

# Description of a widely distributed but overlooked amphipod species in the European Alps

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Amphipods are keystone species in many freshwater ecosystems. Understanding their distribution and diversity is crucial to ensure and preserve freshwater ecosystem functioning, particularly in the northern hemisphere. For the European Alps information on amphipods has been relatively limited until recently. We describe a new, widely distributed amphipod species, *Gammarus alpinus* sp. nov., found across the Alps and analyse its distribution, biogeography as well as genetic and morphological differentiation. Until now, this species has been reported as *Gammarus lacustris*. Based on genetic and morphometric measurements, we show that *G. alpinus* is highly divergent from *G. lacustris*. The latter has a circumpolar distribution, but may be absent from the Alps. The observed occurrence pattern of *G. alpinus* might be explained by a Pliocene range expansion and vicariance due to climate warming following the Quaternary glaciation. Historical drainage divides suggest a single recolonization route from a distinct southern refugium. While *G. lacustris* is widely distributed and not endangered at a global scale, *G. alpinus* is endemic to the Alps and its habitat is negatively affected by eutrophication, non-native species and possibly climate change.

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ADDITIONAL KEYWORDS: *alpinus* – Amphipoda – biogeography – COI – endemic – *Gammarus* – *lacustris* – morphology.

## INTRODUCTION

Freshwater environments only cover about 2% of the Earth's surface, but nearly comprise equal species richness as marine environments, which cover ~70% of the Earth's surface (Wiens, 2015). Alarming, the high diversity in freshwater systems is severely threatened, as these ecosystems are especially sensitive to environmental changes (Vörösmarty *et al.*, 2010). Thus, from a conservation perspective, the identification of diversity hotspots as well as regions of high endemism is needed. Surprisingly for many major groups of invertebrates, solid databases on

their occurrences as well as possible conservation status are lacking (e.g. Revenga *et al.*, 2005).

For freshwater ecosystems, the European Alps are a biogeographically interesting region. Different major European drainage systems originate in the Alps, such as the rivers Rhine, Rhone, Po and Danube. The European Alps harbour many endemic species (Nagy *et al.*, 2012), which suggests a high priority for conservation of alpine biodiversity (Fontaine *et al.*, 2007; Dirnböck, Essl & Rabitsch, 2011; Jacobsen *et al.*, 2012). The need for such conservation measures is corroborated by the fact that the European Alps are, compared to other mountainous regions, densely populated, and heavily affected by anthropogenic changes, such as habitat alteration and climate warming.

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The current species distributions of freshwater organisms in Europe are strongly shaped by the Pleistocene ice ages as a major palaeoclimatic phenomenon (Hewitt, 1999; Foulquier *et al.*, 2008). Refugia in ice-free regions acted as ‘safe harbours’ during glacials, especially for temperate-adapted species (e.g. Stewart *et al.*, 2010). After deglaciation and recolonization this can lead to divergence of species which often remains cryptic due to the geologically short time scale (e.g. Williams, Ormerod & Bruford, 2006). This phenomenon is prevalent in amphipods and isopods throughout Europe (Müller, Partsch & Link, 2000; Vainio & Väinölä, 2003; Fišer *et al.*, 2015; Sworobowicz *et al.*, 2015) but their species status often remains unclear. The conjuncture of a highly variable morphology within species (Karaman & Pinkster, 1977a) combined with cryptic diversity between species resulted in a long lasting debate about the phylogeny and taxonomy in the genus *Gammarus* (Fabricius 1775). Even on the family level (Gammaridae) many uncertainties remain (see Hou & Sket, 2016 for an overview).

Amphipods belong to the most important freshwater organisms with respect to biomass as well as ecosystem processes in many freshwater systems (Holsinger, 1976; Väinölä *et al.*, 2008). They have an important ecosystem function in aquatic foodwebs, linking terrestrial detritus input to higher trophic organisms, such as fish (Rogers, Covich & Thorp, 2010). Furthermore, they are often used as indicator taxa for ecological monitoring (e.g. Stucki, 2010) and ecotoxicological tests (Gerhardt, 2011). Surprisingly, existing knowledge about amphipods in the European Alps is diametrically opposed to their ecological importance. For example, while there are individual species identification keys for Italy (Karaman, 1993), Germany (Eggers & Martens, 2001) or a genus identification key for France (Tachet *et al.*, 2010), an identification key integrating all alpine drainage areas and covering the whole alpine range is lacking. Furthermore, for some countries, first checklists of amphipods have been only recently published (e.g. Switzerland: Altermatt *et al.*, 2014) or are not up to date (e.g. Austria: Vornatscher, 1965). Through the recent use of genetic tools it became clear that many amphipod species in mountainous regions form highly diverse species groups, and that the level of undescribed species is relatively high, for example within the genus *Gammarus* (Müller, 2000; Westram *et al.*, 2011; Mamos *et al.*, 2014, 2016; Weiss *et al.*, 2014; Katouzian *et al.*, 2016). Recent colonizations by invasive species add to the complexity of the status but also change within amphipod communities.

A prominent member of the family Gammaridae is the species *Gammarus lacustris* (Sars 1863). It has an exceptionally wide, circumboreal distribution that

can maintain gene flow across three continents (MacDonald, Yampolsky & Duffy, 2005; Hou *et al.*, 2011; Hou, Sket & Li, 2014; but see Katouzian *et al.*, 2016). This stands in contrast to most freshwater amphipods having small, endemic distributions (Väinölä *et al.*, 2008). In Europe, *G. lacustris* has been reported from northern Germany, Denmark and Fennoscandia in many publications (Supporting Information, Table S1) and is commonly reported to be present in the Alps and prealpine lakes (e.g. Hynes, 1957; Meyran & Taberlet, 1998; Altermatt, Alther & Mächler, 2016; Supporting Information, Table S1). Past studies analysing the global distribution of *G. lacustris*, however, almost completely overlooked populations from the European Alps, and up to now there is no conclusive overview on its distribution as well as taxonomic identity throughout the European Alps in general, and for some countries in particular there are only few historical reports that date back to a pre-molecular biology era (Schellenberg, 1934; Hynes, 1957). Given its highly disjunct occurrence in the Alps, the species status and relationship of alpine populations to all other populations of *G. lacustris* worldwide potentially needs revision (see also Katouzian *et al.*, 2016).

Here, we studied the distribution and diversity of the *G. lacustris* complex in alpine lakes. Thereby, we identified a clear genetic and morphological distinction between circumpolar *G. lacustris* and its alpine populations. Given that alpine populations are genetically and morphologically differentiated and geographically stable isolated from all other populations we raise the alpine group to the species level, describing it as *Gammarus alpinus* sp. nov. We present the first overview of its distribution throughout the Alps and give remarks on its ecological and morphological characteristics. This new gammarid species, probably endemic to the Alps, poses some major implications for the conservation of alpine lakes and the introduction of amphipods or fish in these lakes.

## MATERIAL AND METHODS

### DATA SOURCE AND FIELDWORK

We used a combination of literature research and our own fieldwork to get a comprehensive understanding of the occurrence of the *G. lacustris* complex in the European Alps and prealpine areas. First, we conducted a Web of Science query with the keywords ‘gammarus’ and ‘lacustris’ (also ‘g.’ and ‘lacustris’). In addition, we performed the same search within the ETH internal library search (<http://www.library.ethz.ch/en/>) and Eawag internal library search (<http://www.lib4ri.ch/>), which includes additional unpublished records and reports specifically for

Switzerland, and followed up references given in the studies read. We in total screened 139 publications and reports (107 publications in Supporting Information, Table S1), from which we extracted all existing information on occurrence and (when available) known absences of the *G. lacustris* complex in alpine and prealpine freshwater habitats. Given that there was a lack of literature records especially from the Swiss Alps, we additionally queried the collections of 18 major natural history museums of Switzerland (among others, the Natural History Museum of Basel, Lausanne and Zurich), as well as the Natural History Museum in Berlin with a large amphipod collection for records of *G. lacustris*.

