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Research

Niches within a niche: ecological differentiation of subterranean amphipods across Europe's interstitial waters

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Species that successfully colonized subterranean environments are subject to two opposing selection processes. Stringent abiotic factors select for convergent adaptations, such as loss of eyes and pigments, while interspecific competition drives between-species divergence. Subterranean species can resolve opposing selection by adaptation to physically different microhabitats. Yet, species frequently co-occur in physically homogeneous subterranean habitats, like interstitial. These co-occurrences in such a narrow ecological context can be explained either by equalizing mechanisms, in which neither of the co-occurring species has a competitive advantage, or by more complex niche models that include species' differentiation along a trophic niche axis. We tested these hypotheses using the amphipod genus *Niphargus*. We analysed Europe-wide co-occurrence records of *Niphargus* species from interstitial habitats, split into six independent large-scale regions. Firstly, we addressed whether species' pairwise co-occurrences are random using a probabilistic model. Secondly, we tested whether species cluster into distinct functional–morphological groups and whether ecologically or phylogenetically distinct species are more likely to co-occur. We found that 68% of species co-occurrences were not different from random expectation, indicating that most species had access to most sites within each region. The remaining 32% co-occurred either significantly more or less often than expected by chance. Cluster analysis of functional morphological characters showed that interstitial species belong to two feeding types, micro- and macrofeeders, likely representing two peaks of the interstitial adaptive landscape, and hinting that niche divergence, as a mechanism allowing coexistence, is favoured. Finally, we found that the number of co-occurrences increases with increasing differentiation of functional morphology, but not phylogenetic differences. We conclude that ecological differentiation may be important in shaping such interstitial communities.

Keywords: ecological differentiation, European groundwater, interstitial community



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Introduction

Subterranean environments are characterized by permanent darkness, absence of short-term climatic fluctuations, and a shortage of food resources (Culver and Pipan 2009). Consequently, the boundary between the surface and subterranean ecosystems is seen as a strong ecological filter (Gibert and Deharveng 2002). Species that successfully crossed this barrier have undergone massive evolutionary changes, yielding phenotypic similarity in numerous traits, including loss of pigmentation and eyes, augmentation of extraoptic senses, evolution of starvation resistance, and a shift towards K reproductive strategy (Langecker 2000, Christiansen 2005, Hervant 2012). The effects of the subterranean environment presumably extend beyond the organism's level of the biological organization. On an ecosystem level, limited resources presumably favour trophic generalists yielding truncated trophic structures in subterranean communities, generally lacking primary producers and having only a few top predators (Gibert and Deharveng 2002).

Species adapted to the subterranean environment often encounter each other. This co-occurrence of two or more, often closely related, species has been commonly observed at different spatial scales, even within the same compartment of a single cave (Fišer et al. 2012, Trontelj et al. 2012, Vergnon et al. 2013). In such a resource-limited environment, co-occurring conspecific and heterospecific individuals are likely to compete for resources. The relative strengths of intra- and interspecific competitive interactions shape mechanisms of long-term co-occurrences (Chesson 2000). Yet, co-occurrence mechanisms among subterranean species are still poorly understood.

Co-occurrence of ecologically similar species can be explained by multiple mechanisms. Neutral mechanisms are thought to be important in communities of ecologically similar species (McPeck and Gomulkiewicz 2005), when intra- and interspecific competition has similar impacts on the population dynamics of the respective species, and when neither of the co-occurring species has a competitive advantage over another. In such a scenario, environmental selection and interspecific competition act hand in hand and drive overall ecological similarity among co-occurring species. Consequently, the difference between intra- and interspecific competition diminishes, which is a basis of equalizing mechanisms of co-occurrence (Chesson 2000, Scheffer and van Nes 2006, Siewpielski and McPeck 2010). Eventually, species' co-occurrence depends on random demographic fluctuations rather than competitive differences (Siewpielski et al. 2010, Little and Altermatt 2018). Alternatively, co-occurrences could be stabilized by species' differentiation along another, yet unexamined, ecological niche dimension (Ingram and Shurin 2009, Barabás et al. 2013). In this last scenario, interspecific competition leads to ecological differentiation (Schluter 2000, Martin and Pfennig 2009, Pfennig and Pfennig 2010), reducing the relative importance of interspecific competition as compared to intraspecific competition

(Chesson 2000). In subterranean species, possible differentiations include adaptations to physical–chemical conditions of subterranean microhabitats, spatial segregation within a single locality (Trontelj et al. 2012, DeliĆ et al. 2016, Mammola and Isaia 2016), and differential resource-use (Vergnon et al. 2013, Hutchins et al. 2014, Francois et al. 2016). In such an ecological niche differentiation scenario, subterranean organisms may experience both convergent and divergent selection due to similar environmental conditions and inter-specific competition for the limited resources, respectively. Understanding various selection pressures and the outcome between the opposing selection forces is vital for the study of eco-evolutionary dynamics underlying species co-occurrences and community assembly (Weber et al. 2017). These mechanisms, however, have received little attention in the field of subterranean biology, although subterranean communities are relatively simple and offer an excellent model system to study general factors shaping species co-occurrences.

Here, we tested whether the co-occurrence of subterranean species in interstitial habitats can be explained by differentiation along the resource-use niche axis. We analysed interstitial communities of subterranean crustaceans of the genus *Niphargus*. *Niphargus* is a species-rich group of amphipods with its main distribution in Europe. It lives in all types of subterranean aquatic habitats (Sket 1999, Fišer 2012), including interstitial habitats, which are physically homogeneous habitats within unconsolidated sediments in river alluvia, with small void space and almost stagnant water (Malard and Hervant 1999, Culver and Pipan 2009, Dole-Olivier et al. 2009, Larned 2012). Co-occurrences of *Niphargus* species in interstitial habitats are relatively frequent, with a literature review suggesting that up to six species may live in a single locality (Fišer et al. 2012). The mechanisms mediating their co-occurrences, however remain unknown.

