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Is subterranean lifestyle reversible? Independent and recent large-scale dispersal into surface waters by two species of the groundwater amphipod genus *Niphargus*

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Abstract

Groundwater is an extreme environment due to its absence of light, resource scarcity and highly fragmentary nature. Successful groundwater colonizers underwent major evolutionary changes and exhibit eye and pigment loss (troglomorphies). Consequently, their chances of dispersal and survival in the well-connected surface waters are greatly decreased, resulting in significant endemism. The West Palaearctic subterranean amphipod genus *Niphargus* comprises hundreds of narrowly endemic and troglomorphic species. Nevertheless, a few are known to occur in surface waters, two of which, *N. hrabei* and *N. valachicus*, have extremely large ranges that even exceed those of many surface-water amphipods. We studied whether this pattern results from a secondary colonization of the relatively well-connected epigean environment, and that this ecological shift promoted the large-scale dispersal of these species. Results showed that despite their ecological and zoogeographic similarities, *N. hrabei* and *N. valachicus* are not closely related and independently colonized surface waters. Their phylogeographic patterns indicate Middle to Late Pleistocene dispersal episodes throughout the Danube lowlands, and relatively modest yet significant genetic differentiation among populations. Clustering based on morphology revealed that the two species are phenotypically closer to each other than they are to most other epigean congeners. We presume that the ecological shift to surface environments was facilitated by their ability to thrive in hypoxic waters where rheophilic competitors from the family Gammaridae cannot survive. In conclusion, our results indicate that adaptation to groundwater is not a one-way evolutionary path and that troglomorphic species can occasionally recolonize and widely disperse in surface waters.

1. Introduction

Groundwater macrofaunal species represent a substantial part of freshwater diversity in Europe (Zagmajster et al., 2014). Among the most remarkable features of groundwater fauna is high endemicity (Trontelj et al., 2009; Eme et al., 2017). The key mechanism underlying narrow endemism is weak dispersal, presumably reflecting the physical and ecological properties of groundwater habitats that are fragmented and poorly connected (Strayer, 1994; Lefébure et al., 2006, 2007; Eme et al., 2013). There are only a few widely distributed groundwater taxa, usually meiofaunal species (< 1 mm), living in better connected environments such as hyporheic alluvial habitats along rivers (Ward and Palmer, 1994). Furthermore, groundwater is a challenging environment due to its permanent darkness and resource scarcity (Gilbert et al., 1994; Hüppop, 2000). Consequently, groundwater species display a suite of convergent adaptations (troglomorphies) such as eye loss, depigmentation, body and appendage elongation, low metabolic rates, and resistance to hypoxia (Malard and Hervant, 1999; Hüppop, 2000). The apparent cost of their specialization is a lower ability to cope with the ecological conditions of photic environments. These are stressful for groundwater inhabitants, due to, e.g., damage from ultraviolet light because of depigmentation (Ginet, 1960; Maguire 1960; Langecker, 2000) or strong interspecific competition from well adapted and more prolific surface-water relatives (Fišer et al., 2007; Sket, 2008; Luštrik et al., 2011). Therefore, subterranean species seem to be restricted to the fragmented subsurface, their dispersal through the better connected surface waters is limited, and species ranges greater than 200 km are exceptional (Trontelj et al., 2009). Thus, these patterns from groundwater are an excellent case illustrating how evolutionary processes can shape macroecological patterns (discussed by Weber et al., 2017).

Niphargus is the most diverse genus of freshwater amphipods, comprising over 400 species distributed in West Palaearctic groundwaters (Väinölä et al., 2008; Esmaeili-Rineh et al., 2015; Horton et al., 2017). *Niphargus* species are ecologically diverse, inhabiting almost all types of aquatic subterranean habitats, from deep cave lakes to small pores in the epikarst (Fišer, 2012; Fišer et al., 2006, 2014). Several species are not strictly limited to groundwater and, in addition to permanent subterranean populations they also have stable populations in surface or ecotonal habitats (Karaman, 1977; Fišer et al., 2006, 2014; Copilaş-Ciocianu et al., 2017a). The great majority of *Niphargus* species are narrow-range endemics, most being known only from their type localities (Fišer et al., 2008; Eme et al., 2017). Genetic evidence suggests that most of the presumably widespread taxa within *Niphargus* are actually complexes of endemic cryptic species (Lefébure et al., 2006, 2007; Delić et al., 2017).

Two similar species from the middle and lower Danube lowlands (Southeast Europe) remarkably break this general pattern: *N. hrabei* Karaman, 1932 and *N. valachicus* Dobreanu and Manolache, 1933 (Copilaş-Ciocianu et al., 2017a). They inhabit the muddy, dimly lit and densely vegetated substrate near the shores of slow-flowing or stagnant water bodies and have rarely been reported from groundwater habitats (Mejering et al., 1995; Copilaş-Ciocianu et al., 2017a and references therein). This, coupled with the seasonal life-cycle of *N. valachicus* (Copila□-Ciocianu and Boro□, 2016), implies they are more tied to the surface than to the subterranean environment. The two species have the widest known geographical ranges of any niphargid (>1300 km), rivalling those of many epigean amphipods (Neseman et al., 1995; Borza et al., 2015; Copilaş-Ciocianu et al., 2017a). They exhibit a shallow genetic divergence among distant populations (Copilaş-Ciocianu et al., 2017a) in comparison with other amphipods from the same region (Meleg et al., 2013; Copilaş-Ciocianu and Petrusek 2015, 2017), suggesting relatively efficient dispersal in the recent past, though details about their phylogeographic histories and dispersal mechanism are unknown.

Based on previous phylogenetic studies (e.g. McInerney et al., 2014; Esmaeili-Rineh et al., 2015; Delić et al., 2016), it appears that surface-water affinity in *Niphargus* might not be the ancestral condition, indicating that surface dwelling species could be derived from subterranean ancestors. However, this assumption has neither been postulated nor tested. The epigean lifestyle of *N. hrabei* and *N. valachicus* coupled with their troglomorphic phenotypes further point out that they secondarily colonized surface-waters. This secondary transition to ecotonal / epigean habitats might explain the large geographic ranges of both species. Therefore, the first aim of our study was to investigate if indeed surface-water *Niphargus* species are derived from groundwater ancestors and if so, to infer how many surface colonization events have occurred during the evolutionary history of the genus. Second, we examined the phylogeographic consequences of surface colonization by inferring the spatio-temporal dispersal patterns of *N. hrabei* and *N. valachicus*. Finally, we tested whether the similar ecology of these species is reflected in their morphological similarity.

2. Material and methods

2.1. Sampling, laboratory protocols, sequence alignment and assembly of datasets

Specimens were collected throughout the distribution range of both species between 2009 and 2016 by sweeping a hand net through the dense riparian vegetation of various water bodies. After collection, animals were fixed in 95% ethanol. Depending on sample size, between one and six individuals per sampling locality were molecularly analysed. A total of 19 and 38 localities for *N. hrabei* (54 individuals) and *N. valachicus* (111 individuals), respectively, were investigated (Fig. 1, Table S1).

Genomic DNA was extracted using the Genomic DNA Mini Kit for tissue (Geneaid Biotech Ltd, Taipei). For phylogeographic purposes we used fast evolving mitochondrial and nuclear markers as they can provide phylogenetic resolution at fine spatio-temporal scales. As such, we sequenced a part of the mitochondrial gene for cytochrome c oxidase subunit I (COI) and a substantial fraction of the nuclear internal transcribed spacer (ITS1, 5.8S rRNA and ITS2). These markers proved useful in a preliminary study of the genetic variation in the two focal species (Copilaş-Ciocianu et al., 2017a). Amplification of the COI and ITS fragments followed protocols of Copilaş-Ciocianu et al. (2017a) and Flot et al. (2010a), respectively. For phylogenetic purposes we additionally sequenced two parts of the large ribosomal subunit (28S) and the histone H3 gene (H3), following the protocols in Fišer et al. (2013). These nuclear markers are more conserved and provided sufficient resolution for uncovering the niphargid phylogenetic relationships (e.g. Trontelj et al. 2012, Fišer et al., 2013). Details about primers are provided in Table S3. The length of amplified fragments, and numbers of variable and parsimony informative sites are presented in Table S4.

The protein-coding COI and H3 sequences were aligned with MUSCLE (Edgar, 2004) in MEGA 6 (Tamura et al., 2013) and checked for possible evidence of pseudogenes (i.e., presence of stop codons or reading frame shifts) by subsequent amino acid translation. The ITS and 28S fragments were aligned with MAFFT (Katoh and Standley, 2013) with the Q-INS-i option (Katoh and Toh, 2008). Indels and regions of questionable homology in the 28S marker were identified and removed with GBLOCKS 0.9 (Talavera and Castresana, 2007). Double peaks in the ITS chromatograms (indicating heterozygosity or multiple gene copies) were coded according to the IUPAC ambiguity codes and haplotypes were phased with SeqPHASE (Flot, 2010) and PHASE (Stephens et al., 2001). Contigs were assembled using DNA Baser 4 (Heracle BioSoft 2013; www.DnaBaser.com).

For the analyses of phylogeography, we complemented the newly obtained dataset of both species (41 and 100 individuals of *N. hrabei* and *N. valachicus*, respectively) with additional COI and ITS data from previous studies (Flot et al., 2014; Copilaş-Ciocianu et al., 2017a) (see Table S1). For phylogenetic analyses, we gathered a large dataset comprising 157 ingroup taxa from 21 previous studies (see Table S2). We used the family

Pseudoniphargidae as an outgroup since it is a sister clade to niphargids (Jurado-Rivera et al., unpublished). The concatenated supermatrix contained 28% missing data.

2.2. Phylogeny, topology tests and ancestral state reconstruction

Phylogenetic analyses were carried out to investigate the phylogenetic position of *N. hrabei* and *N. valachicus* within the genus and to infer whether their presence in surface waters is due to a secondary colonization from subterranean habitats. Potential loss of phylogenetic signal due to substitution saturation at the COI marker was inspected using the test of Xia et al. (2003) implemented in DAMBE 5.3 (Xia and Xie, 2003). PartitionFinder 1.1.1 (Lanfear et al., 2012) was used to determine the best fitting evolutionary models and partitioning schemes by employing the greedy algorithm and the Bayesian information criterion. Models and partitions are shown in Table S4.

We used Bayesian inference (BI) and maximum-likelihood (ML) approaches to reconstruct phylogenetic relationships within *Niphargus* using the concatenated supermatrix approach in BEAST 1.8.0 (Drummond et al., 2012) and RAxML-HPC 8.2.9 (Stamatakis, 2014). For the BEAST analysis we used the initial alignment from which poorly alignable regions in the 28S marker were removed (see previous section). For RAxML we used an alignment which kept these regions and was produced with SATé 2.2.7 (Liu et al., 2009). Further details on analysis settings and evolutionary models are provided in Supplementary Information.

