a kind of system of
21 453 reservoirs to control water level. Thus, occasionally, the water from Lake Bienne flows backwards
22 454 through the Thielle and Broye canals in order to retain the overflow from the river Aare (Cioc,
23 455 2009). Whatever the scenario of *D. villosus* introduction to these lakes, either these direct
24 456 connexions or the high touristic pressure and boat traffic on the Lake Neuchâtel (de Ventura *et al.*,
25 457 2016) may explain intense genetic flow within this group of lakes. The observed bottleneck
26 458 supports a scenario with a small initial population, probably a single settlement in one of these
27 459 lakes. However, the results of the migration pattern analysis did not allow to identify any particular
28 460 lake as being the one colonized first. In the 'Zurich group' of lakes, the lack of differentiation and
29 461 symmetrical migration pattern also suggests very large or/and multiple transfers of the killer shrimp
30 462 among these lakes and also prevents, based on the current data, identification which of the lakes
31 463 was colonised first. Even if there is no direct hydrological connection between these lakes, their
32 464 geographic proximity suggest that the secondary spreads may have occurred through overland
33 465 transport. Considering the Neuchâtel group, the similarity in haplotype or allele frequencies among
34 466 lakes also suggests an intense and continuous gene flow among these lakes since their first
35 467 colonization.
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57 469 *Overall reduction in genetic diversity*
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The overall genetic diversity of *D. villosus* populations in the Alpine Lakes was significantly lower in comparison to populations from the western route. The bottleneck effect was detected in 25 % to 75 % of the twelve investigated lakes. The proportion of lakes where bottlenecks were detected was highly dependent on the method used for detection. The relatively low number of Msat available for *D. villosus* is probably the cause for the variation observed in the proportion of bottleneck detection. No correlation was found between allelic richness of populations inhabiting Alpine Lakes and the age since colonization. For example, Lake Traun, sampled eight years after its colonisation, still had very low genetic diversity. Additionally, loss of genetic diversity does not seem to be the only result of lake isolation from the western route. For example, populations from the isolated Lake Garda and Lake Iseo did not suffer large reduction of genetic diversity. Lake Garda has the highest level of allelic richness among the studied lakes. No correlation was found in the Alpine Lakes between allelic richness and (i) the number of boats, (ii) the boat density, which could have explain higher propagule size at the time of introduction. The diverse patterns of genetic diversity found in our study illustrates the complex colonization pattern of the Alpine Lakes compared to the invasion highways.

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

Conservation implications

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

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2 503 *D. villosus* on the native ecosystems in already known. It includes displacing the native amphipod
3 504 *Echinogammarus stammeri* (S. Karaman, 1931) in lake Garda (Casellato, Visentin & La Piana,
4 505 2007), *Gammarus pulex* (Linnaeus, 1758) and *Gammarus fossarum* Koch, 1836 in the Lake Zurich
5 506 (Steinmann, 2008) and *Gammarus roeselii* Gervais, 1835 in Lake Constance (Gergs, Schlag &
6 507 Rothhaupt, 2013). Decrease of the whitefish *Coregonus lavaretus* (Linnaeus, 1758) population in
7 508 the Lake Garda also followed invasion of the killer shrimp (Casellato, Visentin & La Piana, 2007).
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12 509 Currently, at least twelve lakes are invaded by the killer shrimp in the Alpine region (Bącela-
13 510 Spsychalska *et al.*, 2013). High touristic pressure and intense overland transport of boats and water
14 511 sport gear may promote its further spread (Bącela-Spsychalska *et al.*, 2013; de Ventura *et al.*, 2016).
15 512 As clearly evidenced for the Zurich and Neuchâtel lakes groups, such secondary spread may be a
16 513 fast process.
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21 514 Eradication of invasive aquatic macroinvertebrates such as *D. villosus* without heavy damage to the
22 515 entire ecosystem is extremely difficult, expensive, or even impossible (Genovesi, 2005). Only few
23 516 successful examples of eradication of aquatic invertebrates are known e.g. rusty crayfish
24 517 (*Orconectes rusticus* (Girard, 1852)) from the Sparkling Lake, Wisconsin (LTER, 2014). Thus,
25 518 some measures should be implemented to avoid further expansion. Some countries like United
26 519 Kingdom, New Zealand, Canada and the United States of America have implemented programs
27 520 which may prevent or reduce the chance of transferring invasive species with relocation of yachts,
28 521 and other sport gear (Anderson *et al.*, 2015). These programs are generally called 'check, clean and
29 522 dry' and they are addressed to tourists, anglers etc., to aware them about the threats from invasive
30 523 species (Aldridge, Ho & Froufe, 2014; StateDept, 2015). These procedures are easy to implement
31 524 and should be forced immediately in the Alpine marinas to prevent spreading of the killer shrimp
32 525 and other invasive species. Stopping the expansion of invasive species in lacustrine ecosystems
33 526 should be a primary objective due to their detrimental impact on local biota.
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46 528 **Acknowledgment**