Firstly, we tested for every species pair within a region whether the frequencies of pairwise co-occurrences deviate from a random expectation using a probabilistic model proposed by Veech (2013 and 2014). Secondly, we explored whether observed species' co-occurrence could be explained by a differentiation along a trophic niche axis. We used functional morphology, phylogenetic relatedness and co-occurrence data to address whether and how interstitial communities are structured with respect to resource-use. We first predicted that community members diminished competition by ecological differentiation into few distinct feeding types, replicated across communities (Scheffer and van Nes 2006, Vergnon et al. 2012, 2013). We explored whether interstitial species segregate into feeding types (i.e. trophic groups) using functional morphology. Second, we tested whether pairs of ecologically similar species co-occur more or less frequently than ecologically dissimilar counterparts, implying the prevalence of equalizing and stabilizing mechanisms of species' co-occurrences, respectively. We employed functional morphology and phylogenetic relatedness as two alternative measures of ecological dissimilarity, and tested whether and how these measures predicted pairwise species co-occurrences.

Material and methods

Community data and definition of interstitial regions

We analysed the European Groundwater Crustacean Dataset (EGCD) (Zagmajster et al. 2014), and selected all the records of interstitial communities, i.e. localities from which at least two *Niphargus* species were reported. Questionable records were omitted (e.g. records containing non-interstitial species, or records only labelled as '*Niphargus* sp.')

 and supplemented with our own unpublished data (five localities). We obtained a list of 155 localities in total, with 48 different *Niphargus* species, spread throughout most of the European continent (Fig. 1, Supplementary material Appendix 1 Table A1, A2). Then, we divided the sites into six major regions (Northern Atlantic Ocean, North Sea, Celtic Sea and Channel, Black Sea, Eastern and Western Mediterranean Sea, Fig. 1), corresponding to the sea bodies the rivers drain into (Vogt et al. 2007). Each region therefore presents a subset of sites connected through dispersal across geological time scales, and isolated from other such regions. These different regions are regarded as natural replicates allowing

us to assess the repeatability of the observed patterns (see also Mammola 2018).

Organisms

Our analysis was based on interstitial amphipod species of the genus *Niphargus* and the genus *Carinurella*, which is phylogenetically nested within *Niphargus* (Fišer et al. 2008, Esmaili-Rineh et al. 2015). Interstitial animals are rare, in low abundances, and often damaged due to the collection methods. For this reason, we performed all analyses on species level (i.e. no information on within-species variation was included).

In our morphometric analyses, we included 44 species of the 48 species from all over Europe, which encompass all common interstitial species. We measured morphological traits of 33 species, from 111 individuals (1–13 per species). For the additional 11 species, we retrieved the morphological data from the original descriptions (Supplementary material Appendix 1 Table A3). For molecular analyses, we had access to 29 interstitial *Niphargus* species, the phylogenetic position of which was analysed together with 86 non-interstitial *Niphargus* species (see below).