To test whether the two focal taxa that show many ecological, morphological and biogeographic similarities are sister species that represent a single surface water colonization event, an alternative topology where they were constrained to monophyly was compared with the unconstrained phylogeny by applying the Shimodaira-Hasegawa (SH;

Shimodaira and Hasegawa, 1999) and the approximately unbiased tests (AU; Shimodaira, 2002) implemented in the software Treefinder (Jobb, 2011).

We evaluated if, and how many times, groundwater *Niphargus* species secondarily colonized the epigean / shallow subterranean habitat by mapping species habitat onto a phylogenetic tree. The habitat preferences were inferred from available publications and unpublished field data; we treated them as a binary character, simplified to "subterranean" and "surface". The state "subterranean" refers to an exclusively stygobiotic lifestyle, while "surface" encompasses a broad array of habitats at the boundary between the surface and subterranean environments, including roots of submerged plants, forest ditches, *Sphagnum* moss, springs, and shallow subterranean habitats (hypotelminorheic). Ancestral states were inferred with likelihood and Bayesian methods using 1000 post burn-in trees from the BEAST analysis to account for phylogenetic uncertainty. Likelihood mapping using Markov k-state 1 parameter model was performed in Mesquite 3.04 (Maddison and Maddison, 2015). The character state at the root was estimated from the model (Mesquite default setting) and not constrained as in the original method of likelihood reconstruction (Schluter et al., 1997). Bayesian mapping was performed using Bayes Traits v. 3 (Meade and Pagel, 2016). Priors for evolutionary rates from "surface" to "subterranean" and from "subterranean" to "surface" were drawn from a uniform distribution between 0 and 100. The selection of the optimal model of the evolutionary rates was made by comparison of Bayes factors (function *stepping stone*). The best performing models allowed for traits to vary their rate of evolution within and between branches (function *covarion*, adds one additional parameter to the model) (Table S5). Among these models, there was no significant difference in marginal likelihoods if the transition rates from "surface" to "subterranean" and *vice versa* are identical or not. For this reason, we selected a simpler model (function *restrict*, one parameter less, i.e., forward evolutionary rate is equal to backward rate); the final model had two parameters (a single evolutionary rate modified by covarion). We ran 1 010 000 iterations, which were sampled every 1000th generation with burn-in of 10 000. The analyses were repeated four times to check for the consistency of the results. Acceptance rate between 20-40% was achieved by an automatic tuning method implemented in Bayes Traits v.3 as a default.

2.3. Phylogeographic analyses

The number of haplotypes (H), segregating sites (S), haplotype (Hd) and nucleotide diversity (π) and mean number of pairwise nucleotide differences (K) were calculated for *N. hrabei* and *N. valachicus* and their intraspecific clades (see Results) with DnaSP 5.1 (Librado and Rozas, 2009) using both the COI and ITS markers.

In order to explore intraspecific patterns of genetic diversity, we constructed haplotype networks and time-calibrated phylogenetic trees. The models and partitions were estimated with PartitionFinder 1.1.1 and are shown in Table S4. Haplotype networks were constructed for both COI and phased ITS sequences with Haploviewer (Salzurger et al., 2011); maximum likelihood trees inferred with MEGA 6 with the models presented in Table S4 were used as input. Unique haplotypes were selected with the online tool FaBox (Villesen, 2007; http://www.birc.au.dk/software/fabox) and were used to build time-calibrated COI gene trees for both species using BEAST 1.8.0. The best fitting coalescent (constant size, logistic, expansion and exponential growth) and clock models (strict and relaxed) were selected using the modified Akaike information criterion (AICM) with moment's estimator (Baele et al., 2012) in TRACER 1.6 (Drummond and Rambaut, 2007) with 1000 bootstrap replicates. The best clock and coalescent models are shown in Table S6. The MCMC chain was run for 20 million generations and sampled every 1000 generations. Effective sample size of parameters was checked using TRACER 1.6; values of at least 200 were considered appropriate. The first 20% of trees were discarded as burn-in and the maximum clade credibility tree was built using TreeAnnotator 1.8.0 (Drummond and Rambaut, 2007).

The main goal of the dating analysis was to provide an approximate estimation of the time frame of dispersal (i.e. Pleistocene vs. Pliocene or Miocene) and not to pinpoint specific historical factors responsible for the dispersal events. Because the rates of molecular evolution seem to be time-dependent, intraspecific rates can evolve faster than interspecific ones (Ho et al., 2005, 2011; but see Emerson and Hickerson, 2015). This phenomenon has been observed in malacostracans as well, where the intraspecific clock rates vary from 6.58% Ma^{-1} (mantis shrimp *Haptosquilla oulchella*, Crandall et al., 2012) to very fast post-glacial rates of up to 27% Ma^{-1} (mysid *Mysis salemaai*, Audzijonyte and Väinölä, 2006). However, because we do not have any strong *a priori* assumption of fast post-glacial rates, we prefer the 6.58% Ma^{-1} rate as it is intermediary between the interspecific rate of 2.3% Ma^{-1}, commonly used in dating amphipod divergence (e.g. Lefébure et al., 2006; Copilaş-Ciocianu and Petrusek 2015), and the other extreme of very fast intraspecific post-glacial rate of 27% Ma^{-1}. Furthermore, the magnitude of divergence and the time scale of our study are also intermediary (see Results).

The following landscape genetic analyses were performed on the COI marker because it had a greater variability than ITS and sufficient sample size. To test for a pattern of isolation by distance (IBD), we performed a Mantel test in the software Alleles In Space (Miller 2005) using pairwise p-distance values as a measure of genetic distance and 1000 replicates. The genetic population structure was examined using an analysis of molecular variance (AMOVA; Excoffier et al., 1992) in ARLEQUIN 3.5 (Excoffier and Lischer, 2010) by grouping the sampling sites according to the main river drainages. Tests were performed with Tamura-Nei distances and significance was assessed using 10 000 permutations. Population differentiation among drainages was investigated using pairwise fixation indices (Φ_{ST}; Weir and Cockerham, 1984) computed with the Tamura-Nei model in ARLEQUIN. Significance levels were assessed using 10 000 permutations. Correction for multiple testing was performed with the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) in the software SGoF+ (Carvajal-Rodriguez and de Uña-Alvarez, 2011). In the case of *N. hrabei*, four individuals were removed from the AMOVA and Φ_{ST} analyses as they were the only representatives of their respective drainages.

The historical demographic patterns of both species were explored using three approaches based on the COI data. First, to examine if a demographic expansion took place recently, we employed three neutrality tests: Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997), implemented in ARLEQUIN, and *R2* (Ramos-Onsins and Rozas, 2002) in DnaSP 5.1. Their statistical significance was evaluated using 10 000 simulated samples. As a second test for demographic expansion, we calculated mismatch distributions (Rogers and Harpending, 1992) under a sudden-expansion model in ARLEQUIN with 1000 bootstrap replicates. The validity of this model was assessed using the sum of squared deviations (SSD) and Harpending's raggedness statistic (Hri; Harpending, 1994). Third, Bayesian skyline plot analyses (BSP; Drummond et al., 2005) were employed in BEAST 1.8.0 in order to visualize demographic changes through time. The same clock rate and evolutionary models were used as for the time calibrated phylogenetic analyses (see above). The MCMC chain was run for 50 million generations, sampled every 1000 generations and the first 10% of trees were discarded as burn-in. The analyses were repeated three times in order to ensure convergence on the same result. Effective sample sizes were checked with TRACER 1.6.

To explore the origin and dispersal history of each species while accounting for phylogenetic uncertainty, we used continuous Bayesian phylogeographic diffusion models (Lemey et al., 2010) implemented in BEAST 1.8.0. The analysis was based on the COI marker and we used the same settings as for the time-calibrated phylogenetic analyses. Best fitting diffusion models are presented in Table S7 and further details are provided in the Supplementary Information.

2.4. Detection of cryptic lineages

Considering the wide and fragmented range of the studied species, we used the COI and ITS markers to investigate if they are composed of independently evolving cryptic lineages. For the COI dataset (encompassing all individuals of both species) we used a Bayesian implementation of the Poisson tree process on the bPTP server (http://species.h-its.org/ptp) (Zhang et al., 2013). The input phylogenetic tree was generated with PhyML 3.0 (Guindon et al., 2010) using the evolutionary models in Table S4. We ran the analysis for 300 000 MCMC iterations with a thinning of 100 and 20% burn-in. Due to the fact that putative heterozygous individuals were present in the ITS dataset (see Results), potential cryptic lineages were investigated using Doyle's concept of species as fields for recombination (FFRs; Doyle 1995), i.e. assuming that species are characterized by mutual allelic exclusivity. For this purpose, we transformed the haplotype network of phased ITS sequences (obtained as indicated above) into a haploweb by connecting the haplotypes which were co-occurring in heterozygous individuals (Flot et al., 2010b). Interconnection among all haplotypes indicates a common gene pool, and therefore conspecificity under the FFR concept, while groups of haplotypes which are not interconnected might indicate cryptic species or cessation of gene-flow among geographically isolated populations.

2.5. Morphological analyses

In order to test the hypothesis that similar ecology leads to morphological similarity of the two focal species, we analyzed a dataset of 81 *Niphargus* species, of which 15 live in similar habitats as the two focal ones (Tables S2, S8). We measured between 1 and 10 individuals per species. For the analyses we used only adult males; females were considered only in those species that show no sexual dimorphism and only when males were not available. We analyzed 35 traits describing in detail the body shape and size, appendage length, and spine patterns (Fišer et al., 2009) (Table S8). We calculated mean values for the traits for the species and prior to the analysis transformed the data as follows: 1) the number of spines and the body lengths were log-transformed, and 2) in order to remove the impact of body size, all length-measures were regressed onto body lengths, and standardized residuals were calculated. We inferred morphological similarity from cluster analyses using squared Euclidean distances and Ward's agglomeration method. All analyses were run using SPSS ver. 20 (IBM Corp 2011).

3. Results

3.1. Phylogeny, topology tests and ancestral state reconstruction

The substitution saturation test indicated no significant saturation at the COI marker ($p<0.0001$). Both ML and BI analyses on the concatenated dataset revealed similar results. The removal or retention of poorly alignable regions in the 28S marker did not influence the overall phylogenetic pattern (Fig. S1). The topology of the phylogeny is largely congruent with the recent phylogenetic reconstructions of the genus by recovering the same major clades (e.g. McInerney et al., 2014; Esmaeili-Rineh et al., 2015; Delić et al., 2016). All the species inhabiting surface-water habitats, including *N. hrabei* and *N. valachicus*, were recovered in the same major clade; however, the two focal species are not in a sister relationship (Figs 1, S1). The position of *N. valachicus* is recovered at the base of a clade that mostly contains surface-water species, though the support for the clade is not high in the ML analysis. In contrast, *N. hrabei* is clustered together with strong support with two subterranean species, *N. plateaui* and *N. puteanus* (Figs 1, S1). The sister relation of both species (monophyly) was rejected by both the SH and AU tests at $p = 0.036$ and $p = 0.023$, respectively (likelihood unconstrained = -60408.49, AICc = 121554; likelihood constrained = -60430.26, AICc = 121598).