We complemented these data with a field sampling campaign, covering in total 102 lakes in France, Switzerland, Italy and Austria (Supporting Information, Table S2), which were chosen to be suitable for the *G. lacustris* complex based on available information regarding lake size, elevation and accessibility. The sampled lakes are located between 258 and 2381 m a.s.l. At each lake we collected amphipods with the kicknet technique. We sampled the littoral zone (max. depth sampled: 1.5 m) until reaching a threshold of at least 20 sampled individuals, but at least 15 min per site or 60–120 min per lake. If lake size and habitat structure indicated the absence of amphipods we nevertheless sampled at least 30 min. In all lakes where we found the *G. lacustris* complex, individuals were found within 45 min, in most lakes within 5–10 min of search efforts. Sampled individuals were pre-sorted in the field and preserved in molecular-grade ethanol (80%) for subsequent identification. Individuals were identified to species level according to Karaman (1993) and Eggers & Martens (2001) using a Nikon SMZ1500 (Nikon AG, Egg, Switzerland) or Leica M205C (Leica Microsystems, Heerbrugg, Switzerland) stereo microscope. For comparative purposes, ten additional specimens from Fennoscandia (Finland and Norway) were kindly provided by Dr Risto Väinölä (University of Helsinki).

#### MOLECULAR ANALYSIS

DNA was extracted from a total of 86 individuals (Supporting Information, Table S3; 76 alpine and ten Fennoscandian specimens). We dissected tissue from left pereopods 5–7, and extracted DNA using the DNeasy Blood & Tissue kit (Qiagen AG, Hombrechtikon, Switzerland). We applied the spin column protocol for animal tissue as provided by the manufacturer's handbook and incubated the proteinase reaction overnight. We then added 5  $\mu\text{L}$  RNase A (10 mg  $\text{mL}^{-1}$ ) and incubated the reactions at 37 °C for 30 min to yield better results as

suggested by our preliminary tests. For small individuals we also used pereopods 3–4, to ensure enough sample material. The final elution step was performed twice, using only 50  $\mu\text{L}$  of AE buffer and eluting into different microcentrifuge tubes. PCR was conducted using the second elution of the DNA extraction. The final reaction volume of 15  $\mu\text{L}$  resulted from 2.5  $\mu\text{L}$   $\text{H}_2\text{O}$ , 1 $\times$  Qiagen Multiplex Mastermix (7.5  $\mu\text{L}$ ), 0.5 $\times$  Q-Solution (1.5  $\mu\text{L}$ ), 0.5  $\mu\text{M}$  of each primer (2 $\times$  0.75  $\mu\text{L}$ ) and 2  $\mu\text{L}$  DNA template from the second elution from the previous extraction. Primers used were 'LCO1490' and 'HCO2198' (Folmer *et al.*, 1994) for the mitochondrial COI (Cytochrome c oxidase subunit I) gene fragment. Primers for the mitochondrial 12S rRNA gene were 5'-GCCAGCAGCCGCGTTA-3' and 5'-CCTACTTTGT-TACGACTTAT-3' (Trontelj & Utevsky, 2005). The PCRs were conducted on a Biometra TProfessional/Standard thermal cycler (Biometra GmbH, Göttingen, Germany). For COI amplification we used the following protocol: initial denaturation of 15 min at 95 °C followed by 35 cycles of 30 min at 94 °C, 90 min at 40 °C and 60 min at 72 °C. A final annealing time of 10 min at 72 °C was applied before the steady state at 4 °C. For 12S amplification we used the following protocol: initial denaturation of 3 min at 94 °C followed by 50 cycles of 30 min at 94 °C, 60 min at 45 °C and 60 min at 72 °C. A final annealing time of 10 min at 72 °C was applied before the steady state at 4 °C. After checking the resulting fragments on gel pictures, all positive PCR products were purified with Exo I nuclease (Exo I) and shrimp alkaline phosphatase (SAP) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove primers and dNTPs. The master mix consisted of 1.6 U  $\mu\text{L}^{-1}$  Exo I and 0.15 U  $\mu\text{L}^{-1}$  SAP in a total volume of 1.1  $\mu\text{L}$  per reaction. We then added the mix to 7.5  $\mu\text{L}$  of the PCR product and incubated it at 37 °C for 15 min, followed by 15 min at 80 °C. Cycle sequencing was performed using BigDye Terminator v3.1 (Thermo Fisher Scientific). The same sequences as amplification primers and the same thermal cycler were used. Cycling conditions were according to the following protocol for both markers: initial denaturation of 1 min at 96 °C followed by 25 cycles of 10 min at 96 °C, 5 min at 50 °C and 4 min at 60 °C. The final steady state was at 10 °C. DNA precipitation of cycle sequencing products was done using an EDTA-ethanol precipitation protocol. HiDi formamide was added to the precipitated cycle sequencing products to resuspend them. Subsequently the products were sequenced on an Applied Biosystems 3130xl Genetic Analyzer at the Genomic Diversity Centre of ETH Zurich.

Raw sequence reads were verified and aligned using Sequencher version 5.3 sequence analysis

software (Gene Codes Corp., Ann Arbor, Michigan, USA). COI sequences were only included into further analysis when longer than 500 bp with sufficient sequence read quality (at least 65% when combined, but mostly above 95%) and containing no stop codons or pseudogenes. The same holds for the 12S data, where the minimum length was 450 bp and sequence read quality usually above 90%. COI sequences were aligned to GenBank nucleotide sequences using the online Standard Nucleotide BLAST (blastn: [https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE\\_TYPE=BlastSearch&LINK\\_LOC=blasthome](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)) application and checked for taxonomic consistency with own identification. The subsequent sequence alignment of 82 newly generated COI sequences (Supporting Information, Table S3; 74 alpine and eight Fennoscandian specimens), combined with 16 previously generated sequences from an earlier project on Lake Constance (Altermatt *et al.*, 2016) and 23 publicly available COI sequences from *G. lacustris* (accession numbers in Supporting Information, Table S4) was done in MEGA6 (Tamura *et al.*, 2013) using the implemented ClustalW algorithm (Larkin *et al.*, 2007). This resulted in 121 COI sequences for the *G. lacustris* complex (91 alpine, 30 from Holarctic). An additional 77 outgroup sequences from other *Gammarus* species and amphipod genera were generated in our lab following the described protocols. In detail, we compiled ten sequences from *Crangonyx pseudogracilis* (Bousfield 1958), 22 sequences of *Gammarus roeseli* (Gervais 1835), 21 sequences of *Gammarus fossarum* (Koch 1836) and 24 sequences of *Gammarus pulex* (Linnaeus 1758). For 12S there are currently no sequences available on GenBank and the alignment for subsequent analysis is based on our 83 newly generated sequences solely (Supporting Information, Table S3; 73 alpine and ten Fennoscandian specimens).

We tested the monophyly of the entire *G. lacustris* complex using a maximum-likelihood (ML) analysis. The phylogeny for COI based on ML was inferred in MEGA6 using standard settings (K2P, GTR+G+I, 100 bootstraps). The substitution model was selected among 88 possible models using jModeltest 2.1.7 (Guindon & Gascuel, 2003; Durrira *et al.*, 2012) based on logL. The underlying data were 198 untrimmed COI sequences (121 sequences of the *G. lacustris* complex and 77 outgroup sequences), of which MEGA6 used 519 positions to reconstruct the phylogenetic tree (data not shown). The phylogenetic tree based on the 12S alignment consisted of 83 sequences and was computed analogous to compare the tree topology consistency with COI-derived results (data not shown).