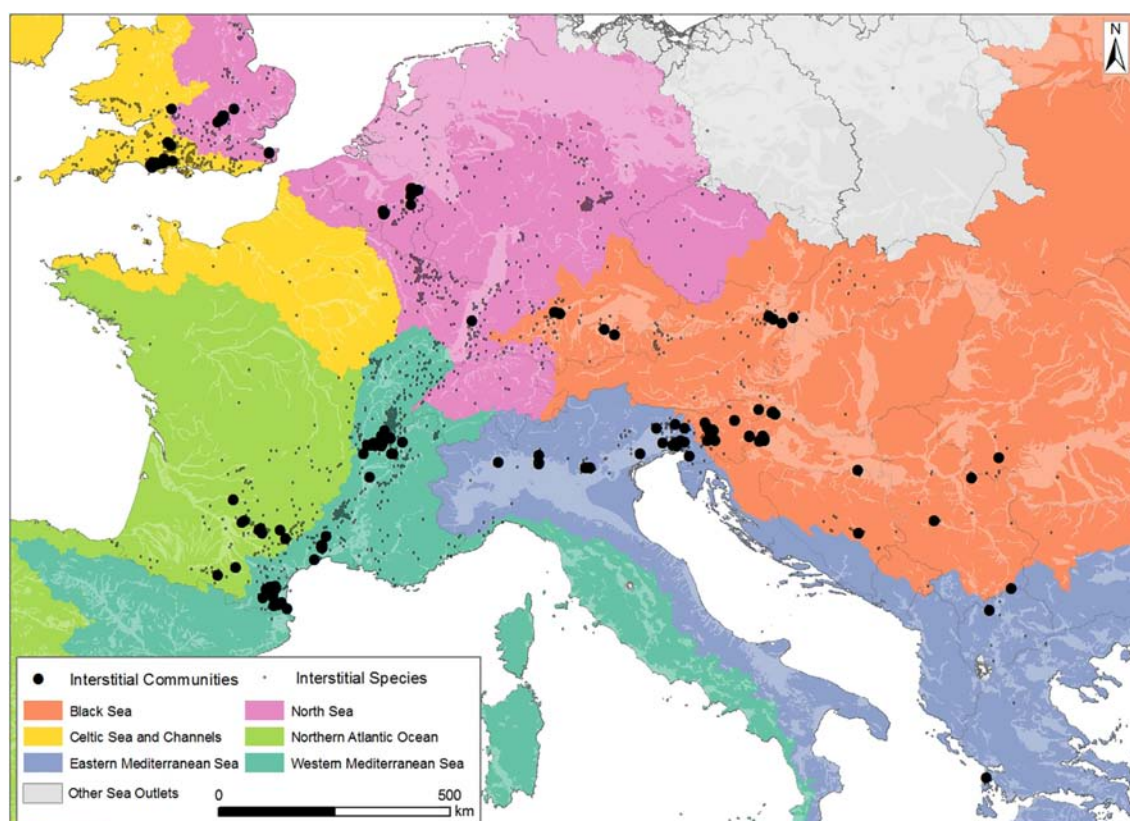


Figure 1. Records of co-occurring interstitial species (black dots) and localities with single species records only ('species' – grey dots) in Europe ($n=155$ communities, source: European Groundwater Crustacean Dataset, Zagmajster et al. 2014). Regions are defined as sea outlets joining major river catchments. Brightly coloured areas within each region present the interstitial habitats suitable for *Niphargus* spp., that is aquifers in unconsolidated sediments with large pore size (as defined in Cornu et al. 2013).

Analysis of functional morphology

We analysed functional morphology related to general feeding properties in amphipods. Amphipods are commonly grazers, but also employ filter feeding and predation (Dahl 1977, Macneil et al. 1997). Firstly, we considered variation in body size of adults (i.e. females with developed oostegites and males with penial papillae). Body size may evolve in response to various selection forces (Fišer et al. 2013), including differences in trophic niches (Vergnon et al. 2013). Secondly, we measured the shape of the first two trunk appendages, called gnathopods. In *Niphargus* these are used for feeding (Ginet 1967), and their size and shape may be subject of resource use-selection (i.e. size of particles they can handle). We analysed three properties: 1) the size of the grip, determining the maximum size of handled food particles, 2) the size of the gnathopods themselves, which is related to the size of the muscles and the strength of the grip, and 3) the length of the carpal article, used for filtering particulate food (Fig. 2). We measured all three properties on both pairs of gnathopods, resulting in six traits (Fig. 2, Supplementary material Appendix 1 Table A3).

Morphometric analyses followed established protocols (Fišer et al. 2009; Supplementary material Appendix 1 Table A3). Propodus circumference was expressed as proportion of total body length. The length of carpus was expressed as a proportion of propodus length (distal–proximal distance). Although these proportions do not completely correct for all aspects of body size, the choice of these proportions allowed us the inclusion of species from descriptions. Palm inclination was expressed as a cosine of a palmar angle. We used a hierarchical cluster analysis based on standardized Euclidean distances and Ward's method to explore whether species cluster into distinct morphological classes (Murtagh and Legendre 2014).

Molecular protocols and phylogenetic analyses

To address whether co-occurrence patterns are associated with phylogenetic differences (Kembel 2009, Best and Stachowicz 2014, Gerhold et al. 2015, Narwani et al. 2015), we assembled a dataset of 115 *Niphargus* (29 interstitial and 86 non-interstitial species) and three *Pseudoniphargus* species as outgroup (Jurado-Rivera et al. 2017). Information on the species/specimens used, including sampling localities, specimen vouchers and GenBank accession numbers are available in Supplementary material Appendix 1 Table A4.

We amplified three nuclear fragments DNA (28S rRNA fragment I, 28S rRNA fragment II, histone 3 subunit A) and one mitochondrial gene fragment (cytochrome oxidase I, COI). Each of the four gene fragments were aligned separately. Alignments were concatenated in Geneious 6.0.5 (Biomatters, New Zealand), partitioned in PartitionFinder 2.1 (Lanfear et al. 2012) and the best-fitted evolutionary model was selected for each partition. Details on laboratory protocols, oligonucleotide primers, alignment procedure and selected evolutionary models are available in Supplementary

material Appendix 1 (supplement to 'Phylogenetic and reconstruction analyses' and Supplementary material Appendix 1 Table A5). Phylogenetic relationships among selected taxa was inferred using Bayesian inference as implemented in MrBayes ver. 3.2 (Ronquist et al. 2012), details on settings are available in Supplementary material Appendix 1. We also reconstructed the evolution of feeding types using several analyses, which are not central to our main hypotheses, and as such presented in the Supplementary material Appendix 1 (supplement subsection 'Phylogenetic and reconstruction analyses').

Patterns of co-occurrence

All analyses described below were conducted using R ver. 3.3.2 (R Development Core Team). We analysed species co-occurrence records using a probabilistic model that estimates whether species' pairwise co-occurrences are more or less frequent than co-occurrences expected by chance, controlling for the number of sampling sites and species rarity (Veech 2013, 2014). We applied Veech's test to species pairs for each region separately using the R package *cooccur* (Griffith et al. 2016). This model assumes that communities are in equilibrium, that is, communities are not at an early stage of colonization, hence biotic and/or abiotic factors have already shaped and stabilized community structure. It also assumes that dispersal should not be limiting. Both assumptions have been supported (see Discussion; Ward and Palmer 1994, Coineau 2000, Lefébure et al. 2007), and are likely fulfilled in our study system.