All ancestral state reconstruction methods support the hypothesis that the ancestor of Niphargidae and Pseudoniphargidae was a subterranean species (Fig. 1, Table S9). Both families apparently diversified in the subterranean environment. Both likelihood and Bayesian mapping indicate that the probability of ancestors having lived in a subterranean environment is above 0.95 across all basal splits. Bayesian mapping suggests that the surface and/or shallow subterranean environments were colonized at least four times independently (Fig. 1; nodes 7, 9, 11 and 13), when the probability for a subterranean ancestor abruptly fell below 0.58. However, the likelihood analysis was less conservative and indicated that ecological change took place along terminal branches, i.e. there might have been even more transitions to surface / shallow subterranean environments (Table S9). In the case of *N. hrabei*, the transition occurred along the terminal branch of the tree, while in the case of *N. valachicus*, the transition probably took place in the common ancestor of clade 11. In any case, these results coupled with the topology of the tree indicate that both focal species colonized surface habitats independently from each other.

3.2. Phylogeography

The COI marker was represented by 12 and 42 haplotypes in *N. hrabei* and *N. valachicus*, respectively, and the ITS marker by 6 and 23 haplotypes (Table 1). Eight out of the 22 analysed individuals (36%) were heterozygous at the ITS locus in *N. hrabei*, and 26 out of 40 individuals (65%) were heterozygous in *N. valachicus* (Table 1). The geographical distribution of haplotypes is shown in Fig. 2. Haplotype and nucleotide diversity, and mean number of pairwise nucleotide differences were higher in *N. valachicus* than in *N. hrabei* at both markers (Table 1).

The haplotype networks based on COI indicate that both species have a geographically structured genetic variation, most haplotypes apparently being endemic in relatively narrow parts of the species' ranges (Fig 2A, B). This pattern was less pronounced at the ITS marker where a single haplotype was widespread across the entire range in each of the species (nH1 in *H. hrabei* and nV1 in *N. valachicus*). However, groups of locally restricted haplotypes could also be observed (Fig. 2C, D). The intraspecific COI time-calibrated trees (assuming the COI rate of 6.58 Ma^{-1}) indicate that *N. hrabei* has a shorter mean coalescence time (103 ka, 95% HPD: 56–152 ka) than *N. valachicus* (353 ka, 95% HPD: 214–504 ka) (Fig. 3A). No strongly supported intraspecific lineages could be observed in *N. hrabei*; however, *N. valachicus* was composed of two distinct clades: clade A distributed in the Pannonian lowlands and clade B distributed in the SE parts of the Pannonian lowlands (where it partly overlaps with clade A), Wallachian Plain, Danube Delta and northern Turkey (Fig 1B).

The IBD test revealed a highly significant relationship between geographic and genetic distances in both species (*N. hrabei*, $r^2=0.43$; *N. valachicus* $r^2=0.37$; $p<0.0001$ in both species), indicating that dispersal is limited (Fig. S2). The AMOVA analysis indicated that most of the observed variation is explained by differences among drainages (*N. hrabei*: 80.05%, $\Phi_{\mathrm{CT}} = 0.80$, $p=0.007$; *N. valachicus*: 58.5%, $\Phi_{\mathrm{CT}} = 0.58$, $p<0.0001$) (Table 2). Drainages are listed in Tables S10 and S11. Pairwise Φ_{ST} distances indicated a significant genetic differentiation at COI among most of the main drainages, with 90% and 82% of all pairwise comparisons being statistically significant in *N. hrabei* and *N. valachicus*, respectively (Tables S10 and S11).

The three demographic tests (Tajima's *D*, Fu's *Fs* and R_2) were applied to each species and to each of the two clades of *N. valachicus*. In the case of *N. hrabei*, only Fu's *Fs* was statistically negatively significant ($p = 0.031$), however, the other two tests were close to the significance threshold (Tajima's *D* $p = 0.056$; R_2 p=0.083), indicating recent population expansion (Table 1). In *N. valachicus* overall and in its clade A none of the tests were significant, suggesting a stable populations size, but population expansion was indicated in clade B as Fu's *Fs* was significantly negative (p<0.0001), while the other two tests were close to statistical significance (Tajima's *D* $p = 0.061$; R_2 $p = 0.06$) (Table 1). The mismatch distribution of haplotype pairwise differences was unimodal in *N. hrabei* and the SSD and Hri tests indicate no significant departure from the assumption of rapid population expansion (Fig. 3B, Table 1). In the case of *N. valachicus*, the overall mismatch distribution was bimodal with the SSD and Hri having no statistically significant values (Fig. 3B, Table 1). Clade A had a multimodal mismatch distribution while clade B was unimodal (Fig. 3B). The SSD and Hri tests were not significant in the former, but the Hri was significant in the latter (Table 1).

Taken together the evidence points to a relatively recent and rapid population expansion in *N. hrabei* and *N. valachicus* clade B and a stable population size in clade A. The BSP indicates a population growth in *N. hrabei* during the last 50 ka with an increase in the last 10-15 ka (Fig. 3B). In *N. valachicus* a population growth took place ca. 100 ka, followed by a decline during the Last Glacial Maximum (ca. 25 ka) and another growth during the last 5-10 ka (Fig. 3B).

The different Bayesian phylogeographic diffusion models produced congruent results regarding the presumed past dispersal patterns. The analyses suggest the origin of

N. hrabei's dispersal is in the lower Danube lowlands, ca. 90 ka (Wallachian Plain, S Romania). From there, it has subsequently spread to the west and east multiple times along the Danube River and only recently, likely postglacially, arrived to the upper parts of the river basin (Fig. 4A). In contrast, the dispersal of *N. valachicus* apparently started earlier, in the southwest of the Pannonian lowlands in northern Croatia, ca. 350 ka. It spread to the east, along the Sava and Drava rivers, reaching the Danube and the Wallachian Plain somewhere between 100 and 200 ka. It reached northern Turkey and the north of the Pannonian Plain only in the last 100 ka, possibly postglacially (Fig. 4B). Although we acknowledge a substantial uncertainty of the absolute values, the results based on a conservative mutation rate strongly suggest that dispersal of these species is relatively recent (the second half of the Pleistocene) and that *N. valachicus* has occupied the Danube lowlands before *N. hrabei*.

3.3. Absence of cryptic lineages

Despite the fact that both species have wide and fragmented ranges, the COI and ITS data do not indicate the existence of reproductively separated lineages. The bPTP analysis based on COI supports the conspecificity of all analysed populations in both species (posterior probability of 0.5 and 0.8 in *N. hrabei* and *N. valachicus*, respectively) (Fig. S3A). Furthermore, the haploweb analysis based on ITS indicates that many of the analysed individuals share the same haplotype (nH1 in *H. hrabei* and nV1 in *N. valachicus*) and all haplotypes co-occurring in heterozygous individuals are interconnected (Fig. S3B), indicating a common gene pool.

3.4. Morphological analyses

The morphological analysis split all 81 analyzed species into two major clusters (Fig. 5). One cluster comprises species living in the stagnant water of permanently flooded parts of karstic massifs while the second includes species found in flowing water, interstitial and surface habitats. The first cluster is further split into three subclusters corresponding to lake, lake giant and daddy-longlegs ecomorphs (Trontelj et al., 2012). The second cluster has a more complex structure and its two principal subclades are split even further. Species occurring in surface habitats were clustered into four separate groups. *N. hrabei* and *N. valachicus* along with *N. elegans* from northern Italy were clustered together with small bodied, interstitial and epikarst taxa (small pore ecomorph *sensu* Trontelj et al., 2012). Apparently, they are not very similar to the other species occurring in surface habitats that were rather clustered together with cave stream species (cave stream ecomorph *sensu* Trontelj et al., 2012) (Fig. 5).

4. Discussion

Our results indicate that, despite their ecological and morphological similarities, *N. hrabei* and *N. valachicus* are not closely related. Their phylogenetic position and reconstructed ancestral states imply independent colonization of surface waters from subterranean ancestors. Their phylogeographies reveal large-scale dispersal across the Danube lowlands throughout the Pleistocene, and their morphologies are more similar to each other than to most other congeners known to occur in surface-water habitats. These results indicate that the habitat shift from ground- to surface waters enabled the dispersal and range expansion of these species. Below we discuss the possible factors that have facilitated this ecological shift and examine the biogeographical histories of these two species.

4.1. Reversal to surface waters

It appears that the ecological barrier between subsurface and surface is weaker than previously thought for troglomorphic species. Reversal to surface habitats from subterranean ancestors has been proposed so far in typhlochactid scorpions (Prendini et al., 2010) and in phalangopsid crickets (Desutter-Grandcolas, 1997), but both studies relied on morphology only, and did not test this phenomenon on molecular phylogenies. To our knowledge, our study is the first to use a molecular phylogeny to test whether troglomorphic subterranean species can recolonize surface habitats. In the case of *Niphargus*, all of the surface-water species belong to a large, morphologically and ecologically diverse clade.

Although most of the surface-water *Niphargus* species have known populations both in subterranean and surface / ecotonal habitats (Fišer C. et al., 2006; 2010b; 2014; Fišer Ž. et al., 2015), *N. hrabei* and *N. valachicus* are probably the most detached from the subterranean environment as they have much larger ranges and a far greater number of documented occurrences in surface versus groundwater (Copilaş-Ciocianu et al., 2017a). Therefore, it appears that there were at least two independent colonization events of truly surface-waters and multiple shifts to ecotonal habitats from subterranean ancestors during the evolutionary history of *Niphargus*.

According to the molecular dating analysis of McInerney et al. (2014), the clade that contains the surface-water species has radiated during the Late Eocene. This is in accordance with the amber fossil evidence which indicates that some species of *Niphargus* were already living in surface freshwaters during this time period (Coleman and Myers, 2000; Jażdżewski and Kupryjanowicz, 2010). Thus, the evidence indicates that there were recurrent colonization events of surface waters during the evolutionary history of the genus. Moreover, we cannot rule out a scenario that the ancestors of some clades for a certain period lived and dispersed in ecotonal habitats, leading to a secondary colonization of subterranean waters.