As we noted a deep divergence between alpine and non-alpine *G. lacustris*, we explored the relationship

between the two clades in more detail and also estimated the age of the split by reconstructing a rooted time tree with a Bayesian approach in BEAST 2.3.0 (Bouckaert *et al.*, 2014). The phylogenetic tree for COI was inferred based on all 121 untrimmed sequences (see Wiens & Moen, 2008) without outgroup species (Drummond & Bouckaert, 2015). The data were partitioned according to codon position (Brandley *et al.*, 2011) previous to Bayesian analysis, using the same model for all partitions. Preliminary, different evolutionary models for COI sequence data were compared by Effective Sample Size (ESS) values, log-traces and ultimately Akaike's information criterion through Markov chain Monte Carlo (AICM) values (Baele *et al.*, 2012). ESS values >200 were interpreted as adequate for further analysis (Drummond & Bouckaert, 2015). Tested site substitution models were HKY and GTR with gamma distributed rate variation among sites (+G) and different gamma category counts. Furthermore, the presence of invariant sites (+I) was tested according to ESS and AICM. Frequencies of substitutions were taken from empirical values, whereas rates were estimated in BEAST and compared to equal rates. Subsequently, we selected the molecular clock model by comparing a strict molecular clock to a relaxed clock and a random local clock (Drummond & Suchard, 2010) to select the best model for inferring the chronogram. As clock rate we implemented a mean value of 0.0115, previously reported from other gammarid species (Lefébure *et al.*, 2007; Hou & Li, 2010; Copilaş-Ciocianu & Petrussek, 2015). As population function for the tree prior, we employed the simpler Yule model and compared it to a coalescent constant population model, a birth-death model, and a coalescent exponential population model. The final Bayesian inferred (BI) phylogeny for COI was based on HKY as site model, a relaxed log-normal molecular clock model and a coalescent constant population model prior. The length of the Monte Carlo Markov chain (MCMC) was set to 10 000 000 with a pre burn-in of 100 000 steps and trees stored every 10 000 steps. The trace logs from the MCMC runs were inspected in Tracer 1.6. Subsequently, six MCMC runs were combined in LogCombiner 2.3.0 and resampled every 10 000 steps. Combined tree logs were annotated in TreeAnnotator 2.3.0 by the maximum sum of clade credibilities with a burn-in of 10%, visualized in TreeGraph 2.7.1 (Stöver & Müller, 2010) and edited in Adobe Illustrator CS6 (Adobe Systems, Inc., San Jose, California, USA). Due to the limited geographical coverage of 12S sequences we did not compute a time tree in BEAST.

Finally we tested whether the alpine populations deserve species status. Species delimitation based on the barcoding gap was inferred from pairwise inter-

and intraspecific distances for COI and 12S, calculated in MEGA6 as nucleotide substitutions corrected with the Kimura two-parameter model (Kimura, 1980), again with transitions and transversions and Gamma distributed rates among sites (parameter = 1). The respective histograms were plotted in R version 3.1.3 (R Core Team, 2015).

Species delimitation based on COI data by a Poisson Tree Processes (PTP) model (Zhang *et al.*, 2013) was conducted using the server-based Bayesian implementation bPTP as provided on <http://species.hits.org/>. Here, the data basis was a rooted tree generated in MEGA6 inferred from ML (standard settings: K2P, GTR+G+I) and based on all sequences available for the *G. lacustris* complex (total: 121) and 24 newly generated *G. pulex* sequences as outgroup (same data as for the COI ML-phylogeny but with only one outgroup species). The bPTP analysis ran for 100 000 MCMC generations, 100 thinning runs and a burn-in of 0.1.

The COI haplotype network was constructed in popART (Leigh, Bryant & Steel, 2015) as a median-joining network (Bandelt, Forster & Röhl, 1999) and is based on the previously used 121 sequences of the *G. lacustris* complex.

COI and 12S sequences were uploaded to the Barcode of Life Database (BOLD, <http://v3.boldsystems.org/>), automatically given barcode status after approval and assigned to separate BINs (Barcode Index Numbers) by implemented sequence cluster algorithms. Pairwise distances and geographical distribution can directly be viewed online.

#### MORPHOMETRIC ANALYSIS

We dissected 86 adult individuals from 20 locations (same individuals as for molecular analysis), and prepared microscopic slides with the dissected Antennae 1 and 2, Gnathopods 1 and 2, Pereopods 3–7, the first Pleopod, Uropod 1 and Uropod 3, the Telson and the mouthparts. Body parts were generally taken from the right lateral side of the body. The removed parts were mounted in Kaiser's glycerol gelatine (Carl Roth GmbH, Arlesheim, Switzerland) as aqueous mounting medium. The mounted parts were covered with a glass slip and the edges sealed with transparent nail polish. Separate photos of all body parts were taken using a Nikon SMZ1500 stereomicroscope and a mounted Canon EOS 5D Mark III under the highest possible magnification. Photos were analysed with ImageJ 1.48v (Rasband, 2014) using pixel to  $\mu\text{m}$  ratios deduced from photos of a microscopic scale bar (Wild Heerbrugg AG, Heerbrugg, Switzerland). For all individuals, 37 morphological characters were recorded (Supporting Information, Table S5). We also noted the sex of the

individuals, as not only male individuals were used for analysis. All analysed photos were taken from the lateral side. All length measures were standardized to the mean of the total body length (three measurements). Plots showing the different characters/lengths against the standard were computed to see putative differences between clades in a visual examination. A principal components analysis (PCA) as ordination method was applied to the raw and log-transformed data (data not shown). Furthermore, analysis on single sex data was conducted. Additionally, the package missMDA (Josse & Husson, 2012) was used to handle missing values. All analyses were done in R version 3.1.3 (R Core Team, 2015).

#### ECOLOGICAL ANALYSIS

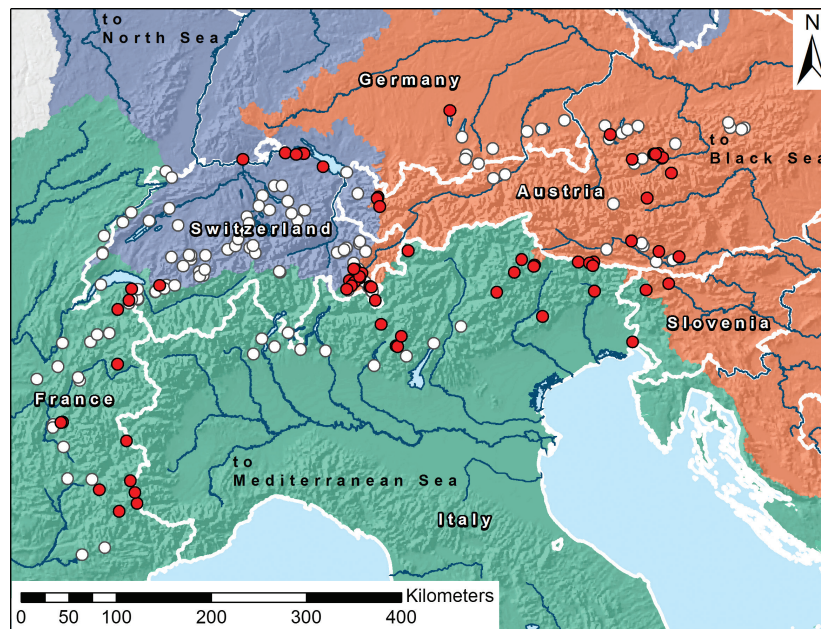
The distribution map was generated in ArcMap 10.2.2 (ESRI 2014. ArcGIS Desktop: Release 10). To determine the habitat properties and ecological requirements of the alpine *G. lacustris* complex we compared whether lakes where individuals were found share common ecological properties. Therefore, we explored the size of lakes and their altitudinal distribution. Based on our experience when collecting samples, we assumed that species live either in rather large lakes at lower elevation or in small higher elevation lakes. The contour plot was generated in R version 3.1.3 and based on complete cases of ecological data of sampled specimens. Elevation data and lake sizes were used in an individually written script, including the function 'kde2d' from the package MASS (Venables & Ripley, 2002) and the function 'image' from package graphics (core distribution).