To test whether the observed co-occurrences might be favoured by the ecological differences or phylogenetic differences among the species, we constructed three matrices for each region, summarizing frequencies of co-occurrences, ecological differences and phylogenetic differences for all species pairs. Species co-occurrences were obtained from the European Groundwater Crustacean Dataset (Supplementary material Appendix 1 Table A6). Ecological differences were inferred from functional morphological traits, and expressed as standardized Euclidean distances between species pairs (Supplementary material Appendix 1 Table A7). Phylogenetic distances were inferred from nuclear marker 28S rRNA I, which unlike other markers, is available for all the studied species. Phylogenetic distances were measured using patristic distances (Supplementary material Appendix 1 Table A8), calculated in R package *ape* (ver. 3.4) (Paradis et al. 2004).

We first tested whether species within a feeding type are more similar to each other compared to species between feeding types. We did this by pairwise comparisons of all species within and between functional groups with respect to functional morphology and phylogenetic relatedness using analyses of similarity (ANOSIM), implemented in the R package *vegan* (Oksanen et al. 2017). Thus, the ANOSIM analysis exploring functional morphology was based on the matrix of standardized pairwise Euclidean distances, while the ANOSIM analysis on phylogenetic relatedness was based

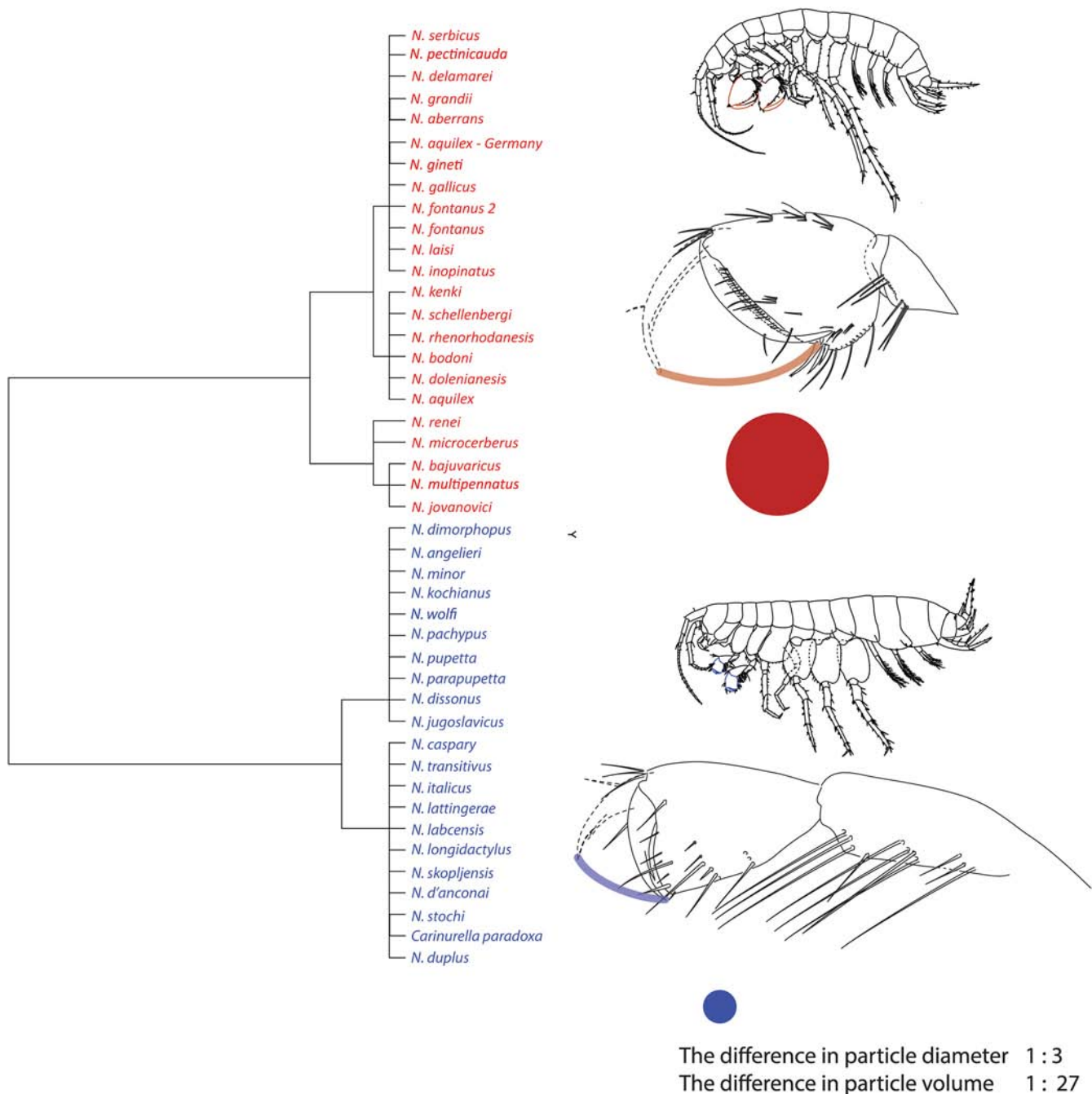


Figure 2. Results of morphometric analysis and functional morphology of gnathopods in interstitial *Niphargus*. Left: cluster analysis identified two morphological groups, one with large gnathopods having a large grip (red), and one with small gnathopods with more setae (blue). Both feeding types were found in all six biogeographic regions. Gnathopods are the main appendages used for handling food particles in *Niphargus*. The shapes of carpus and propodus show trade-offs for feeding on large and small particles. Right: two different feeding types, scaled to the same size. Note the difference in sizes of gnathopod propods related to the body size. Propods of feeding types differ in shape. Detailed illustrations of gnathopods are scaled to the same size in order to illustrate the functional differences due to different shapes. Macrofeeders (red colour) have almond shaped propodus that increases grip size. Microfeeders (blue colour) have long carpus with numerous setae to brush microparticles. Two equally sized individuals manage very different particle sizes.

on pairwise patristic distances. For the ANOSIM, we used the default settings with 999 permutations.