The occurrence of a high number of troglomorphic groundwater species has been long documented at the surface/subsurface boundary (shallow subterranean habitats; Culver and Pipan, 2014). It is generally thought that they are ancestral forms which represent the initial stages of colonization of the deeper subterranean realm (Culver and Pipan, 2009, 2014). However, our results add to the growing body of evidence which indicates that the opposite is also possible. Nevertheless, the factors that promoted the ecological shift to shallow subterranean and eventually surface habitats are unknown. The persistence of troglomorphic species in ecotonal habitats is probably determined by the greater availability of nutritional resources (assumed by Sket, 2008; Culver and Pipan, 2009, 2014; Fišer et al., 2010a), but their presence in epigean habitats can be realized in rare circumstances in which competitors are absent or scarce (Humphreys, 2000; Prendini et al., 2010). Indeed, the distribution of troglomorphic species in surface environments seems to be limited by competition, predation and risk of ultraviolet radiation exposure due to irreversible adaptations such as lack of eyes and pigment (reviewed in Fišer C. et al., 2014; Fišer Ž. et al., 2016).

N. hrabei and *N. valachicus* are bound to the muddy, dimly lit and densely vegetated bottom of stagnant or slow flowing lowland waters (Copilaş-Ciocianu et al., 2017a and references therein). These habitats are characterized by eutrophic conditions, high temperature and frequent hypoxia (Junk et al., 1989; Parr and Mason 2004; Graeber et al., 2013) and are avoided by the presumably ecologically closest competitors, the oxyphilic and mostly rheophilous gammarid amphipods (Mejering, 1991; Mejering et al., 1995; Henry and Danielopol, 1999; Copilaş-Ciocianu et al., 2014; Copilaş-Ciocianu and Boroş, 2016; Borza et al., 2017; Mauchart et al., 2017). Experimental evidence has shown that niphargids can tolerate hypoxia and other adverse environmental conditions much better than surface-water gammarids (Danielopol et al., 1994; Hervant et al., 1995; Malard and Hervant, 1999; Coppellotti Krupa and Guidolin, 2003; Simčič et al., 2005, 2006; Flot et al., 2014). Therefore, it seems that *N. hrabei* and *N. valachicus* might have a competitive edge over gammarids in these habitats; however, experimental proof is needed in this particular case.

Furthermore, it appears that gammarids of the genus *Gammarus* survived Pleistocene glaciation episodes in montane refugia from where only a few species regionally dispersed to lower elevations (Copilaş-Ciocianu and Petrusek 2015, 2017; Copilaş-Ciocianu et al., 2017b). *G. roeselii* and some invasive Ponto-Caspian species, which are common in the Danube lowlands, have dispersed only postglacially or even in historical times from the Balkans or the Black Sea (Barnard and Barnard, 1983; de Vaate et al., 2002; Cristescu et al., 2004; Rewicz et al., 2015). We therefore hypothesise that the colonization of surface waters was possible because of the general and historical scarcity or even absence of gammarid competitors in the habitats in which *N. hrabei* and *N. valachicus* thrive.

4.2. Morphological evolution

The similarity of species living at the boundary between the surface and subterranean ecosystems was noted earlier and some authors even considered them as members of own subgenus or species group (Karaman 1950; Sket 1958, Straškraba 1972a). Our results imply that this similarity is of convergent origin due to multiple independent colonization events of the epigean / ecotonal environment. However, this ecological shift does not always lead to the same phenotype: secondary colonizers of the surface-subterranean boundary are grouped into two morphological clusters. Interestingly, the two focal species cluster together with *N. elegans*, another nearly entirely surface-water species with a large range, widespread across the northern Italian lowlands (Karaman 1977). The astonishing similarities in their morphology, ecology, and range-size deserve further attention. A key question arising from this pattern is whether the large range size is a result of an enhanced possibility of passive dispersal connected with transition to better-connected habitats (see below), or whether some morphological traits may be (indirectly) linked with dispersal capacity. Thus, the functional links between morphological similarities, ecological conditions and dispersal ability remain yet to be explored.

4.3. Phylogeography and dispersal

According to our phylogeny, *N. hrabei* is a sister species to *N. plateaui*, a taxon known from western France. Such a vast distance (ca. 1500 km) between these species might indicate a long-range dispersal in the past, or that additional related lineages remain undiscovered or went extinct. On the other hand, *N. valachicus* belongs to a clade of northern Dinaric species which are known to occur in surface-water habitats. This agrees with its SW Pannonian dispersal origin, which is in geographical proximity to its relatives.

If we consider the scenario of relatively conservative mutation rates, coalescence times for both species correspond with periods of warm interglacial stages. Haplotypes of *N. hrabei* coalesce ca. 100 ka (Eemian), roughly corresponding with the Marine Isotopic Stage 5d (ca. 109 ka), while coalescence time for *N. valachicus* is ca. 350 ka, corresponding with the Marine Isotopic Stage 9 (ca. 337 ka) (Lisiecki and Raymo, 2005), suggesting that their dispersal may have started during these warmer periods. In the case of *N. hrabei*, its initial Eemian expansion would coincide with the expansion of *Fagus sylvatica* in Central and Southern Europe (Magri et al., 2006). The presence of *N. valachicus* in isolated streams along the Black Sea coast is likely explained by the lower water levels and freshwater conditions during the Last Glacial Maximum (Ryan et al., 1997; Bahr et al., 2006; Georgievski and Stanev, 2006). Our data reveal that *N. valachicus* has reached northern Turkey very recently, possibly during the Holocene, but definitely before the last connection with the saline Mediterranean ca. 7-9 ka (Federov, 1971; Ryan et al., 1997; Badertscher et al., 2011). During this time the shelf of the Black Sea was exposed, forming vast deltaic systems that probably facilitated coastal dispersal (Federov, 1971; Ryan et al., 1997). The presence of this species along the Caspian Sea shores in Iran (Karaman, 1998) might be explained by the frequent Pleistocene connections with the Black Sea which have facilitated biotic interchange (Leonov et al., 2002; Grigorovich et al., 2003; Badertscher et al., 2011). However, it is not yet known if the Iranian populations are indeed conspecific with *N. valachicus*.

Although we urge caution in interpreting absolute dates, these results confidently refute previous hypotheses according to which *N. hrabei* and *N. valachicus* invaded freshwater from the brackish Paratethys Sea during the Late Miocene (Straškraba 1972b; Sket 1981) and are in accordance with the preliminary data presented in our previous study (Copilaş-Ciocianu et al., 2017a). A faster mutation rate would, of course, indicate an even more recent expansion, and even two to three times slower rate would not push coalescence times further back than the Pleistocene. The Pannonian clade of *N. valachicus* (clade A) seems to have had a stable demographic history throughout the Late Pleistocene as opposed to clade B and *N. hrabei* which exhibit substantial recent demographic growth. The stable demography of clade A is in agreement with the emerging view that the Pannonian Basin functioned as a glacial refugium for a wide array of taxa, ranging from aquatic invertebrates and vertebrates to terrestrial plants and mammals (Neumann et al., 2005; Verovnik et al., 2005; Fussi et al., 2010; Antal et al., 2016; Vörös et al., 2016).

The significant correlation between genetic and geographic distances and genetic differentiation among drainages indicates that despite the wide ranges of the focal *Niphargus* species, their dispersal is limited. This apparent contradiction might be explained by the fact that their dispersal is passive and happened throughout relatively long periods of time (ca. 100 ka in *N. hrabei* and 350 ka in *N. valachicus*), although in some parts of their ranges they have very likely experienced postglacial demographic expansion. This indicates that chances for dispersal are not equal in time and the highest probability for spreading could be restricted to favourable climatic periods or linked to rare long-distance dispersal events. Given that freshwater amphipods are usually poor and passive dispersers, their dispersal among different water bodies could be achieved either by animal vectors – especially waterfowl and aquatic mammals (Peck, 1975; Swanson, 1984; Rachalewski et al., 2013) – or during flooding episodes (e.g. Van Leeuwen et al., 2013). In any case, the large expanses of flat and homogeneous relief with interconnected water bodies of the Danube floodplains seem to have facilitated the range expansion of these species. The same pattern might be true for *N. elegans*, a species which is widespread throughout the floodplains of the Po River in northern Italy (Karaman, 1977) and also exhibits low intraspecific genetic divergence (Fabio Stoch, pers. comm.).

Considering that groundwater habitats are poorly connected in comparison to surface ones, the occurrence of at least two independent large-scale dispersal events of troglomorphic species in surface waters indicates that habitat connectivity might play a greater role in limiting dispersal than species' ecology or biology.

5. Conclusion

Our results strongly indicate that more than one invasion of surface waters and even more shifts to the surface/subsurface boundary from subterranean ancestors have occurred during the evolutionary history of *Niphargus*. Furthermore, we show for the first time that troglomorphic species are capable of relatively rapid and large-scale dispersal in surface waters. This indicates that adaptation to groundwater might not be a one-way evolutionary path and subterranean troglomorphic species can occasionally recolonize and widely disperse in surface waters.

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Supporting information

Tables S1-S11 Details about samples used in the phylogeographic and phylogenetic analyses, GenBank accession numbers, geographic coordinates, PCR primers, evolutionary, coalescent and clock models, morphological data, ancestral states, and population differentiation based on Φ statistics.

Figures S1-S3 Fully annotated BI and ML phylogenies, isolation by distance plots and cryptic lineage delimitation results.

Table 1. Genetic polymorphism (COI and ITS) and historical demographic results (COI) for the studied species and clades. For the neutrality tests, p-values are provided in parentheses and significant values are shown in bold.

Species/clade	***N***	***S***	***H***	***Hd*** (SD)	π (SD)	***K***	***D*** (*p*)	F_s (*p*)	R_2 (*p*)	τ	θ_0	θ_1	***SSD*** (*p*)	***Hri*** (*p*)
COI														
N. hrabei	54	15	12	0.768 (0.046)	0.0028 (0.0004)	1.76	-1.376 (0.056)	-4.160 (**0.031**)	0.108 (0.083)	0.68	1.23	3451.34	0.003 (0.522)	0.034 (0.275)
N. valachicus overall	111	42	42	0.962 (0.007)	0.0157 (0.0004)	7.13	-0.413 (0.406)	-5.742 (0.117)	0.092 (0.402)	12.26	0.00	16.48	0.009 (0.470)	0.008 (0.719)
N. valachicus A	49	19	14	0.877 (0.030)	0.0079 (0.0006)	3.56	-0.724 (0.252)	3.044 (0.880)	0.107 (0.385)	3.21	1.54	8.32	0.004 (0.766)	0.015 (0.867)
N. valachicus B	62	36	28	0.956 (0.011)	0.0071 (0.0006)	4.42	-1.385 (0.061)	-14.238 (**0.000**)	0.102 (0.06)	3.00	1.90	3414.97	0.006 (0.078)	0.020 (**0.028**)
ITS														
N. hrabei	30 (22)*	7	6	0.736 (0.056)	0.00047 (0.00008)	1.26								
N. valachicus	66 (40)*	28	23	0.73 (0.060)	0.00339 (0.00045)	4.96								

N – sample size (no. of sequences); S – number of variable sites; H – number of haplotypes; Hd – haplotype diversity; π – nucleotide diversity; K – mean number of pairwise nucleotide differences; D – Tajima's D statistics; Fs – Fu's Fs statistics; R2 – Ramos-Onsins and Rozas's R2 statistics; τ – coalescence time in mutational units; θ_0, θ_1 – effective population size at the start and the end of the expansion; SSD – sum of squared deviations; Hri – Harpending's raggedness index;* – parentheses refer to no. of analysed individuals

Table 2. Analysis of molecular variance (AMOVA) in the studied species based on COI with populations grouped according to the major river drainages (listed in Tables S10 and S11). Significant p-values are shown in bold for the fixation indices (*Φ*-statistics).

