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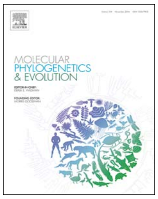

Is subterranean lifestyle reversible? Independent and recent large-scale dispersal into surface waters by two species of the groundwater amphipod genus *Niphargus*

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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 tested if this pattern results from a secondary colonization of the relatively well-connected epigean environment, and whether 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 macrofauna represents a substantial part of freshwater diversity in Europe (Zagmajster et al., 2014) and among its most remarkable features 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 which 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).

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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 (Meijering et al., 1995; Copilaş-Ciocianu et al., 2017a and references therein). This, coupled with the seasonal life-cycle of *N. valachicus* (Copilaş-Ciocianu and Boroş, 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 across), rivalling those of many epigean amphipods (Nesemann 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., 2017). 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