Next, we tested for a relationship between number of co-occurrences and degree of morphological and phylogenetic

differentiation, respectively. The response variable for these analyses was a matrix describing the number of co-occurrences of all species. The explanatory variables were, for the separate analyses, matrices describing the Euclidean distance

(i.e. morphological differentiation) and the patristic distance (i.e. genetic differentiation) among all species pairs and a matrix describing the presence of each of these species in each region. We run multiple regression on distance matrices (MRM), following Lichstein (2007) using the R package *ecodist* (Goslee and Urban 2017). These MRMs account for interdependence in the distance matrices using permutation tests of significance for regression coefficients. Non-significant terms (region, interaction) were removed, and final analyses were run using the simplified model. We run this model using the whole dataset as well as excluding data of no co-occurrence.

Data deposition

All sequences are deposited on GeneBank. Data available from the Dryad Digital Repository: <<https://doi.org/10.5061/dryad.kd3g03r>> (Fišer et al. 2019).

Results

Species richness varied from 3 to 21 species per region (Celtic Sea drainage: 3 species, Northern Atlantic Ocean drainage: 4 species, North Sea: 7 species, Western Mediterranean Sea: 9 species, Eastern Mediterranean Sea: 19 species and Black Sea: 21 species). At the local scale, communities consisted of two to eight species. In total, this resulted in 376 pairwise co-occurrences, that is, a specific species co-occurring with another species at a given number of independent sites (Celtic Sea drainage: 16 pairwise co-occurrences, Northern Atlantic Ocean drainage: 16 pairwise co-occurrences, North Sea: pairwise 22 co-occurrences, Western Mediterranean Sea: pairwise 64 co-occurrences, Eastern Mediterranean Sea: pairwise 74 co-occurrences and Black Sea: pairwise 184 co-occurrences).

The probabilistic analysis suggested that pairwise species co-occurrences were in 68% of the cases not different from random expectation (45–100% per region; Table 1, Supplementary material Appendix 1 Fig. A1). In the remaining 32% of the cases, however, they were significantly more (0–18% per region, Table 1) or less frequent (0–36% per region, Table 1) than random co-occurrences.

Cluster analyses of morphological traits identified two distinct groups of species, corresponding to two main feeding types (Fig. 2, cophenetic correlation coefficient 0.695). The first group comprised species with small propods of gnathopods (small grip) and long carpal articles with numerous setae. These species are unlikely to handle large particles, but may use numerous setae on carpal articles as brushes to gather food. We refer to them as microfeeders. The second group comprised species with moderately sized to large propods and short carpal articles, likely handling large particles (large grip). We refer to them as macrofeeders. Both feeding types most likely evolved several times independently (Fig. 3, details in Supplementary material Appendix 1 subsection ‘Phylogenetic and reconstruction analyses’).

Phylogenetically related species of the same feeding types were not regionally segregated in a predictable pattern (Fig. 3). Some clades or even species of the same feeding types are shared among different regions. For instance, some macrofeeders occur both in the Eastern Mediterranean Sea and in the Black Sea region. Also, feeding types of the same region may belong to different phylogenetic lineages (e.g. macrofeeders of the West Mediterranean Sea are composed of unrelated *N. rhenorhodanensis* complex, *N. delamarei* and an undescribed member of *N. fontanus* species complex). Finally, species of the same feeding types may occur in different regions (e.g. macrofeeders of the Eastern Mediterranean Sea are different than macrofeeders of the Celtic Sea, Western Mediterranean Sea, North Sea and North Atlantic Ocean).

An analysis of co-occurrence of feeding types showed that both feeding types are present in all regions. Both feeding types were represented with a roughly similar number of species in all regions. The feeding types had on average equal proportions also at the local scale, although some records substantially deviated toward either type (Table 1). We found that morphological differences between feeding types were higher than those within feeding types (ANOSIM statistic $R=0.701$, $p<0.001$; Fig. 4A). Feeding types do not differ with respect to phylogenetic distances (ANOSIM statistic $R=0.022$, $p=0.3$; Fig. 4B).

The relationship between number of co-occurrence records and morphological differentiation (i.e. Euclidean distance) was positive in five out of six regions for the full dataset (Fig. 5) as well as when excluding species pairs with zero co-occurrences (Supplementary material Appendix 1 Fig. A2). In the analysis of multiple regression on distance matrices we found a marginally significant effect for the positive relationship between Euclidean distance and number of co-occurrences when all data were considered ($F=158$, $p=0.098$), and a significant effect when the no co-occurrence data were removed ($F=46.8$, $p=0.037$). No relationship was found between phylogenetic (i.e. patristic) distances and number of co-occurrences ($F=61.3$, $p=0.24$) and the removal of the no co-occurrences did not affect this conclusion ($F=21.5$, $p=0.25$).