#### RESULTS

Our fieldwork and literature research resulted in the first conclusive overview about the occurrence of the *G. lacustris* complex throughout the Alps, covering France, Italy, Switzerland, Germany, Austria and Slovenia (Fig. 1).

#### MOLECULAR ANALYSIS

Alpine and non-alpine specimens of *G. lacustris* constitute strongly supported monophyletic clades (Fig. 2, Supporting Information, Fig. S1). The alpine populations form a highly distinct and well-supported clade separated from circumpolar *G. lacustris* populations. Whereas sequences from as far apart as East China (Beijing) and North America (Vancouver Island) cluster together with sequences from Fennoscandia that we generated ourselves, all alpine sequences are clearly separated and form a



**Figure 1.** Distribution map of *Gammarus alpinus* sp. nov. Red circles indicate lakes with populations of *G. alpinus*, while white circles indicate sampled lakes without *G. alpinus*. The figure summarizes data from the literature and our own sampling. The major alpine drainage areas are given in colour (blue = Rhine/North Sea; orange = Danube/Black Sea; green = Rhone, Po/Mediterranean Sea).

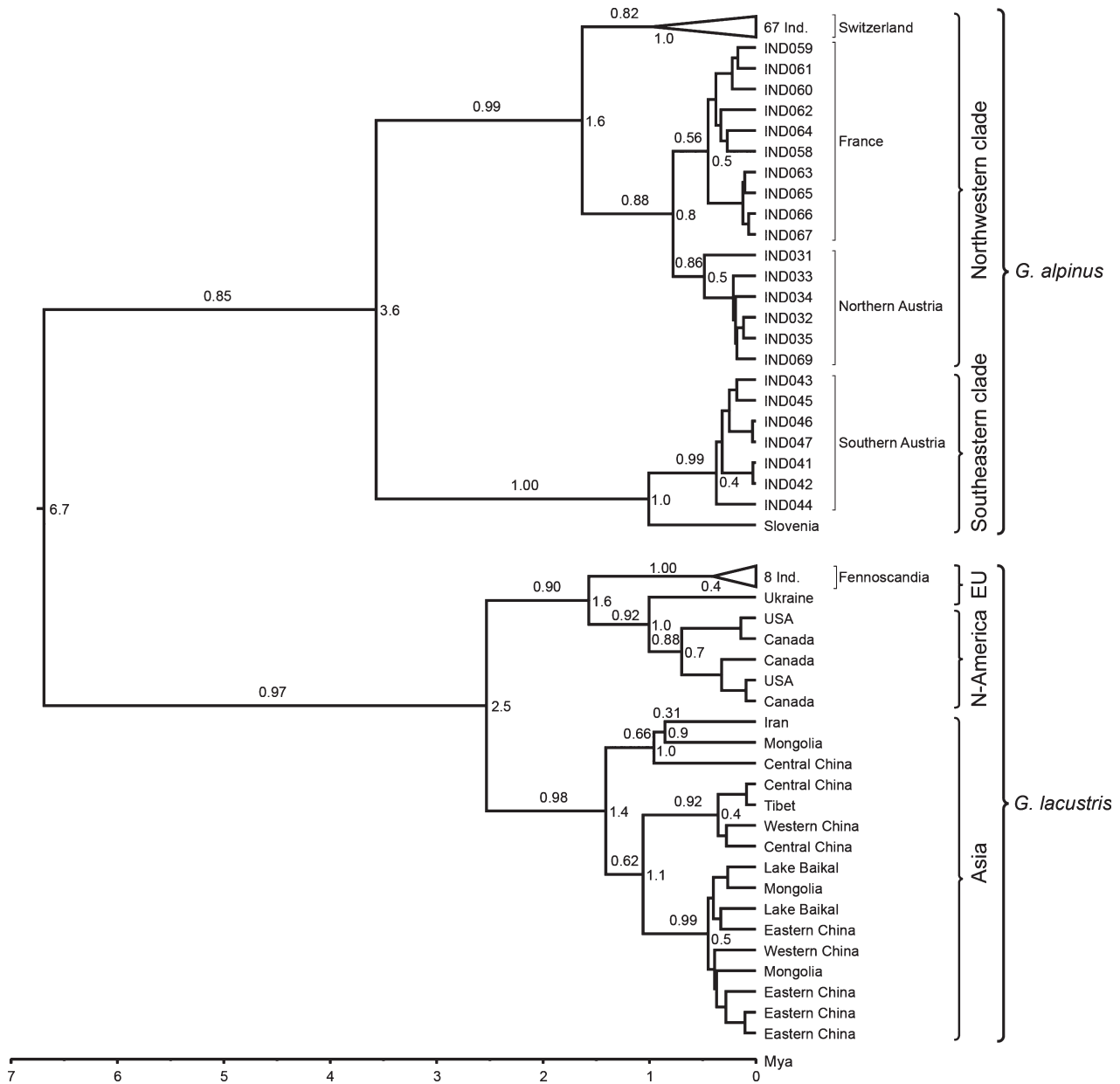
distinct clade. The alpine clade is further divided into two distinct, well-supported subclades (Fig. 2, Supporting Information, Fig. S1). The resulting phylogeny shows a consistent topology regardless of the gene (COI or 12S) and method used (ML or BI). Moreover, the general topology of three distinct clades is robust even to different prior parameters in the BI tree. The estimated age of the alpine *G. lacustris* clade is around 6.7 Myr (95% interval: 2.3–13.6 Myr).

Furthermore, pairwise distances of the COI sequences (K2P) within and between the initial *G. lacustris* specimens and the two alpine clades show a clear difference (Fig. 3). The comparisons between the *G. lacustris* specimens and the alpine specimens show differences greater than 5.2% (mean 6.5%,  $\pm 0.02\%$ ). By contrast, all 3866 pairwise distances of COI sequences within the three clades (*G. lacustris* and two alpine ones) are below 5% [mean 0.65%,  $\pm 0.01\%$  (SE)]. Exceptions are three comparisons between Asian and North American specimens that are just above 5% (maximal distance 5.8%), but these also reflect the largest geographical distances. Within *G. lacustris* from Fennoscandia, North America and Asia mean pairwise distance ( $\pm$  SE) is 2.2% ( $\pm 0.3\%$ ), compared to 0.8% ( $\pm 0.2\%$ ) within the alpine clades. Data for 12S sequences in general show less diversification, but with the same clear cut-off ( $5.2 \pm 1.3\%$  between *G. lacustris* and

alpine specimens,  $0.3 \pm 0.1\%$  within alpine specimens,  $0.8 \pm 0.2\%$  in *G. lacustris*; Supporting Information, Fig. S2). These findings are consistent with results from *G. fossarum*, in which the threshold for discriminating molecular operational taxonomic units is found to be around 4% with mate discrimination occurring in sympatric populations (Lagrue *et al.*, 2014; Galipaud *et al.*, 2015).