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49 529 The study was financed by the Polish Ministry for Science and Higher Education, grant no. N
50 530 N304350139 and partially from the internal funds of the University of Lodz. Thanks are due to
51 531 Alicja Konopacka for help in sampling, Uta Mürle from HYDRA, Germany for providing us the
52 532 samples from the Constance Lake, and Sandra Casellato for providing us the samples from Garda
53 533 Lake.
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Table 1. Sampling and diversity of *D. villosus* in twelve Alpine Lakes. Acro. = Acronym. Lat. = Latitude. Long. = Longitude. Alt. = Altitude. Nb. Boats = Number of boats that could be scored on an aerial/satellite picture. Dates = year of first record / year of sampling. Msat = microsatellites (7 loci). mtDNA = mitochondrial DNA (2 targets COI and 16S). N = number of individual analysed. Na = total number of alleles or haplotypes. H_E average expected heterozygosity. A_r = average allelic richness estimated with correction for sample size through rarefaction. Bottleneck = results for the Wilcoxon rank test computed in BOTTLENECK under IAM, TPM and SMM microsatellite mutation models. Significant deviation from mutation/drift equilibrium ($P \leq 0.05$) and deviation after the application of false discovery rate procedure (FDR=0.016) are in bold (Benjamini and Yekutieli 2001).

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

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* these lakes are also interconnected through small navigable canals. a = (Holdich & Pöckl, 2007); b = (Mürle, Becker & Seitz, 2003); c = (Casellato *et al.*, 2006); d = (Bącela-Spychalska *et al.*, 2013); e = (Lods-Crozet & Reymond, 2006); f = (Lubini *et al.*, 2006); g = (Steinmann, 2010); h = (Steinmann, Mürle & Ortlepp, 2006); i = (Steinmann, 2008); j = (Bollache, 2004); k = (Grabowski, Bącela & Wattier, 2007*b*)

Table 2 Genetic pairwise differentiation between twelve Alpine Lakes *D. villosus* populations estimated for microsatellite (F_{ST} , below diagonal) and mitochondrial DNA data (Θ_{ST} , above the diagonal). See Table 1 for acronym definition.

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

Fig. 4. Comparison of (a) allelic richness and (b) expected heterozygosity between eleven populations from the western route of invasion and the populations from the Alpine Lakes. The boxes represent 25th–75th percentiles, upper and lower whiskers indicate minimum and maximum values. Square inside each box indicates median value. Non-parametric Wilcoxon rank test (A_r : $Z = 2.93$ $p = 0.003$; H_E : $Z = 2.85$, $p = 0.004$)

Fig. 5. Allele frequency distribution in populations of *D. villosus* from the 12 Alpine Lakes (black bars), compared with population WR19 (grey bars - same population in each diagram) that has not experienced a bottleneck and. Bottlenecks are expected to shift the mode by reducing the number of rare alleles. The population of WR19 is representative of other populations surrounding the Alpine Arch (not showed). It was chosen to illustrate the allele distribution in populations outside the Alps as it is the geographically closest population to the Alpine Lakes.