Species	**Source of variation**	**d.f.**	**Sum of squares**	**Percentage of variation**	***Φ*-statistics**	***P***
N. hrabei						
	Among drainages	4	31.28	80.05	*Φ*ct = 0.80	**0.007**
	Among populations within drainages	10	4.03	11.19	*Φ*sc = 0.56	**0.002**
	Within populations	35	3.4	8.75	*Φ*st = 0.91	**<0.0001**
N. valachicus						
	Among drainages	12	381.68	58.5	*Φ*ct = 0.58	**<0.0001**
	Among populations within drainages	25	131.3	33.1	*Φ*sc = 0.79	**<0.0001**
	Within populations	73	32.75	8.4	*Φ*st = 0.91	**<0.0001**

Figure captions

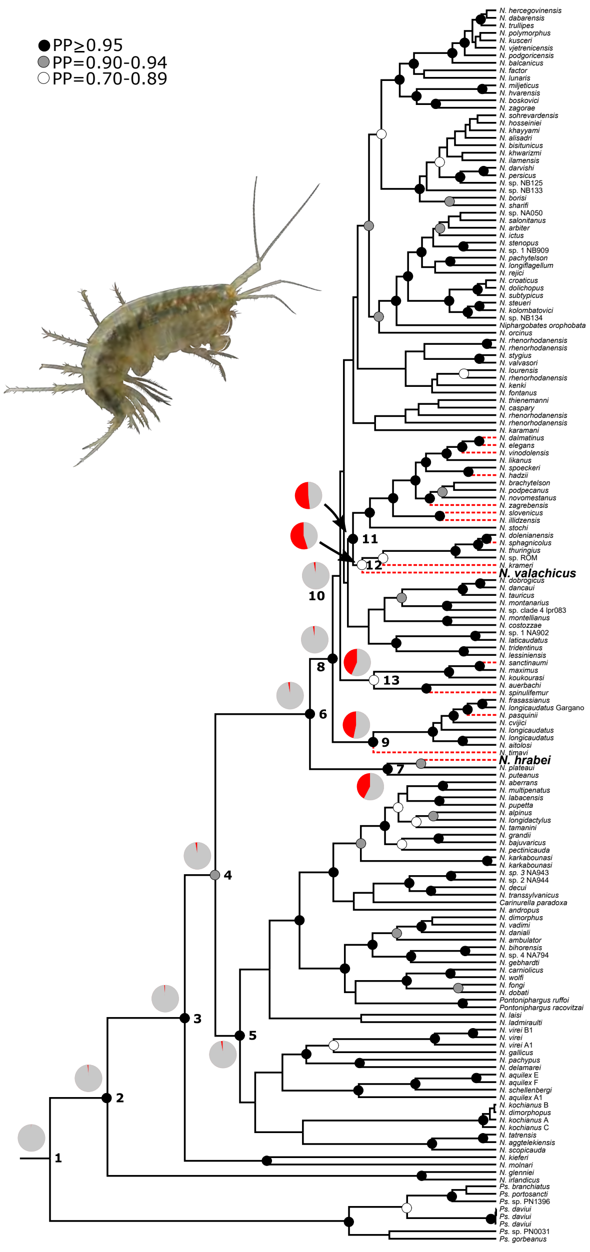
Fig. 1 Bayesian phylogeny of *Niphargus* and ancestral state reconstruction of the species' habitat. Species that occur in surface habitats are highlighted with red, dashed branches. The widely-dispersed focal species, *N. hrabei* and *N. valachicus* are indicated with larger font size. Circles at nodes denote posterior probability for clade support (black ≥ 0.95, grey = 0.90-0.94 and white = 0.70-0.89). The pie charts along the numbered nodes of the tree indicate the probability of subterranean (grey) and surface (red) ecology of the ancestors. The numbers at nodes correspond with those in Table S9. Inset image depicts a male *N. valachicus* (Photograph: Denis Copilaş-Ciocianu).

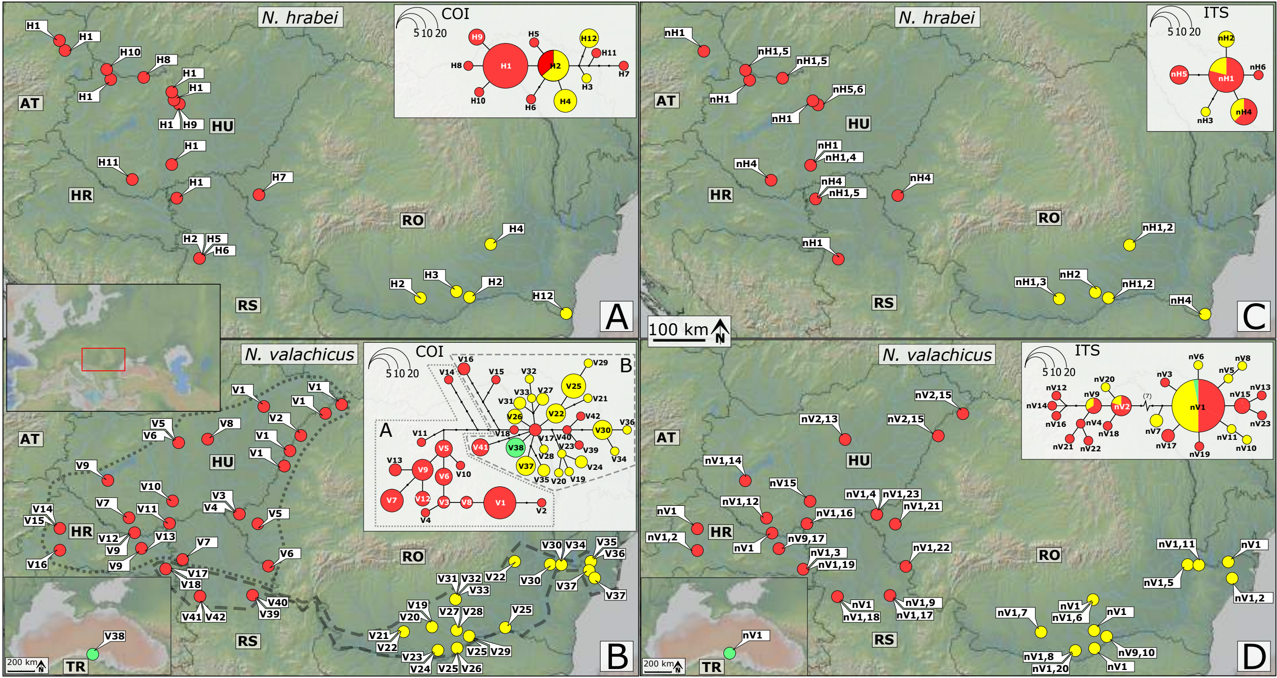
Fig. 2 Geographical distribution and haplotype networks of COI and ITS in *N. hrabei* (A, C) and *N. valachicus* (B, D). Colours indicate middle (red) and lower (yellow) Danube and Black Sea (green) populations. Distribution of clades A and B of *N. valachicus* are indicated by dotted and dashed lines, respectively. The size of the circles in the haplotype networks is proportional to the observed frequency of the corresponding haplotype. Each segment indicates one substitution. Relevant countries are indicated by corresponding 2-letter ISO codes: AT—Austria, HU—Hungary, HR—Croatia, RO—Romania, RS—Serbia and TR—Turkey.

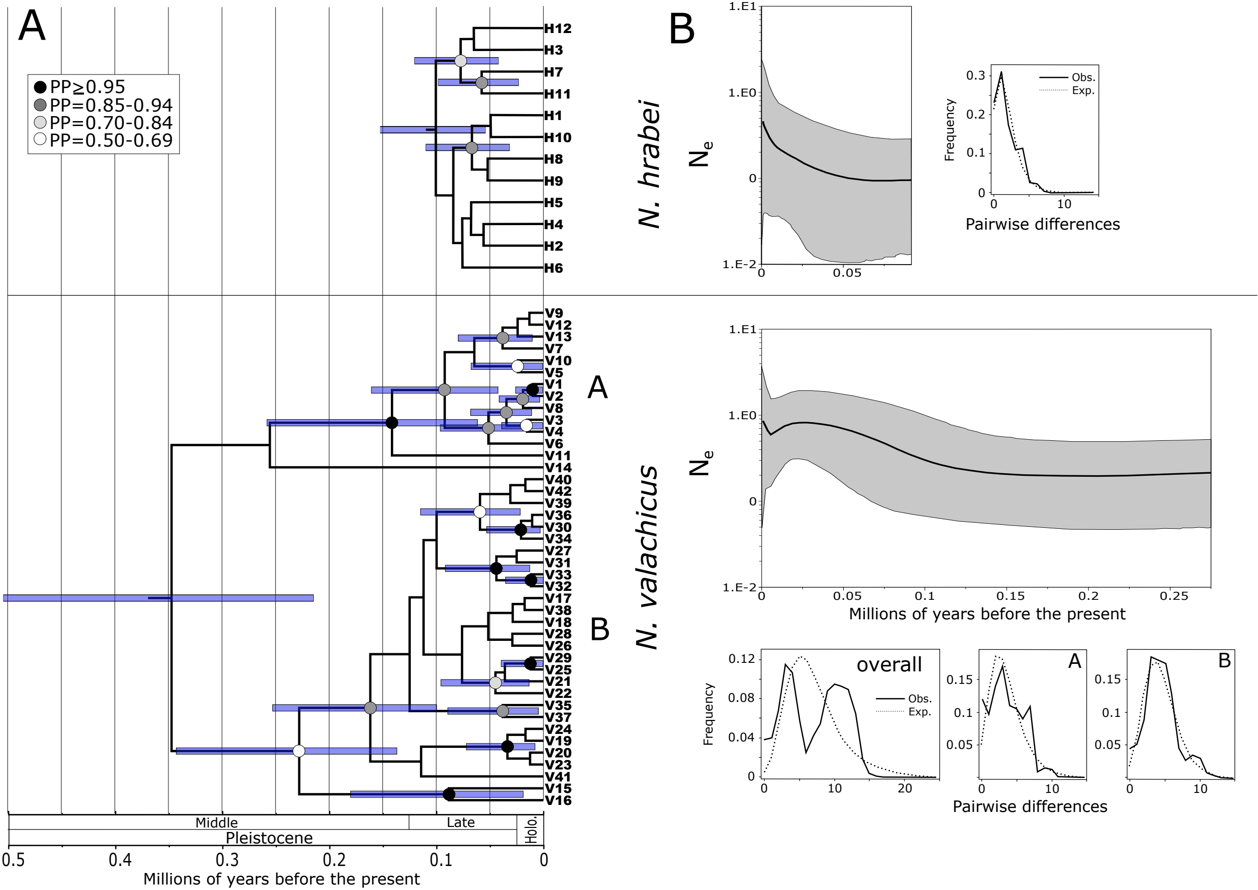
Fig. 3 Coalescence and demography of *N. hrabei* (upper panel) and *N. valachicus* (lower panel). A) Time-calibrated COI trees. Posterior probability at nodes is indicated by circles (black ≥ 0.95, dark grey = 0.85–0.94, light grey = 0.70–0.84, and white = 0.50–0.69). Blue bars at nodes denote the 95% HPD intervals of clade age. B) Bayesian skyline plots (BSP) and mismatch distribution histograms. Mean populations size through time is shown by thick black lines and 95% confidence intervals with grey in the BSP. Mismatch distributions were also calculated separately for each of the two clades (A and B) of *N. valachicus*. Continuous lines indicate the observed frequency of pairwise differences and dotted lines indicate the expected frequency under a model of sudden demographic expansion.