A haplotype network (Fig. 4) based on COI data corroborates the clustering into two separate species (colours in the figure highlight the origin of the specimens, the circles represent distinct haplotypes). The PTP analysis based on COI suggests delimitation of the *G. lacustris* complex into two separate species, both by ML and Bayesian analysis of the sequences. PTP support is 0.956 for the initial *G. lacustris* branch and 0.919 for the new alpine branch (identical values for the Bayesian and ML approaches).

Analysis of the COI barcodes on BOLD resulted in two clear and separated BINs (BOLD:ACH6111 and BOLD:ADB3370) for all alpine specimens, whereas the sequences from the rest of the world were assigned to other, separated BINs. Within the aligned sequences of COI (658 bp) 14 single nucleotide polymorphisms (SNPs) are identified, which are diagnostic for the alpine clade compared to the original *G. lacustris*. One SNP allows the distinction of all three clades (see Description).



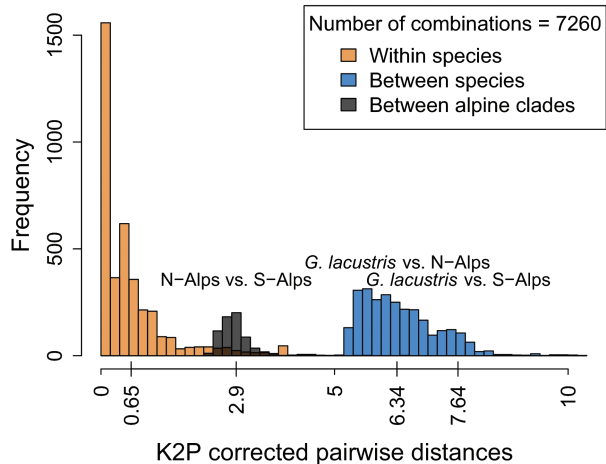
**Figure 2.** Bayesian-inferred phylogenetic time tree of *Gammarus alpinus* sp. nov. and *Gammarus lacustris*. Values above branches (two decimal places) indicate likelihood based on 5406 combined trees. For better readability, individuals from Switzerland and Fennoscandia were also collapsed. Values at nodes (one decimal place) indicate the age of the branch in Myr. Branch lengths indicating divergence time (with a relaxed clock model).

MORPHOMETRIC ANALYSIS

We find consistent morphological differences of the alpine gammarid clades compared to *G. lacustris* specimens from Norway and Finland. Most importantly, the alpine specimens almost exclusively bear one group of setae along the anterior margin of the carpopodite of gnathopod 2, while both sexes of *G. lacustris* always bear two groups of setae (Fig. 5). Further differences are given below in Description of

the novel species. The PCA did not reveal a clear difference (data not shown).

Summarizing the molecular data and adding the morphological differences between our alpine samples and the *G. lacustris* samples from all over the northern hemisphere and given the stable geographical separation, we hereby erect the alpine clade of the previous *G. lacustris* to the species status by naming it *Gammarus alpinus* sp. nov. The geographical distances between *G. lacustris* populations being



**Figure 3.** Pairwise differences of COI sequences support species status. Clade divergence as pairwise differences (K2P-corrected) indicates alpine *G. alpinus* sp. nov. to be different from all other *G. lacustris* sequences published so far. This diagram supports the species status of the alpine gammarid specimens but does not suggest a further distinction into separate alpine species, as pairwise distances fall into the distances observed worldwide.

larger than those between *G. lacustris* and *G. alpinus*, with genetic differences between *G. lacustris* populations being smaller than genetic differences

between *G. lacustris* and *G. alpinus* by an order of magnitude, support this nomenclatural act.

#### DESCRIPTION OF *GAMMARUS ALPINUS* SP. NOV.

SUBORDER GAMMARIDEA (LATREILLE 1802)

FAMILY GAMMARIDAE (LEACH 1813)

*GAMMARUS ALPINUS* SP. NOV. (FIGS 5, 6)

*References (that now most likely have to be considered G. alpinus* sp. nov.)

1, 2, 8, 12, 14–16, 19, 22, 35–38, 45, 46, 49, 52–54, 65, 66, 72, 73, 83–86, 94, 101 and 102 in Supporting Information (Table S1).

#### Type locality

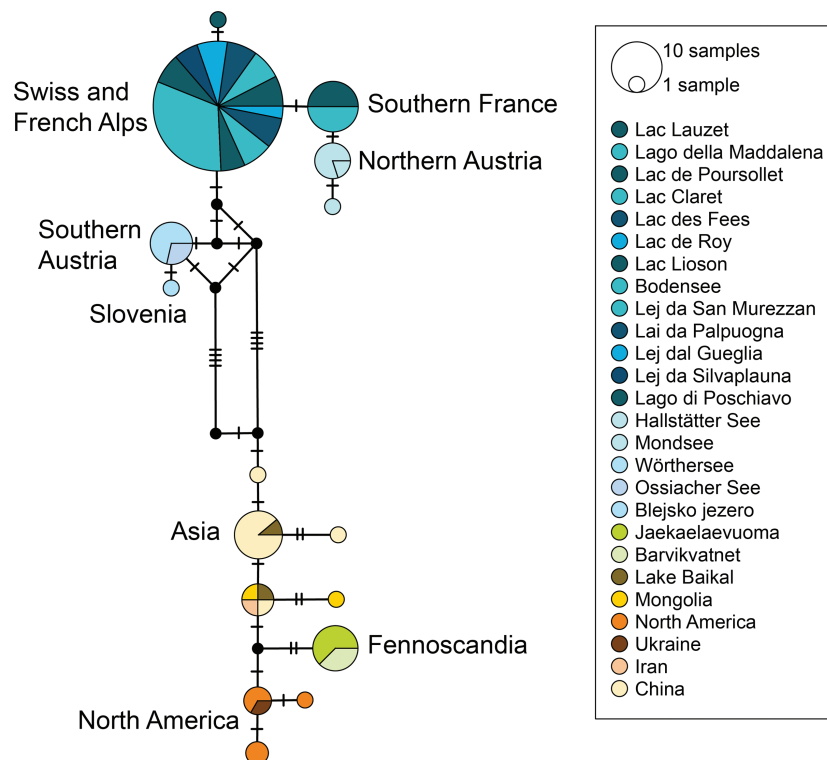
Lej da San Murezzan (Lake St. Moritz), Switzerland, 9°50'51.05"E, 46°29'48.02"N [WGS84], equivalent to 784901 m E, 152323 m N [CH1903/LV03].

#### Material examined

Seventy-seven specimens of *G. alpinus* in detail (the same were used for molecular analysis), another ~30 when studying specific characters.