Illustrations

Fig. 1

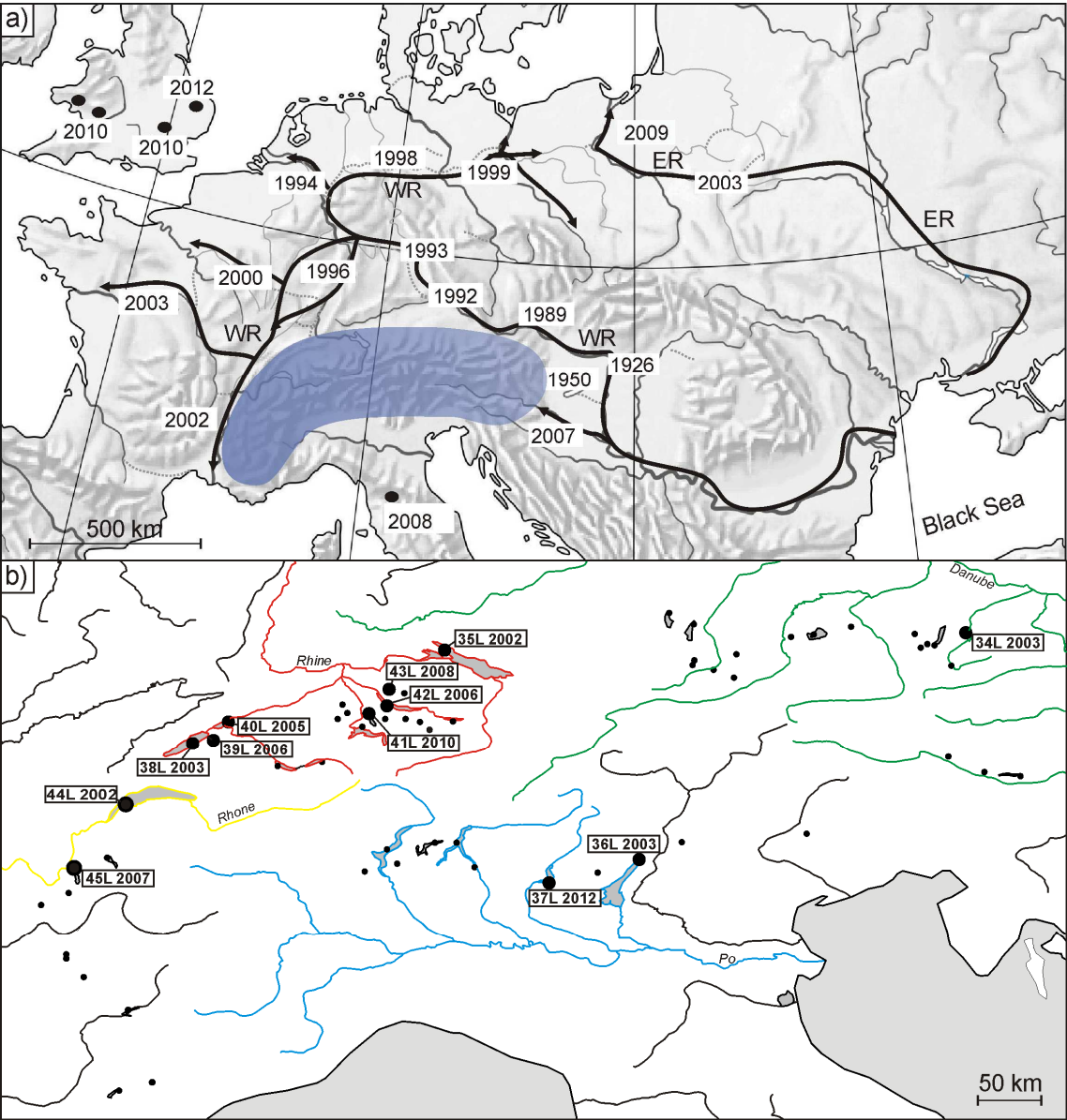


Fig. 2

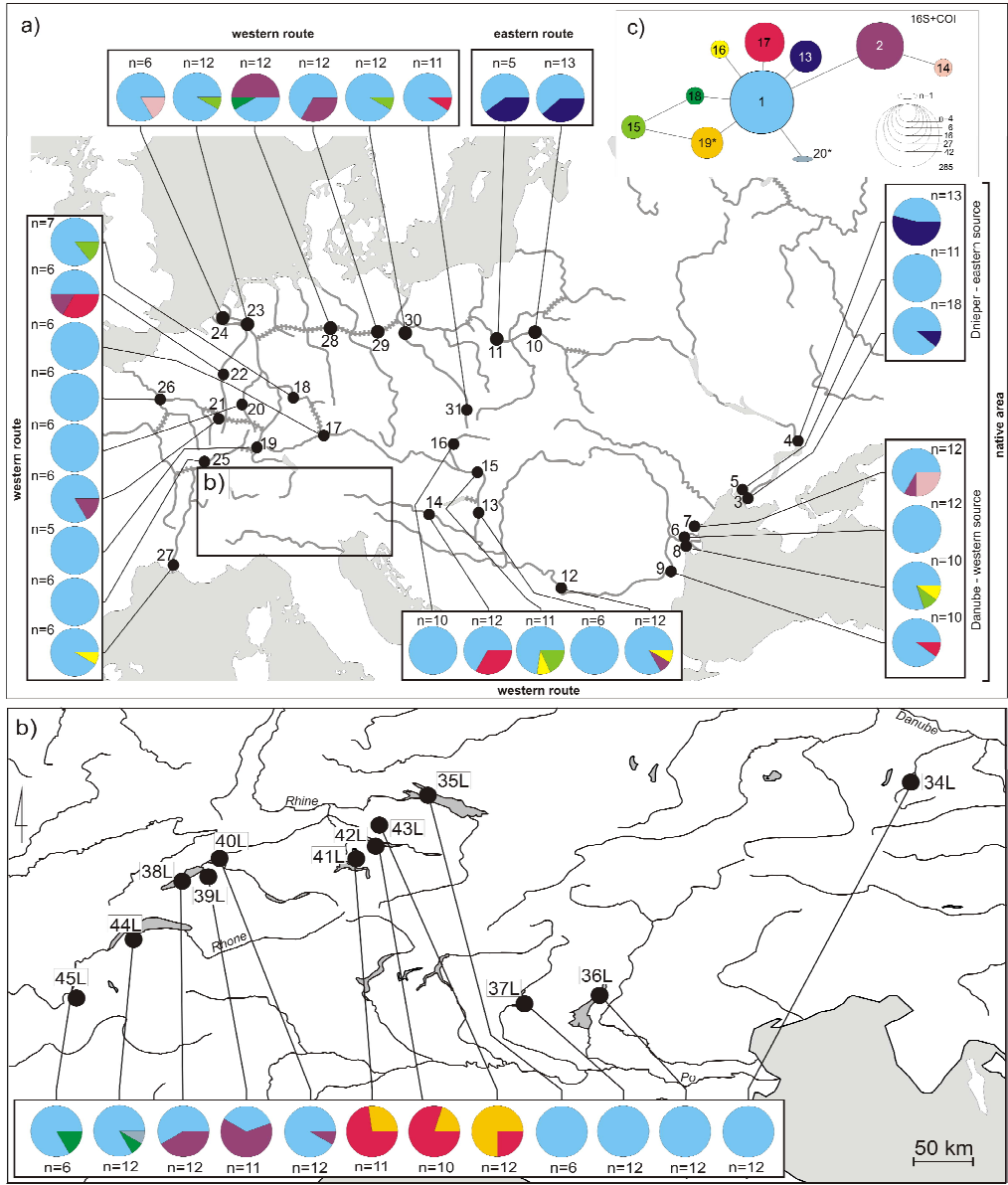


Fig. 3

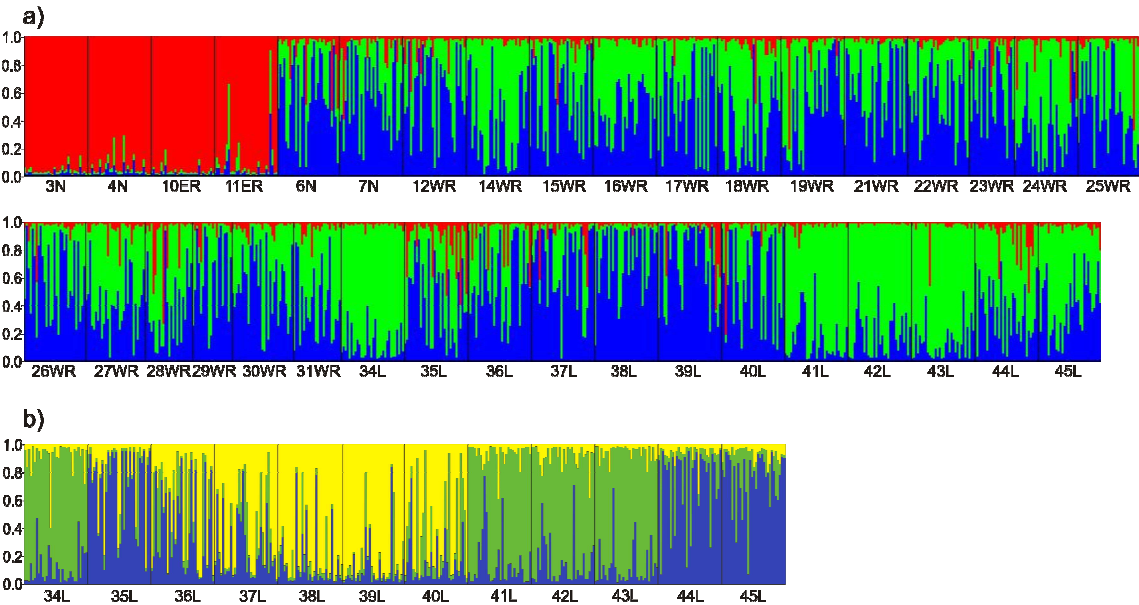


Fig. 4