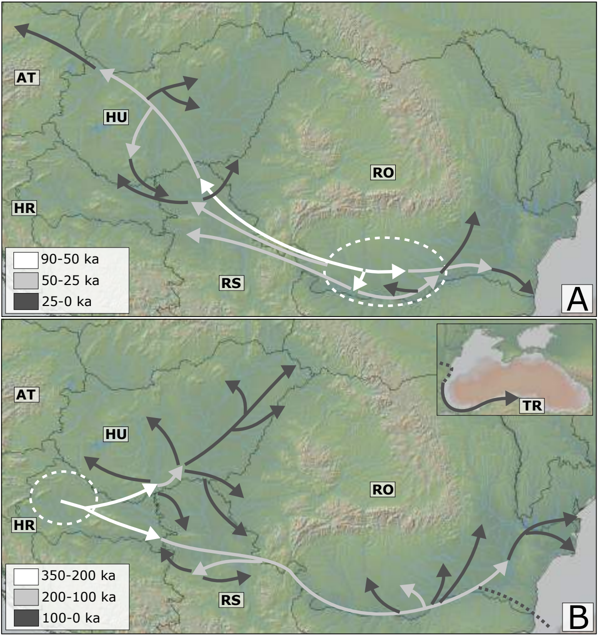
Fig. 4 Dispersal of *N. hrabei* (A) and *N. valachicus* (B) inferred from Bayesian phylogeographic diffusion models. The putative origin of dispersal is shown with a dashed, white line. Dispersal routes at different time intervals are indicated by different line shadings. Country ISO codes are the same as in Fig. 2.

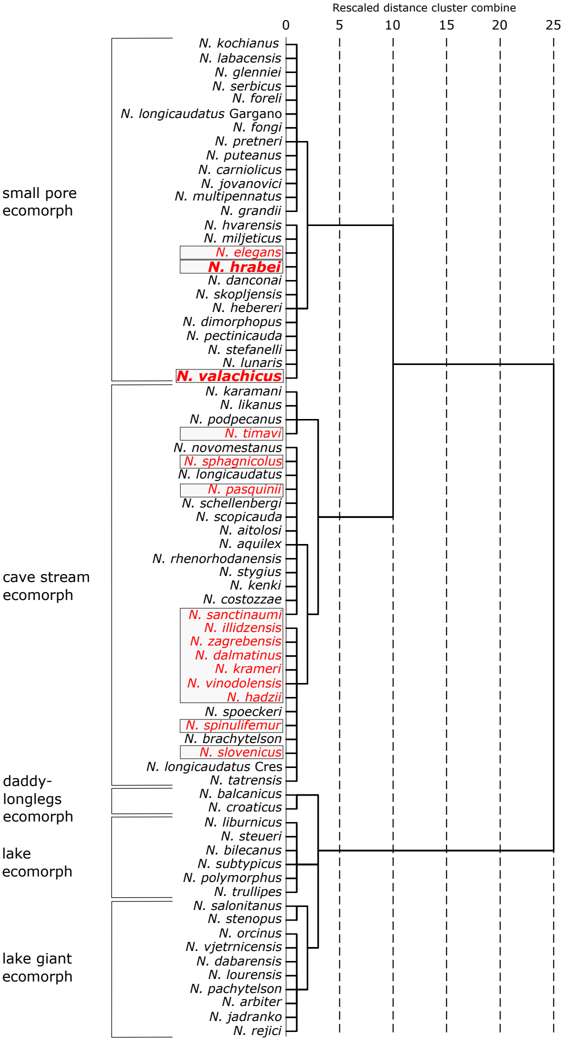
Fig. 5 Clustering of 81 *Niphargus* taxa based on 35 morphological traits. Surface-water species are indicated with red font and grey shading. The focal *N. hrabei* and *N. valachicus* are shown with larger font size. Ecomorph names follow Trontelj et al., (2012).


●PP≥0.95
●PP=0.90-0.94
○PP=0.70-0.89
N. hercegovinensis
N. dabarensis
N. trullipes
N. polymorphus
N. kusceri
N. vjetrenicensis
N. podgoricensis
N. balcanicus
N. factor
N. lunaris
N. miljeticus
N. hvarensis
N. boskovici
N. zagorae
N. sohrevardensis
N. hosseiniei
N. khayyami
N. alisadri
N. bisitunicus
N. khwarizmi
N. ilamensis
N. darvishi
N. persicus
N. sp. NB125
N. sp. NB133
N. borisi
N. sharifi
N. sp. NA050
N. salonitanus
N. arbiter
N. ictus
N. stenopus
N. sp. 1 NB909
N. pachytelson
N. longiflagellum
N. rejici
N. croaticus
N. dolichopus
N. subtypicus
N. steueri
N. kolombatovici
N. sp. NB134
Niphargobates orophobata
N. orcinus
N. rhenorhodanensis
N. rhenorhodanensis
N. stygius
N. valvasori
N. lourensis
N. rhenorhodanensis
N. kenki
N. fontanus
N. thienemanni
N. caspary
N. rhenorhodanensis
N. rhenorhodanensis
N. karamani
N. dalmatinus
N. elegans
N. vinodolensis
N. likanus
N. spoeckeri
N. hadzii
N. brachytelson
N. podpecanus
N. novomestanus
N. zagrebensis
N. slovenicus
N. illidzensis
N. stochi
N. dolenianensis
N. sphagnicolus
N. thuringius
N. sp. ROM
N. krameri
N. valachicus
N. dobrogicus
N. dancaui
N. tauricus
N. montanarius
N. sp. clade 4 Ipr083
N. montellianus
N. costozzae
N. sp. 1 NA902
N. laticaudatus
N. tridentinus
N. lessiniensis
N. sanctinaumi
N. maximus
N. koukourasi
N. auerbachi
N. spinulifemur
N. frasassianus
N. longicaudatus Gargano
N. pasquinii
N. cvijici
N. longicaudatus
N. longicaudatus
N. aitolosi
N. timavi
N. hrabei
N. plateaui
N. puteanus
N. aberrans
N. multipenatus
N. labacensis
N. pupetta
N. alpinus
N. longidactylus
N. tamanini
N. grandii
N. bajuvaricus
N. pectinicauda
N. karkabounasi
N. karkabounasi
N. sp. 3 NA943
N. sp. 2 NA944
N. decui
N. transsylvanicus
Carinurella paradoxa
N. andropus
N. dimorphus
N. vadimi
N. daniali
N. ambulator
N. bihorensis
N. sp. 4 NA794
N. gebhardti
N. carniolicus
N. wolfi
N. fongi
N. dobati
Pontoniphargus ruffoi
Pontoniphargus racovitzai
N. laisi
N. ladmiraulti
N. virei B1
N. virei
N. virei A1
N. gallicus
N. pachypus
N. delamarei
N. aquilex E
N. aquilex F
N. schellenbergi
N. aquilex A1
N. kochianus B
N. dimorphopus
N. kochianus A
N. kochianus C
N. tatrensis
N. aggtelekiensis
N. scopicauda
N. kieferi
N. molnari
N. glenniei
N. irlandicus
Ps. branchiatus
Ps. portosancti
Ps. sp. PN1396
Ps. daviui
Ps. daviui
Ps. daviui
Ps. sp. PN0031
Ps. gorbeanus
11
12
10
8
13
6
9
7
4
3
5
2
1


A
N. hrabei
COI
5 10 20
H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
AT
HU
HR
RO
RS
B
N. valachicus
COI
V1
V2
V3
V4
V5
V6
V7
V8
V9
V10
V11
V12
V13
V14
V15
V16
V17
V18
V19
V20
V21
V22
V23
V24
V25
V26
V27
V28
V29
V30
V31
V32
V33
V34
V35
V36
V37
V38
V39
V40
V41
V42
TR
200 km
N
C
N. hrabei
ITS
nH1
nH2
nH3
nH4
nH5
nH6
nH1,5
nH5,6
nH1,4
nH1,2
nH1,3
100 km
N
D
N. valachicus
ITS
(7)
nV1
nV2
nV3
nV4
nV5
nV6
nV7
nV8
nV9
nV10
nV11
nV12
nV13
nV14
nV15
nV16
nV17
nV18
nV19
nV20
nV21
nV22
nV23
nV2,15
nV2,13
nV1,14
nV1,12
nV1,16
nV1,4
nV1,23
nV1,21
nV1,2
nV9,17
nV1,3
nV1,19
nV1,22
nV1,18
nV1,9
nV1,17
nV1,11
nV1,5
nV1,7
nV1,6
nV9,10
nV1,8
nV1,20
200 km
N


A
● PP≥0.95
● PP=0.85-0.94
● PP=0.70-0.84
○ PP=0.50-0.69
H12
H3
H7
H11
H1
H10
H8
H9
H5
H4
H2
H6
V9
V12
V13
V7
V10
V5
V1
V2
V8
V3
V4
V6
V11
V14
V40
V42
V39
V36
V30
V34
V27
V31
V33
V32
V17
V38
V18
V28
V26
V29
V25
V21
V22
V35
V37
V24
V19
V20
V23
V41
V15
V16
A
B
Middle
Late
Pleistocene
Holo.
0.5
0.4
0.3
0.2
0.1
0
Millions of years before the present
N. hrabei
N. valachicus
B
N$_e$
1.E1
1.E0
0
1.E-2
0
0.05
Frequency
0.3
0.2
0.1
0
0
10
Obs.
Exp.
Pairwise differences
N$_e$
1.E1
1.E0
0
1.E-2
0
0.05
0.1
0.15
0.2
0.25
Millions of years before the present
overall
Obs.
Exp.
Frequency
0.12
0.08
0.04
0
0
10
20
A
0.15
0.1
0.05
0
10
B
0.15
0.1
0.05
0
10
Pairwise differences