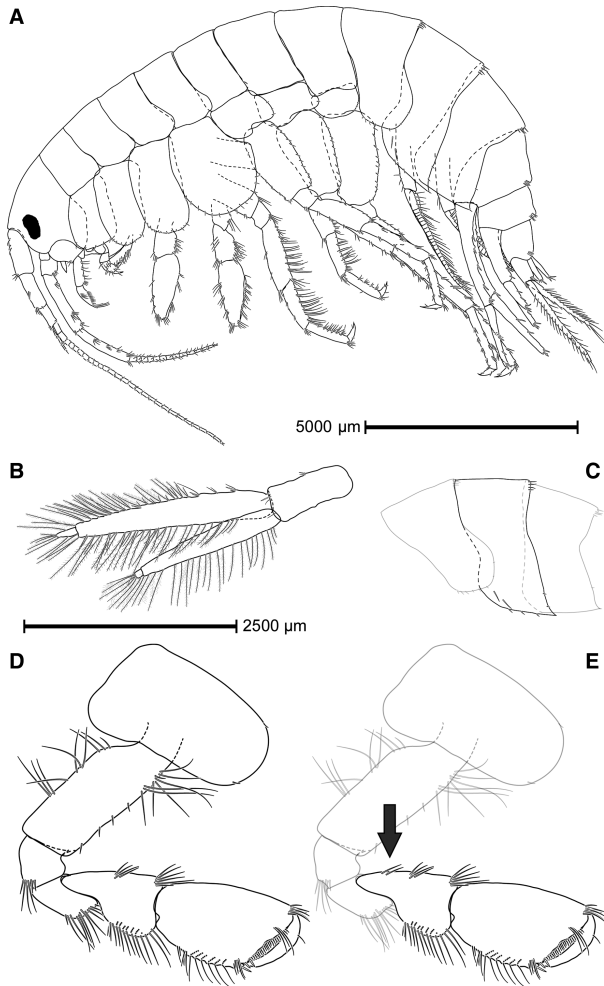
#### Holotype

The male holotype is deposited in the collection of the Musée de Zoologie, Lausanne, Switzerland



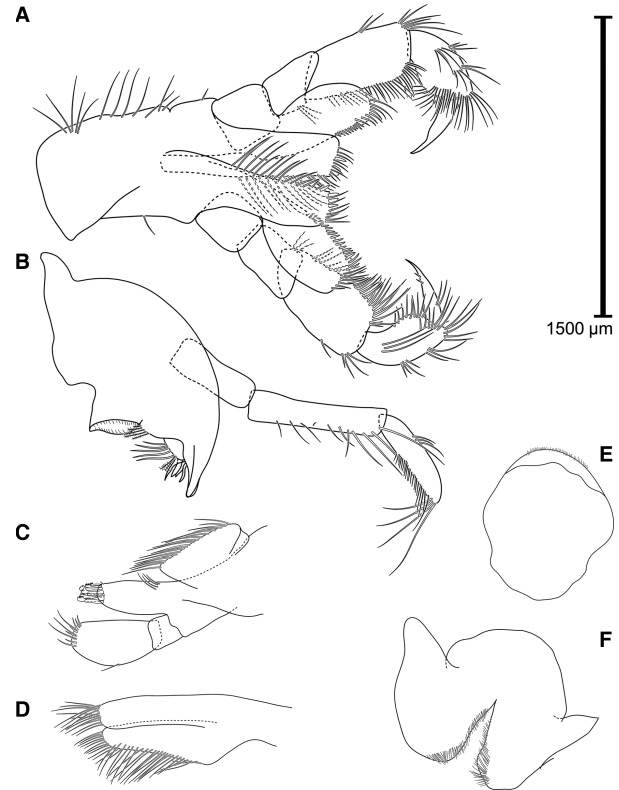
**Figure 4.** COI haplotype network. Haplotype network based on COI sequences calculated as a median joining network. Circles represent distinct haplotypes and different colours indicate sampling localities.





**Figure 5.** Habitus and detail drawings of *Gammarus alpinus* sp. nov. (♂, 21 mm, Lej da San Murezzan, Switzerland). A, habitus; B, uropod 3 with long inner ramus; C, epimeral plate 2 with sharply pointed posteroinferior corner; D, gnathopod 2; E, derived and simplified gnathopod of *G. lacustris* indicating the second group of setae along anterior margin of carpopodite of gnathopod 2 to visualize the distinctive character between the sister species. Drawings by Vid Švara.

(voucher number GBIFCH00329236), along with four paratypes (2♂; GBIFCH00329237 & GBIFCH00329238, 2♀; GBIFCH00329239 & GBIFCH00329240) from the same location, collected by R. Alther, 27.VIII.2014. Ten additional paratypes (5♂, 5♀) from Lej da Silvaplana (9°47026.97 E, 46°27017.89 N), very close to the type locality, are deposited in our own collection at Eawag, Dübendorf. Additionally, DNA vouchers of analysed specimens are deposited in our own collection.



**Figure 6.** Mouthparts of *Gammarus alpinus* sp. nov. (♂, 21 mm, Lej da San Murezzan, Switzerland). A, left and right maxilliped; B, mandibular palp, inner face; C, maxilla 2; D, maxilla 1; E, labium/upper lip; F, labrum/lower lip. Drawings by Vid Švara.

#### Etymology

The species epithet, *alpinus*, refers to its distribution throughout the European Alps, its probable endemic status there and its habitat preference for alpine lakes.

#### Diagnosis

Morphologically nearly identical to *G. lacustris*, it is a comparably large and robust species (Fig. 5A). *Gammarus alpinus* has only one clear group of two to six setae (and very rarely an isolated seta) along the anterior margin of the carpopodite of gnathopod 2 (Fig. 5D), while *G. lacustris* has two distinct groups that harbour five to eight setae in total (Fig. 5E).

#### Description

Body length: maximum length (TOT, see Supporting Information, Table S5) within 77 individuals from 18 localities (Supporting Information, Fig. S3) is 20 mm (♀, Lac de Roy, France). Adding the length of first antenna results in more than 32 mm; maximum length of tip of rostrum to tip of third uropod is 25 mm. Females slightly but significantly larger than

males (based on a Welch two sample *t*-test, lengths with and without first antenna; mean length of ♀♀ = 22.17 and 15.00 mm; mean length of ♂♂ = 20.07 and 13.68 mm; *P* = 0.043 and 0.036, respectively).

Head: eyes roundish or suboval, bean-like form widely found in the genus *Gammarus*; lateral cephalic lobe subrounded.

Antenna 1: seldom much longer than one-third of total body length (TOT + antenna 1), never half of it; setation relatively short; main and accessory flagella with 12–33 and 2–3 (up to 5) segments, respectively; length ratio of peduncular segments 1:2:3 = 1:0.83:0.53; peduncular segments 2 and 3 usually with one tuft of setae each.

Antenna 2: slightly longer than half of the first antenna; prominent and pointy tip of the glandular cone reaches peduncular segment 4; peduncular segments 4 and 5 armed with tufts of setae, usually arranged in three longitudinal rows; short and slender flagellum (4–16 segments, usually about 10) never with flag-like brush or swollen appearance; calceoli may be present but not prevalent and no apparent pattern for presence or absence has been found.

Mandibular palp: third segment (Fig. 6B) armed with one group of A-setae, one or two groups of B-setae, 18–30 D-setae and 4–5 E-setae.

Metasome: segments 2 and 3 (Fig. 5C) can harbour some small setules along their posterior margins.

Urosome: even, without excavations or elevations.

Gnathopod 1: propodus piriform in both sexes; palmar margin with a single but strong medial spine; 3–12 angular spines along inferior margin of the propodus.

Gnathopod 2 (Fig. 5D): propodus with a more rectangular form; no prominent sexual dimorphism in shape; almost exclusively only a single group of setae present along anterior margin of carpopodite (exceptions in 2.6% or two out of 77 individuals), harbouring 2–5 setae (one specimen had 6).

Pereiopods: armature comparable to *G. lacustris*; no enlarged or prominent postero-inferior lobe in basis of pereiopods 5, 6 and 7; dactylus of P3–P7 distinctively more slender than in most other members of the the *Gammarus pulex* group (*sensu* Karaman & Pinkster, 1977b).

Epimeral plates: postero-inferior corner of the first epimeral plate somewhat rounded, with a merely pointed and retracted edge, harbouring one seta; postero-inferior corner of second and third plate always sharply pointed (Fig. 5C); inferior margin of the epimeral plates harbours setae and often spines, with highly variable absolute number and ratio of spines to setae; often setae or spines on the lateral surface of the second epimeral plate; few short setae may be implanted along the posterior margin of the last two epimeral plates, spines were never recorded;

number of spines and setae usually differs between left and right side of the individual.