Discussion

Our results give both a detailed but also a generalizable view on the co-occurrence patterns and possible coexistence mechanisms of interstitial *Niphargus* species. Two thirds of the co-occurrences are not different from what would be expected by chance, while the remaining third significantly deviated from a random expectation. Random co-occurrences can indicate neutral dynamics and equalizing mechanisms of co-occurrence, an early stage of colonization (when environmental filtering and/or biotic interactions did not have sufficient time to shape the communities), or fluctuating environmental filtering driving communities away from their equilibrium. Non-randomly frequent co-occurrences

Table 1. Summary of the number of sites (local scale) and the overall number of interstitial *Niphargus* species (divided in macro- and microfeeders). The within-region ratio of macro- versus microfeeders across all sites is given (the median, 10% and 90% percentiles respectively). Finally, the number of co-occurrences that are significantly less likely than expected by chance (negative), not different from chance (neutral) and significantly more likely than expected by chance (positive) are given based on the co-occurrence analysis by Veech (2013) and Griffith et al. (2016).

Region	Number of sites	Number of <i>Niphargus</i> species	Number of macrofeeders	Number of microfeeders	Median ratio macrofeeders/microfeeders (across all sites)	10% percentile macrofeeders/microfeeders (across all sites)	90% percentile macrofeeders/microfeeders (across all sites)	Negative	Neutral	Positive
Black Sea	39	21	12	9	1	0.15	2.2	8	48	8
Celtic Sea and Channels	12	3	2	1	1	0.15	2.2	1	2	0
E. Medit. Sea	27	18	4	14	1	0.33	1	4	15	2
North Sea	21	7	3	4	1	0.35	1	4	5	2
N Atlantic Ocean	11	4	3	1	1	0.35	1	0	3	0
W Medit. Sea	44	9	6	3	1	1	2	9	18	5

may indicate interspecific interactions, limitations in dispersal or local environmental filtering. Despite some limitations and caution needed in interpreting the results of the co-occurrence model (Veech 2013, see below), we have two overall conclusions: firstly, the number of random co-occurrences would likely not be that high if species were spatially strongly restricted, suggesting that dispersal limitation is not important in assembling communities of interstitial *Niphargus*. Secondly, the random co-occurrences suggest either ecological similar species and equalizing mechanisms of co-occurrence, or ecologically different species and stabilizing mechanisms of co-occurrence. As we discuss in the following, the sum of our results is in support of the latter hypothesis.

All studied interstitial *Niphargus* species clearly classified into two distinct morphological classes (micro- and macrofeeders), reflecting differences in species' trophic ecology (Fig. 2). Both feeding types have been repeatedly found in all regions (Table 1) and have evolved several times independently (Fig. 3, Supplementary material Appendix 1 subsection 'Phylogenetic and reconstruction analyses'), which strongly indicates their selective advantage. A similar conclusion is supported by the ANOSIM analysis, in which feeding types did not explain differences between the phylogenetic relationships (Fig. 4). Hence, we suggest that interstitial species occupy two different adaptive peaks, and that this divergence minimized competitive interactions. The two feeding types can represent a basis of stabilizing mechanisms of species co-occurrence. This is consistent with studies from Edwards aquifer (Texas, USA), showing that different amphipod species monopolize heterogeneous sources of organic food and occupy different trophic levels (Hutchins et al. 2014, 2016).

Furthermore, the regression analyses suggest a positive, albeit weak relationship between niche differentiation and frequency of co-occurrences (note that result was significant only when the no co-occurrence data were excluded). If co-occurrences were predominantly mediated by equalizing mechanisms, ecologically similar species would co-occur more frequently than dissimilar ones, and the overall relationship would be negative. This is not the case here. Although the importance of niche differentiation varies in different regions (Fig. 5), the data suggest that niche differentiation and stabilizing mechanisms of co-occurrence importantly explain the structure of interstitial communities of *Niphargus* (see also Fišer et al. 2012). Phylogenetic differences do not play a role in community assembly. We recognize that our study does not encapsulate all possible mechanisms of co-occurrences, and the importance of equalizing mechanisms should not be completely discarded. At least part of random co-occurrences might have been of species of the same feeding type. However, many other explanations, such as early stage of colonization, low dispersal and fluctuating environmental filtering, seem less plausible. Interstitial is an old habitat, with generally stable environmental conditions (Ward and Palmer 1994, Coineau 2000). *Niphargus* apparently colonized many of these sites a long time ago, mostly after the last glaciation