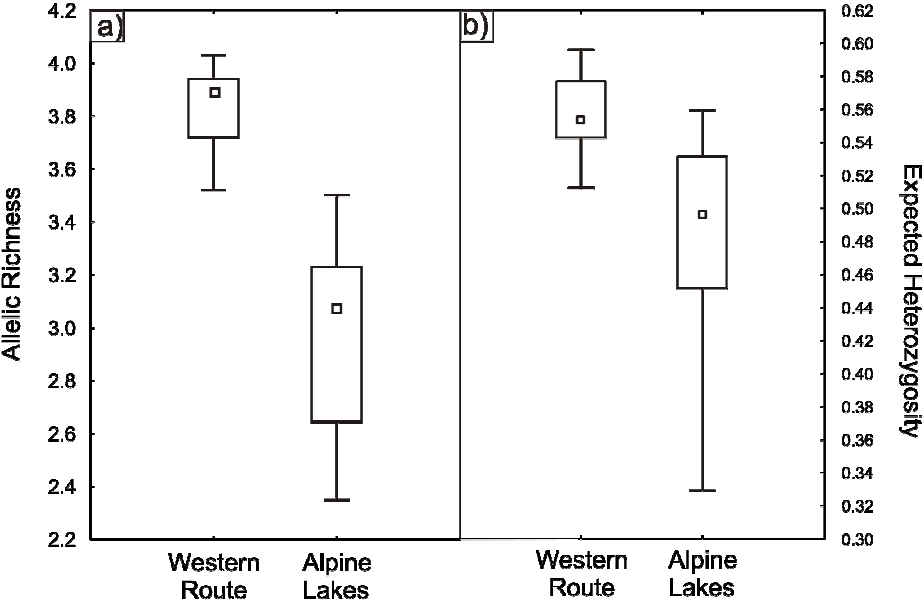


Fig. 5

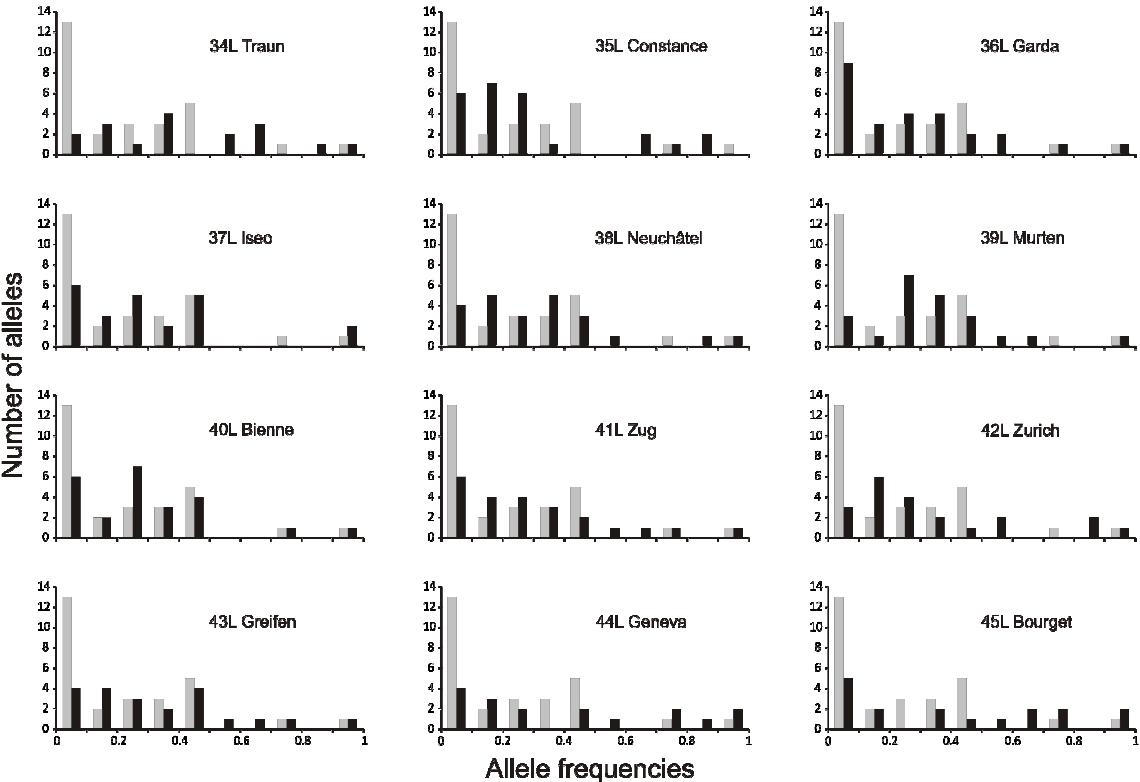


Table S1 Assignment of the individuals to the COI and 16S haplotypes as well as concatenated ones (16S+COI) with GeneBank accession numbers. Code for the individual consist of the lake number as in Figure 1 and number of the individual.

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 μ 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.

Control population	Lake population	Z	P-value
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