Uropod 3: endopoditeneary reaches length of the first exopodal segment (Fig. 5B); second exopodite segment usually well developed; proportion of inner to outer ramus between three-quarters and 1; setae along inner and outer margins in both endo- and exopodite virtually always plumose.

Telson: lobes sparsely armed, usually with no more than one or two terminal spines and some setae, most of them usually at least as long as the spines; sometimes a subbasal spine can be found on the surface of the telson lobe in large individuals of both sexes.

Overall habitus: colour of live specimens is greyish to brown or rather white, depending on habitat in which they are found. Specimens can be covered with algae, especially around tufts of setae, depending on habitat.

Molecular diagnosis: affiliation to BIN BOLD: ACH6111 or BOLD:ADB3370 (<http://www.boldsystems.org>) corresponds to belonging to *Gammarus alpinus*. In detail, within the aligned sequences of COI (658 bp, 5'-GACATTATATTTTGTGTTTGTAG...-CGTTTTAGCCGGAGCTATCA-3') we identified 14 SNPs, which are diagnostic for the alpine clade compared to the original *G. lacustris* (positions 103, 160, 163, 187, 211, 220, 274, 337, 343, 347, 370, 448, 523, 553 in 5'–3' reading direction). One SNP at site 337 even allows the distinction between all three clades. Nucleotide 'T' indicates the initial *G. lacustris*, whereas 'A' indicates the south-eastern alpine clade and 'C' indicates the north-eastern alpine clade.

#### Variability

The pattern of variability observed in *G. alpinus* reveals no significant differences among populations and the variability present is associated with postembryonic developmental changes as well as sexual differences.

#### Distribution

The species is restricted to the European Alps and has been recorded in Austria, France, Germany, Italy, Slovenia and Switzerland. Its possible distribution in the Balkans and Apennines (Pljakic, 1952; Pljakić, 1963; Iannilli & Ruffo, 2002) remains to be confirmed.

#### Remarks and affinities

The morphological distinction from its sister species, *G. lacustris*, comes down to two stable quantitative characters and one qualitative character. One is the absence of a second group of setae (often as a single seta) along the anterior margin of the carpopodite of gnathopod 2 (present in *G. alpinus*). The second is that the anterior group of these setae harboured five

or fewer setae (a single specimen was an exception), whereas in *G. lacustris* all studied specimens had at least five setae implanted (Fig. 5E) when summing up both setae groups (Supporting Information, Fig. S4). Additionally, the number of D-setae on the third segment of the mandibular palp seems to be slightly lower in *G. alpinus*, but without a clear cut-off allowing a reliable distinction. Whereas the structure of the epimeral plates is still a valid and strong discriminating character of the *G. lacustris* complex from other species of the *Gammarus pulex*-group, this character cannot be used in the discrimination of *G. lacustris* from *G. alpinus*. We thus suggest a distinction based on a barcoding approach, by extracting DNA from a pereopod, applying the COI-barcoding protocol provided in this paper and aligning the resulting sequence to our published sequences on BOLD (BIN BOLD:ACH6111 or BOLD:ADB3370). Furthermore, we reason that individuals from France, Switzerland or southern Germany that would have been identified as *G. lacustris* until recently can be assigned to *G. alpinus* with a high degree of certainty.

### Ecology

*Gammarus alpinus* has a bimodal elevational distribution (Fig. 7). It predominantly inhabits alpine lakes above 1500 m a.s.l. but also inhabits lowland lakes at around 300–500 m a.s.l. in close vicinity to the Alps. These two habitat types do not correspond to the molecular clades found in *G. alpinus*. The species has never been recorded in headwater streams. A few specimens were found downstream of Lake Constance as well as in small tributaries to Lake Constance close to the inlets (Altermatt *et al.*, 2016). Whereas the first observation can be explained by passive drift, the findings in the inlets clearly suggest some ability to persist in slow running and small streams and creeks. *Gammarus alpinus* was often found to hide in conifer debris (R.A. & F.A., pers. observa.). It can persist at very low temperatures close to 0 °C for a long time (findings in ice-sealed lakes).

### Zoological nomenclature

In accordance with the ICZN Code of Nomenclature the new species name and status are registered in the Official Register of Zoological Nomenclature (ZooBank). ZooBank Life Science Identifier (LSID) for new species. <http://zoobank.org/urn:lsid:zoobank.org:act:C1A6A35D-6F5E-4BD9-98D8-33AF8917C816>.

## DISCUSSION

### A NEW SPECIES AND ITS DISTRIBUTION

We provide the first overview of the *G. lacustris* complex in the European Alps. The clear pattern of a

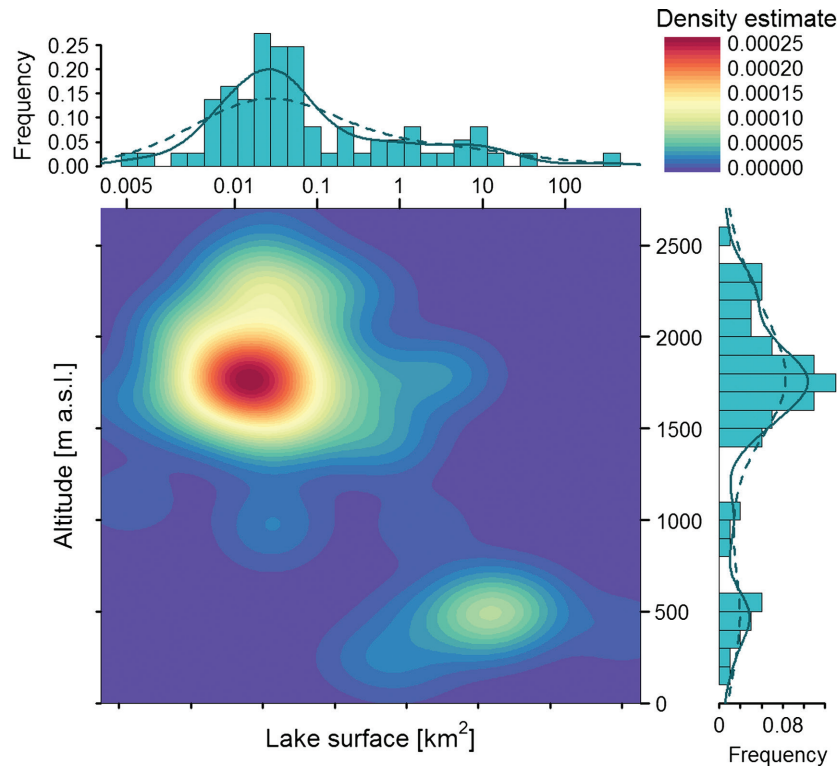
highly divergent lineage compared to the circumboreal *G. lacustris* s.s. is surprising, given the well-known occurrence in the Alps. We raise the alpine populations to species level based on consistent and stable morphological and molecular support, as well as by the clear geographical separation from its sister species. We consider that this separate status is stable and is strongly justified according to the concepts reviewed by De Queiroz (2007).

The newly described *G. alpinus* is probably endemic to the Alps, and does not overlap in its range with *G. lacustris*. Further analysis is needed to determine its distribution in Italy and Austria. Particular focus should be laid on the reported Apenninian populations of *G. lacustris* (Iannilli & Ruffo, 2002). As they were described as genetically similar to alpine specimens, we suspect that they are representatives of *G. alpinus* as well. Sequences from work by Meyran & Taberlet (1998) could not be directly included as their COI fragments were generated with different markers (COI a-H and COI-Gf) and the sequences do not match with the standard Folmer region. On a qualitative level, however, their findings of diverging lineages within the French populations are consistent with our overall findings. Whereas sequences from the northern French Alps match closely together with the Swiss sequences, sequences from the southern French Alps are slightly diverging and match better to sequences from the northern Austrian Alps.