AT
HU
HR
RO
RS
90-50 ka
50-25 ka
25-0 ka
A
AT
HU
HR
RO
RS
TR
350-200 ka
200-100 ka
100-0 ka
B


Rescaled distance cluster combine
0
5
10
15
20
25
small pore ecomorph
N. kochianus
N. labacensis
N. glenniei
N. serbicus
N. foreli
N. longicaudatus Gargano
N. fongi
N. pretneri
N. puteanus
N. carniolicus
N. jovanovici
N. multipennatus
N. grandii
N. hvarensis
N. miljeticus
N. elegans
N. hrabei
N. danconai
N. skopljensis
N. hebereri
N. dimorphopus
N. pectinicauda
N. stefanelli
N. lunaris
N. valachicus
cave stream ecomorph
N. karamani
N. likanus
N. podpecanus
N. timavi
N. novomestanus
N. sphagnicolus
N. longicaudatus
N. pasquinii
N. schellenbergi
N. scopicauda
N. aitolosi
N. aquilex
N. rhenorhodanensis
N. stygius
N. kenki
N. costozzae
N. sanctinaumi
N. illidzensis
N. zagrebensis
N. dalmatinus
N. krameri
N. vinodolensis
N. hadzii
N. spoeckeri
N. spinulifemur
N. brachytelson
N. slovenicus
N. longicaudatus Cres
N. tatrensis
daddy-longlegs ecomorph
N. balcanicus
N. croaticus
lake ecomorph
N. liburnicus
N. steueri
N. bilecanus
N. subtypicus
N. polymorphus
N. trullipes
lake giant ecomorph
N. salonitanus
N. stenopus
N. orcinus
N. vjetrnicensis
N. dabarensis
N. lourensis
N. pachytelson
N. arbiter
N. jadranko
N. rejici

Supplementary Information

Is subterranean lifestyle reversible? Independent and recent large-scale dispersal into surface waters by two species of the groundwater amphipod genus *Niphargus*

Denis Copilaş-Ciocianu, Cene Fišer, Péter Borza and Adam Petrusek

Supplementary Methods

Phylogenetic analyses

Bayesian inference (BI) and maximum-likelihood (ML) approaches were used to reconstruct phylogenetic relationships and examine the position of *N. hrabei* and *N. valachicus* within the genus. Both approaches were applied to the concatenated supermatrix. BI was carried out in BEAST 1.8.0 (Drummond et al. 2012). We employed the evolutionary models in Table S4 for each partition. Random starting topologies were used for each run and speciation was modelled using a Yule prior. As we were not interested in absolute divergence times, we used a relaxed molecular clock with a lognormal distribution and the rate was left at the default value of 1. Clock models and trees were linked across partitions. The MCMC chain was run for 100 million generations with a sampling frequency of 1000 and 30% of the trees were discarded as burn-in. Convergence and effective sample size was assessed using TRACER 1.6 and the maximum clade credibility (MCC) tree was produced with TreeAnnotator 1.8. The alignment used in the BEAST analysis had regions of questionable homology in the 28S marker removed with GBLOCKS 0.9 (Talavera and Castresana, 2007). To evaluate the effect of removal of these regions on the topology of the tree, we used a ML method that simultaneously estimates the sequence alignment and phylogenetic tree in SATé 2.2.7 (Liu et al. 2009), thus retaining the poorly alignable regions. MAFFT 6.7 (Katoh et al. 2005) was used as the initial aligner and OPAL 1.0.3 (Wheeler & Kececioglu 2007) was used to merge the alignment of subproblems into the final alignment. The tree was estimated with RAxML 7.2.6 (Stamatakis 2006) and the GTR+ Γ model. The cycle of alignment and tree estimation was iterated ten times. Because SATé does not calculate bootstrap support on RAxML produced trees and does not handle codon partitions, the alignment with the best likelihood was used for the final tree estimation and bootstrapping in RAxML-HPC 8.2.9 (Stamatakis 2014). A thorough ML tree search was performed with the GTR+Γ model assigned to each partition and 1000 fast bootstrap iterations. We conducted two independent runs of both ML and BI. Analyses were carried out on the CIPRES Science Gateway (Miller et al. 2010).

Bayesian phylogeographical diffusion models

To explore the origin and dispersal history of each species while accounting for phylogenetic uncertainty, we used Bayesian phylogeographic diffusion models (Lemey et al. 2010) implemented in BEAST 1.8.0. The analysis was based on the COI marker and we used the same settings as for the time-calibrated phylogenetic analyses. The coordinates of each locality were used as a quantitative trait and individuals possessing the same haplotype but collected from different locations were retained because their corresponding coordinates contained spatial information. We compared four diffusion models available in BEAST: a random walk model following a homogeneous Brownian diffusion (BD), and three relaxed random walk models (RRW) using gamma, lognormal and Cauchy distributions (Lemey et al. 2010). For each spatial diffusion model and species, we ran two independent runs of 30 million generations which were sampled every 1000 steps with 10% burn-in and convergence was evaluated with TRACER. The best-fit diffusion models were selected by calculating Bayes factors based on marginal likelihoods estimated with path and stepping stone sampling (Baele et al. 2012) and are presented in Table S6. The phylogeographic history of each species was visualized in Google Earth Pro 7.1.5 (https://www.google.com/earth/) by producing and input Keyhole Markup Language (kml) file with SPREAD 1.0.7 (Bielejec et al. 2011).

Analysed morphological traits

A total of 34 quantitative and one qualitative trait were analysed. Landmarks and variation of these traits were presented in a previous study (Fišer et al. 2009). Many of the measured traits are presumably linked to the species ecology (see Trontelj et al. 2012; Fišer et al. 2015; Copilas-Ciocianu et al. 2017). *Body size* is related to the trophic ecology, but also to the pore size of microhabitats the species lives in (Trontelj et al. 2012). *Body shape* relates to hydrodynamic properties of the body (Dahl 1977) and reproductive biology (Fišer et al. 2013). It can be inferred from coxal plates II and III and bases of pereopods V-VII (all measured as width and length). *Appendage length* is a tradeoff between extra-optic sensory capacity and resilience to water flow (Pipan & Culver 2012; Trontelj et al. 2012, Delić et al. 2016). We measured lengths of antennae I-II and pereopods V-VII. *Shape and size of gnathopods I-II* is likely involved into feeding biology. We measured lengths of carpus and propodus, propodus palm and the length of propodus diagonal (Copilas-Ciocianu et al. 2017). *Uropods I and III* are sexually dimorphic in some species. In these species, males have either elongated inner ramus of uropod I, or distal article of exopodite of uropod III, or both. In addition, some males have a strange, flap-like appendix on a base of uropod I. We measured lengths of both rami of uropod I, and both articles of exopodite of uropod III; the flap-like appendix was treated as present-absent. Spines on dactyls of pereopods III-VII, and spines on urosomites I-II, the function of which is not known, vary in number between one and nine, were counted.

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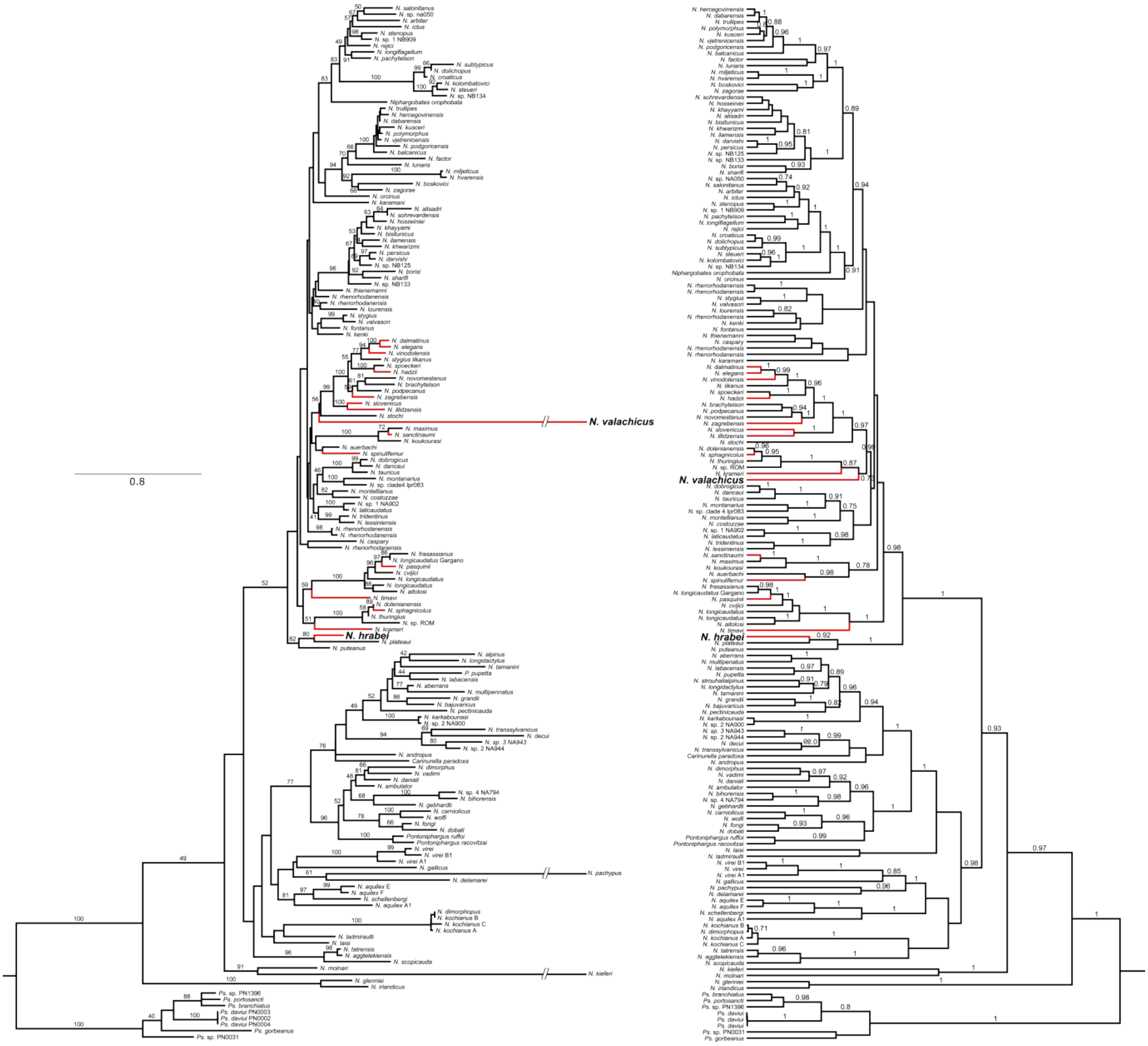
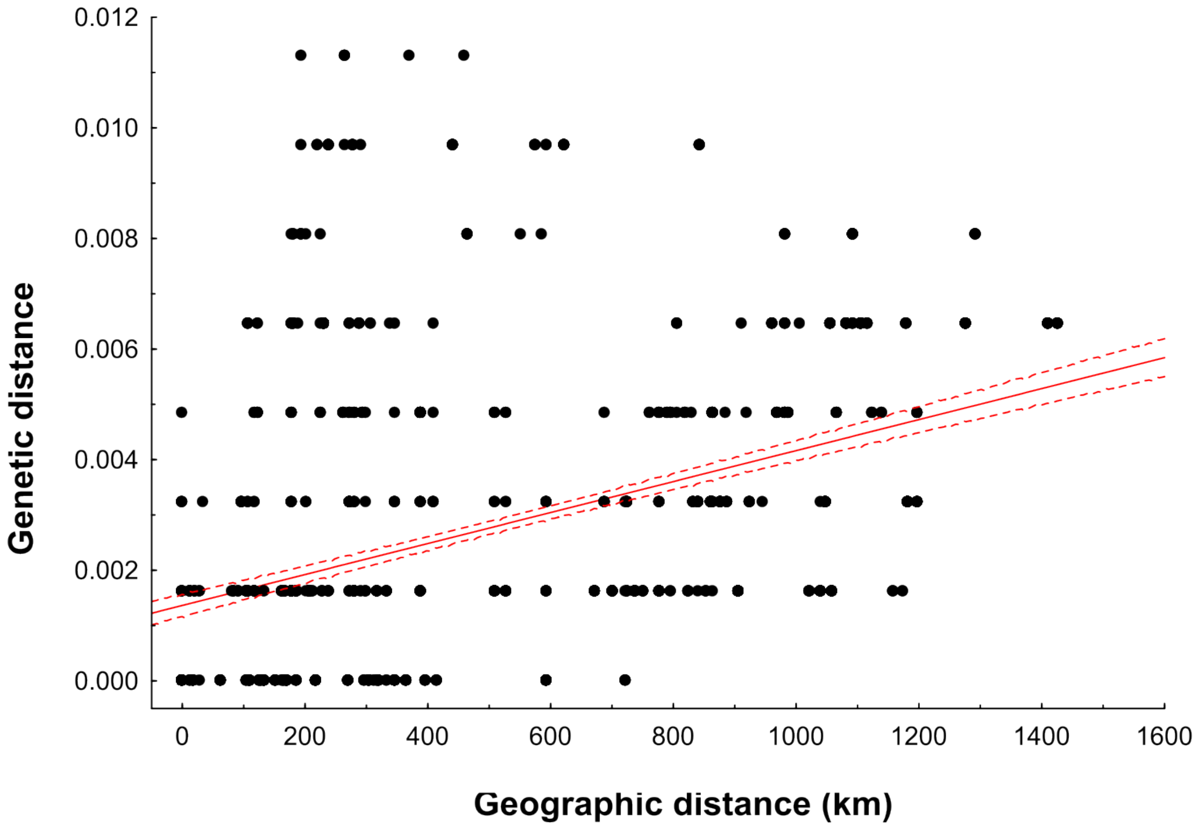