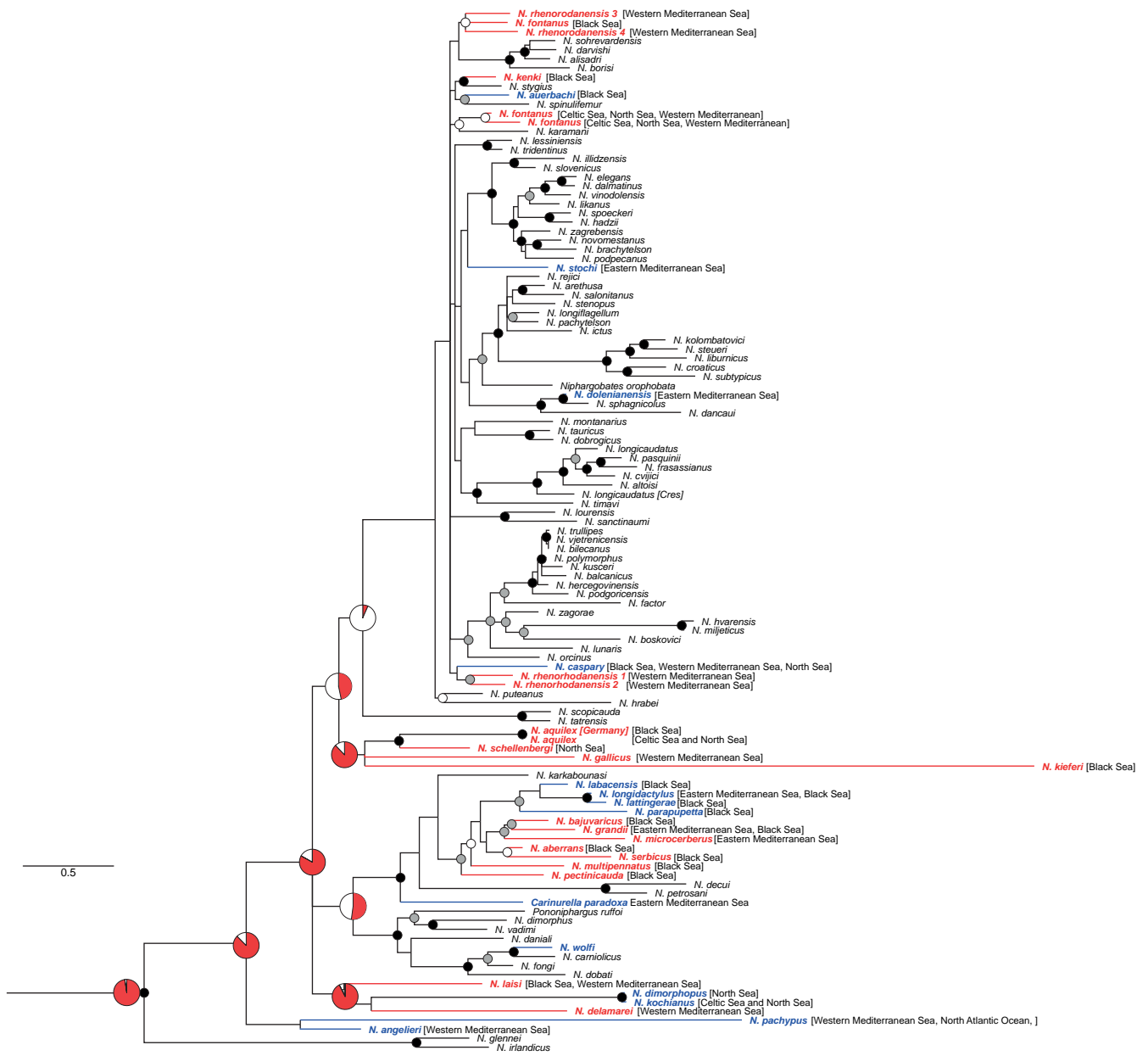


Figure 3. Phylogenetic relationship of *Niphargus* reconstructed using Bayesian inference, and the evolution of micro and macrofeeders. The support for nodes is colour coded (posterior probability values black – 1, grey 0.95, white 0.9), the outgroup is omitted. The interstitial species were apparently ancestors to all *Niphargus* (pie charts show the probability that ancestor was inhabitant of interstitial); several lineages of ‘small feeding type’ apparently evolved secondarily. Similarly, micro- and macrofeeders evolved several times independently, the latter seem to be ancestral (indicated by the red colour in pie charts; see also Supplementary material Appendix 1 section ‘Phylogenetic and reconstruction analyses’).

(Lefebvre et al. 2007). Moreover, because the environmental conditions in the interstitial are generally stable over long periods and across regions, it seems unlikely that fluctuating environmental filtering could generate random co-occurrence pattern (Zagmajster et al. 2014).

While all the results are overall robust and the patterns repeatable, as observed across different and independent drainage basins, our study is still based on a series of assumptions and possible caveats. These caveats are not different

from those commonly assumed in macroecological studies, but are worthwhile to be discussed. Specifically, we identified four possible aspects of our dataset that may affect the conclusions. First, the analysis of co-occurrence data only makes statements about the observed pattern being expected by chance or not (see above). The subsequent interpretations then depend on the validity of the assumption that dispersal is not limiting. This assumption is in accordance with molecular studies, which provided evidence for long-distance

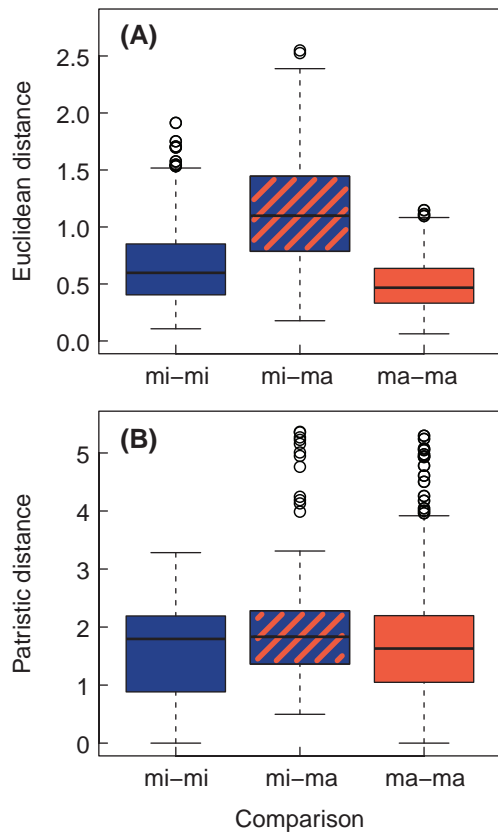


Figure 4. Boxplots showing pairwise comparison of all species within the functional groups (mi–mi and ma–ma; mi = microfeeders, in blue and ma = macrofeeders, in red) as well as between functional groups (mi–ma) for (A) Euclidean distance, as a measure of morphological similarity and (B) patristic distance, as a measure of phylogenetic relatedness. Boxplots give median (thick line), interquartile range (box) and 1.5× interquartile range (whiskers).