### EVOLUTION AND BIOGEOGRAPHY OF THE *G. LACUSTRIS* SPECIES COMPLEX

The previous view of *G. lacustris* as a homogeneous species is no longer justifiable, even though the truly circumboreal distribution still holds (Väinölä *et al.*, 2008). Recent data from Asia Minor (Katouzian *et al.*, 2016) and our data reveal a remarkable fragmentation in some southern and disjunct parts of the *G. lacustris* complex distribution. All fragmentation occurred in montane areas south of 48°N, whereas populations in the north seem to be homogeneous. Previous findings on the worldwide molecular diversity of *G. lacustris* with a maximum value of 3.5% pairwise sequence divergence in COI (Hou, Li & Li, 2009) are now contrasted with two examples of divergence from its sister species in separate regions [Irano-Anatolian/Caucasus (Katouzian *et al.*, 2016) and European Alps (our study) biodiversity hotspots]. This new pattern indicates that geography and ecology of the species complex interact in the speciation process.

As observed in other alpine species, we initially assumed a divergence due to geographical separation during past glacial maxima with different refugia.



**Figure 7.** Habitat preferences of *Gammarus alpinus* sp. nov. Contour plot of the kernel density estimate of the altitudinal vs. lake surface distribution of all 78 lakes (out of 201 lakes) with reported populations of *G. alpinus* throughout the Alps. A slight bimodal pattern in both histograms highlights that the species can occur in large, pre-alpine lakes, such as Lake Constance, but is predominantly found in high-elevation, small alpine lakes. Kernel density estimates for the histograms are based on a smoothing bandwidth of  $1\times$  (solid line) and  $2\times$  (broken line) the SD of the kernel.

However, the estimated age of the split appears to have started before the beginning of the Quaternary in the late Miocene or early Pliocene. But as the split probably pre-dates the Pleistocene, we hypothesize that while glaciation of the Alps may not have started the speciation process, it is likely to have enhanced it, and the disconnection favoured the divergence into two separate species. Notably, during glacial maxima, *G. alpinus* populations were geographically much closer to Holarctic *G. lacustris* populations but they did not merge. This indicates that the two species might have been well differentiated by that time.

*Gammarus alpinus* occurs almost exclusively in areas draining into the Black Sea and the Mediterranean Sea (Fig. 1), indicating a southern refugium during glacial maxima. Moreover, this is supported by the absence of *G. alpinus* for most of the river Rhine drainage basin except around Lake Constance, which supports a split that could be dated back to a geological era when the predecessor of the alpine Rhine and Lake Constance were still draining into the Danube system (before 450 000 years ago,

Berger *et al.*, 2005; Keller, 2009; Heuberger, Büchi & Naef, 2014). Even after warming in the Holocene, populations remaining in the colder Alps remained disconnected from the rest of the boreal populations due to the remoteness of the Alps from other occurrences of *G. lacustris* and warmer regions representing a barrier towards the north for cold-adapted *G. alpinus*.

Further studies in Italy and especially the Apennines could reveal additional information on the biogeographical history of *G. alpinus* in Central Europe. Certainly, the *G. lacustris* complex shows a completely different speciation pattern as detected in *G. balcanicus* and *G. fossarum* with their main speciation occurring 20 Mya in the Paratethys (Mamos *et al.*, 2014, 2016; Copilaş-Ciocianu & Petrusek, 2015). The current distribution and connectivity of habitats play a major role in species diversity and diversification. Whereas a patchy distribution might favour species divergence (Lefébure *et al.*, 2007; Trontelj *et al.*, 2009), a continuous distribution can have a homogenizing effect by dispersal and admixture of populations. Our lacustrine

samples cover a comparable geographical range to the related species *G. fossarum* (e.g. Westram *et al.*, 2011). However, we did not find greater diversity as compared to the better connected species such as *G. fossarum* (e.g. Weiss *et al.*, 2014), but a quite similar level of morphological diversity and even lower genetic divergence within *G. alpinus*. This implies a temporal rather than a spatial explanation for the low diversity found within COI and 12S sequences of the focal complex.

Clearly, more data are needed to explore the enigmatic evolutionary history and biogeography of this species complex. On the one hand, less conservative markers might bring insight into dispersal capacities of the circumboreal *G. lacustris* and perhaps help to identify the role of homogenizing factors such as transport by birds (Segerstråle, 1954) or even human-driven displacement, such as by fishermen or as a result of commercial bait trade. On the other hand, the taxonomic status of populations in mountain lakes of central Asia needs to be revised. Although sequences within GenBank included in our analysis from Tibet and the Himalayan region belong to *G. lacustris*, more cryptic species from Central Asia should not be ruled out.

#### CONSERVATION IMPLICATIONS

Invasive species are a major threat to aquatic species, especially to amphipods, which contain numerous invasive species themselves (Havel *et al.*, 2015). Interestingly, we rarely found another species of amphipod co-occurring in the sampled alpine lakes, suggesting that invasive species are not yet a threat to high-elevation populations of *G. alpinus*. The situation of *G. alpinus* in the lower elevation lakes is different. Recent invasions into many of these lakes (Mürle, Becker & Rey, 2003; Baćela-Spychalska *et al.*, 2013) represent a threat to diversity (Dick & Platvoet, 2000; Bollache *et al.*, 2004). The most prominent example is the Pontocaspian amphipod *Dikerogammarus villosus* (Sowinsky, 1894), which has become an invasive species throughout Western Europe over the last few decades. It is already outcompeting species in larger lowland lakes, for example Lake Constance. Hence we suggest that high alpine lakes can be relatively undisturbed refugia for highly specialized freshwater species. However, once these ecosystems are disturbed and *G. alpinus* becomes locally extinct, recolonization may be strongly limited by the remoteness of the lakes. In summary, the impact of climate warming on cold-adapted *G. alpinus* per se, or in interaction with elevated pressure by invasive species, has unknown effects on this endemic gammarid and opens new questions regarding conservation and species persistence.

#### CONCLUSION

Combining molecular analysis with morphometric measurements we identified a novel species of a specialized and possibly endemic lacustrine *Gammarus*, distinct from its prominent sister species *G. lacustris*. We resolve the cryptic status and describe it as *G. alpinus* sp. nov., providing information on its molecular and morphological identification as well as on its distribution and ecology. The current distribution and its palaeogeographical explanation fit well with molecular data and suggest an early Pliocene diversification from its sister species *G. lacustris*. The species status is justified by its stable geographical separation. Its endemic status highlights the conservation importance of remote and high alpine lakes, which are threatened due to land-use change (especially eutrophication), climate warming and invasive species.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article:

**Figure S1.** ML-inferred phylogenetic tree of Gammarids found in the Alps.

**Figure S2.** Pairwise differences of 12S sequences.

**Figure S3.** Sampled lakes and origin of specimens for molecular analysis.

**Figure S4.** Morphological difference in gnathopod 2 between *G. alpinus* sp. nov. and *G. lacustris*.

**Table S1.** Publications screened for information on the occurrence/records of *G. lacustris* in alpine and pre-alpine freshwater habitats.

**Table S2.** Sampled lakes (102 total).

**Table S3.** BOLD BINs and GenBank accession numbers of newly generated COI and 12S sequences.

**Table S4.** GenBank accession numbers of available and included COI sequences [accessed 12 January 2015].

**Table S5.** Measured morphological characters for morphometric analysis.