Fig. S1. Maximum-likelihood (left) and Bayesian phylogenies (right) obtained from the concatenated dataset of COI, H3 and 28S sequences. The ML tree was obtained from an alignment produced with SATé and contained poorly alignable regions in the 28S marker, while the alignment for the BI tree had these regions removed and was obtained with MAFFT. Numbers above nodes are bootstrap percentages and posterior probabilities, respectively. Only values above 40% and 0.70 are shown. Surface-water species are shown with red branches. *N. hrabei* and *N. valachicus* are indicated with larger font size.

N. hrabei **r = 0.4317, p = 0.00001**



N. valachicus **r = 0.3737, p = 0.00001**

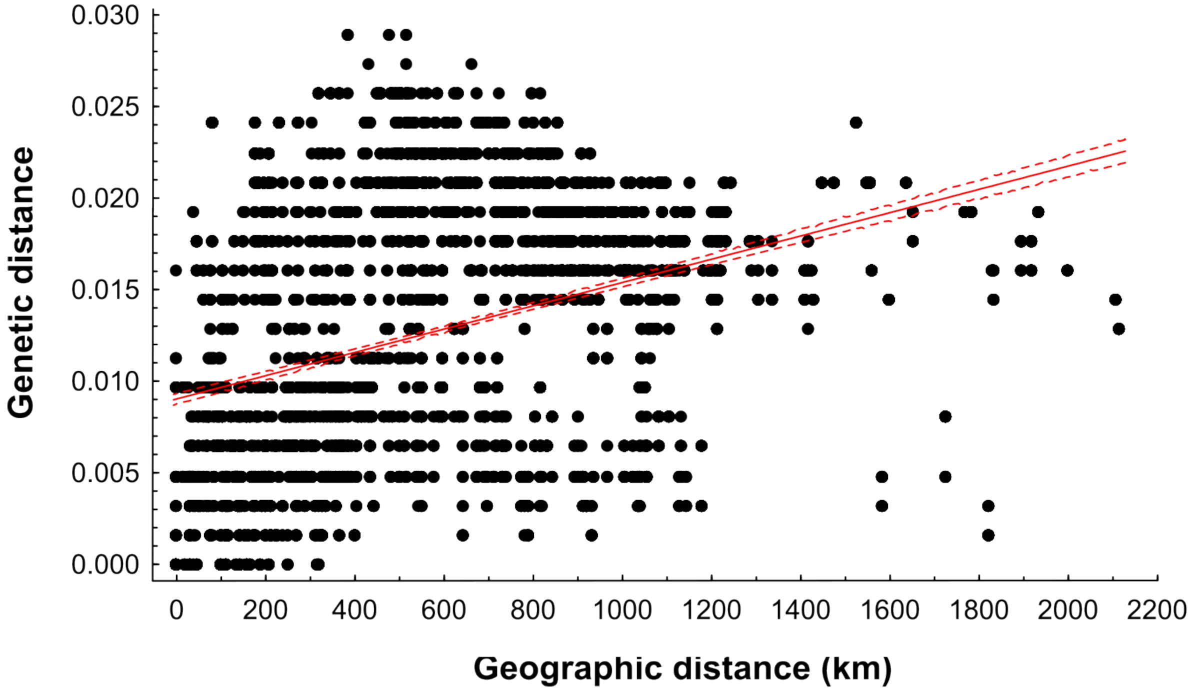


Fig. S2. Correlation between geographic and genetic (based on COI) distance.

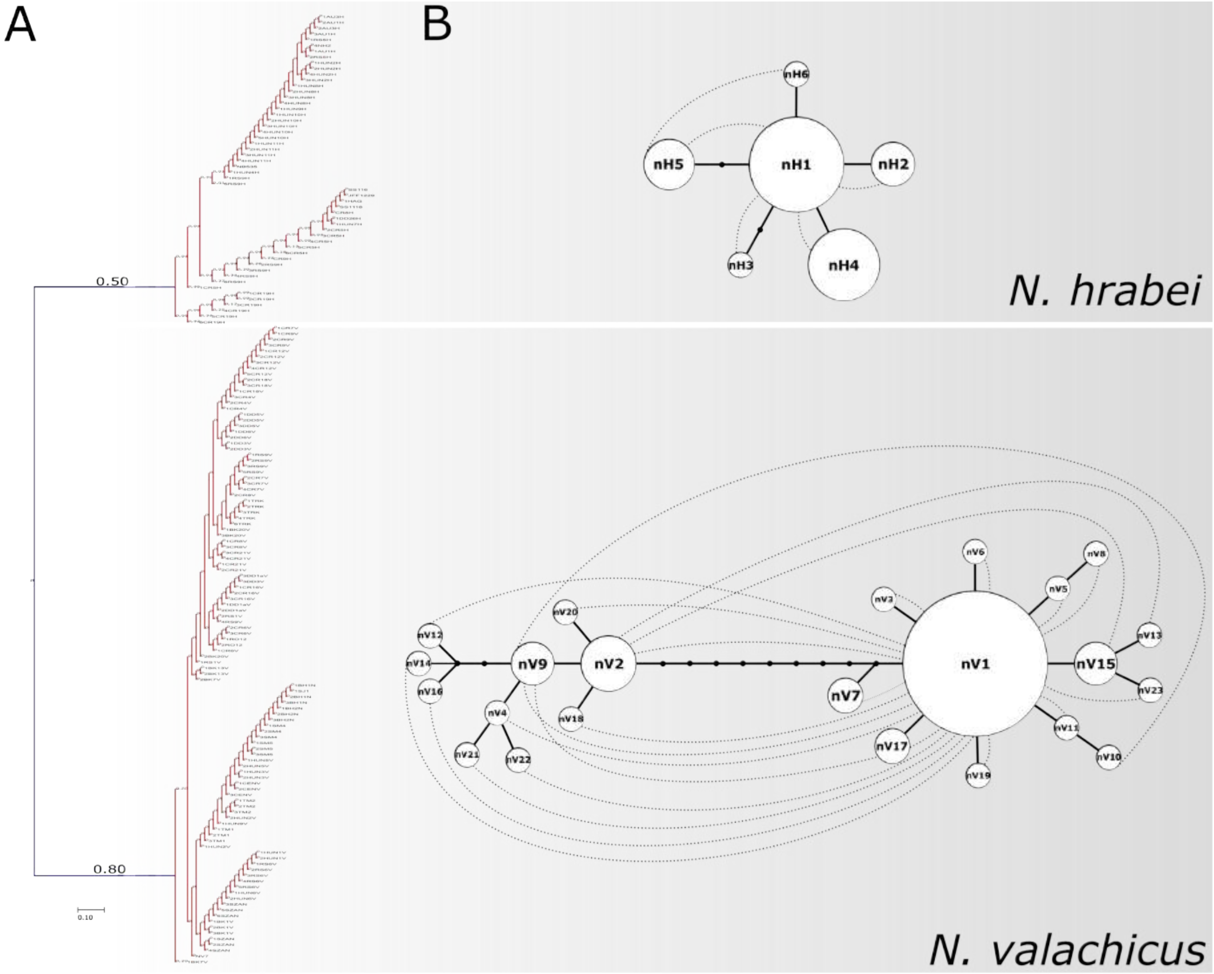


Fig. S3. Cryptic lineage delimitation results based on COI and ITS in *N. hrabei* (above) and *N. valachicus* (below). A) Results from the bPTP analysis based on COI. Clustered red branches indicate putative lineages which correspond to the two focal species, i.e. no independent intraspecific lineages were detected. Numbers above branches indicate the posterior probability for species assignment. B) Haplowebs based on phased ITS sequences. Haplotypes that co-occur in the same individual are connected by thin, dotted lines. All haplotypes are interconnected, indicating a common gene pool.