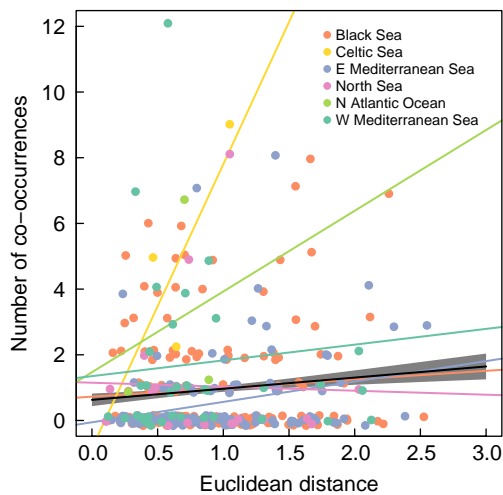


Figure 5. Relationship between morphological distances and number of co-occurrences for the six different regions (colour-coded). For illustration purposes, we show individual linear regression lines fitted to the data of the individual regions. Shaded area represents the SE around the overall fit (black line).

dispersal along ‘the interstitial highways’ (Lefebure et al. 2007). Noteworthy, the assumption of unlimited dispersal is critically needed for the interpretation of non-random species’ co-occurrences, because the same pattern may either emerge due to ecological factors or due to violations of the dispersal assumption. In our study, only one third of co-occurrences are non-random, suggesting that even in the most conservative interpretation only a minor fraction of our results could be misinterpreted. Second, the ecological differentiation was based on differences between mean species phenotypes and does not account for competition-driven character displacement, which has been shown to potentially have an important role in niche differentiation in *Niphargus* (Fišer et al. 2015a, Delić et al. 2016). Therefore, the case-specific, small-scale ecological differences (e.g. local character displacement) may be underestimated. Incorporation of local divergences, if available, might reveal character displacements, increase the differences between locally co-occurring species and even strengthen our conclusions that suggest the importance of species-specific ecological differences. Third, although our dataset is extensive, it is mainly based on co-occurrence data derived from the literature, as again is often the case in macroecological studies. Thus, we cannot control for variation in sampling effort, and have relatively little information on the robustness of ‘absence’ data. The observed co-occurrences include positive records only, while the further unobserved co-occurrences may include also false negatives. The analysis is based on untested assumption that the false-negative absences are unbiased across species or geographical range; therefore, additional data might change the results. However, all of this is not expected to result in any systematic patterns, but rather to erode patterns, and is thus conservative with respect to the interpretations. Seeing that we found clear and repeatable patterns despite the heterogeneity of the data suggests relatively strong signature of the underlying processes. Lastly, our study did not consider all cryptic diversity, that is, morphologically indistinguishable species (Bickford et al. 2007). While we had genetic data for the majority of species, we are missing these data at the population level, and would expect that at least some of the nominal species contain multiple cryptic species. Cryptic species are a common phenomenon in *Niphargus* (Lefebure et al. 2007, Trontelj et al. 2009, Meleg et al. 2013, Delić et al. 2017a, b, Eme et al. 2018) and may be broadly sympatric or even co-occurring (Fišer et al. 2015b, Delić et al. 2017a). Cryptic species may increase the complexity of local communities and underestimate taxonomic structure, possibly affecting the conclusions (Fišer et al. 2018). Importantly, analyses of functional morphology are not sensitive to cryptic species. In summary, even though we cannot completely assess the impact of these four caveats, we do not have any indication that any of them can change the main conclusions.

We conclude that both neutral dynamics and differentiation along a trophic niche axis are likely contributing to species co-occurrences on a local scale (Gravel et al. 2006) and that these processes are reflected over different geographic scales. Our study shows how the species rich and

highly diversified group of interstitial *Niphargus* may offer an interesting model system to evaluate niche differentiation at continental scales, with local communities and respective conditions being naturally highly replicated (Mammola 2018). Communities in a long-term stable environment, such as groundwater, caves, deep sea or deep lakes, seem to be a promising model system for testing the contrasting effects of environmental filtering and interaction processes, driving species differentiation and co-occurrence. Undeniably, exploration of these communities is technically challenging and requires specialized gears. Also, opportunities for experimental manipulation are limited (Larned 2012). However, communities in these environments are less prone to transient phenomena commonly observed in many ecological systems (Hastings et al. 2018). These transient phenomena violate major assumptions underlying many ecological concepts, and are possibly hindering conclusions on ecological or evolutionary processes underlying community assembling. Such transient phenomena are less likely in stable habitats protected from short-term environmental fluctuations (Culver and Papan 2009), and studies of such habitats may give insights into patterns and dynamics otherwise blurred by transient phenomena (Hastings et al. 2018). Also, communities in such habitats may likely have reached a steady-state over evolutionary timescales, offering valuable insights into eco-evolutionary feedbacks. As such, *Niphargus* in interstitial habitats, or other organisms in such stable habitats (including many crustaceans or molluscs in interstitial or deep sea habitats, or fish in deep lakes) may present excellent model systems to study the role of conflicting selection forces over evolutionary timescales. In this study, we explored only a fraction of groundwater communities. An interesting question would thus be whether the results of our study can be generalized to other animal groups in the same communities, to groundwater communities in general, or to other communities that assembled in remote and stable environments. Thus, more systematic explorations of the unseen world, as the interstitial undoubtedly is, could indeed give a new insight into ecological dynamics in a wider sense.

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Supplementary material (available online as Appendix ecog-03983 at www.ecography.org/appendix/ecog-03983).
Appendix 1.