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The Ancient Britons: Groundwater fauna survived extreme climate changes over tens of millions of years across NW Europe

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Abstract

Global climate changes during the Cenozoic (65.5 - 0 Ma) caused major biological range shifts and extinctions. In Northern Europe, for example, a pattern of few endemics and the dominance of wide-ranging species is thought to have been determined by the Pleistocene (2.59 – 0.01 Ma) glaciations. This study, in contrast, reveals an ancient subsurface fauna endemic to Britain and Ireland. Using a Bayesian phylogenetic approach we found that two species of stygobitic invertebrates (genus *Niphargus*) have not only survived the entire Pleistocene in refugia but have persisted for at least 19.5 million years. Other *Niphargus* species form distinct cryptic taxa that diverged from their nearest continental relative between 5.6 and 1.0 Ma. The study also reveals an unusual biogeographical pattern in the *Niphargus* genus. It originated in Northwest Europe ~88 Ma and underwent a gradual range expansion. Phylogenetic diversity and species age are highest in Northwest Europe suggesting resilience to extreme climate change, and strongly contrasting the patterns seen in surface fauna. However, species diversity is highest in Southeast Europe indicating that once the genus spread to these areas (~ 25 Ma), geomorphological and climatic conditions enabled much higher diversification. Our study highlights that groundwater ecosystems provide an important contribution to biodiversity and offer insight into the interactions between biological and climatic processes.

INTRODUCTION

Global climate has changed significantly throughout the Cenozoic (65.5- 0 Ma) with glacial cycles during the Miocene, Pliocene and Pleistocene (Louwye *et al.* 2008; Zachos *et al.* 2001a; Zachos *et al.* 2008). Precipitation also fluctuated from extended arid (13.2 - 11.5 Ma) to very wet conditions (10.2-9.8 Ma; Bohme *et al.* 2008). Fauna, for example ectothermic vertebrates and freshwater Crustacea, experienced major range shifts or extinctions, and ecosystems were dramatically modified (Bohme 2003, Klaus and Grosse 2010). During the Pleistocene glaciations (2.59 - 0.01 Ma), large areas of the northern hemisphere were covered by glaciers or permafrost and were uninhabitable (reviewed in Provan & Bennett 2008), with particularly marked biogeographic impact in northern Europe.

Britain and Ireland are a prime example illustrating this ecological impact, with repeated covering by glaciers and permafrost greatly limiting the persistence of terrestrial species. These islands are likely to have been isolated during interglacials, at least since the formation of the English Channel ~ 0.45 Ma (Gupta *et al.* 2007), preventing dispersal of terrestrial fauna from the continent. Strong palaeontological and genetic evidence indicates that the majority of the current fauna of Britain and Ireland arrived from mainland Europe following the Pleistocene glaciations, dispersing across a land bridge with continental Europe during the short period after ice retreat and before the bridge was submerged by rising sea levels (Hewitt 2004; Yalden 1982). Consequently Britain and Ireland have always been thought to have limited endemic biodiversity. However, the biodiversity of groundwater ecosystems may challenge this orthodoxy, with evidence from North America (Holsinger *et al.* 1983) and Iceland (Kornobis *et al.* 2010), suggesting that groundwater ecosystems may occur under glaciated areas. Moreover species are present in formerly glaciated areas, indicating that they must either have survived in refugia or dispersed there since glaciations (Galassi *et al.* 2009; Martin *et al.* 2009). As with recent advances in our understanding of deep ocean vent ecosystems (Dubilier *et al.* 2008; Lopez-Garcia *et al.* 2003; Van Dover *et al.* 2002), groundwater ecosystems may offer novel insights into fundamental ecological and evolutionary processes. In this study we use a Bayesian phylogenetic approach, which shows that groundwater fauna must have persisted through glacial periods in Britain and Ireland within refugia. Furthermore, we show how groundwater ecosystems may have developed across Europe in response to changing climatic and geomorphological conditions. Finally we demonstrate that the biogeographical pattern of diversity across Europe is unexpected, with increasing phylogenetic diversity at higher latitudes.

Our study focuses on amphipod crustacea, which are a major component of subterranean ecosystems, and offer a tractable model for investigating ecological and evolutionary processes within this challenging environment. The largest genus among them is *Niphargus* (Amphipoda: Niphargidae) with over 300 described species distributed across most of Europe (Vainola *et al.* 2008). *Niphargus* are stygobites (obligate groundwater inhabitants), ~ 0.3-3.0 cm in length, which are adapted to live in subterranean environments. They are blind, lack pigmentation and have elongated appendages (Figure 2a). Previous phylogeographic studies of *Niphargus* have demonstrated high levels of endemism and cryptic diversity at small geographic scales (Fišer *et al.* 2008; Trontelj *et al.* 2009), suggesting limited dispersal and long-term persistence of local populations, as well as morphological convergence for adaptations to the subterranean environment (Trontelj *et al.* 2012). Only six taxa of *Niphargus* are currently known from Britain and Ireland (Robertson *et al.* 2009). Here we show that two species endemic to Britain and Ireland (*N. glenniei* and *N. irlandicus*) are far older than previously thought, suggesting persistence through extreme climatic and geomorphological changes over at least 19 million years. Furthermore, those species thought to have been wide ranging European species (*N. aquilex*, *N. fontanus*, *N. kochianus*) are in fact also ancient British endemics.

MATERIAL AND METHODS

Sampling

A modified Cvetkov net sampler, notenboom sampler, or baited traps were used to collect samples from boreholes, springs and wells. 454 *Niphargus* specimens were preserved in >70% ethanol (Figure 1, Table S1), comprising samples from 63 populations (222 individuals) in Britain and Ireland including five of the six species present. We were unable to obtain sufficient samples for DNA extraction of the rare *N. wexfordensis*. Additionally, 224 individuals from 47 populations and 5 species were collected from Belgium, the Netherlands, Germany and France (Figure 1, Table S1) including all species known to co-occur in Britain and continental Europe (*N. aquilex*, *N. fontanus, N. kochianus*). Furthermore, samples were obtained from two species which occur in the vicinity of Britain located in France, but for which no DNA sequence data existed (*N. pachypus*, 1 population, 2 individuals; *N. forelli*, 2 populations, 4 individuals). Samples from published data sets (see below) covered largely the central and south-eastern part of the distribution and included data of 185 populations from 74 described species (Figure 1, Table S1).

De novo *sequencing and data sets for phylogenetic analysis*

Genetic variation of *Niphargus* was assessed at two mitochondrial genes, cytochrome oxidase subunit I (COI) and 16s rRNA (16S) and the nuclear small subunit 28s rRNA (28S; for details see Supplementary materials). Our analysis combined these new DNA sequence data with all published *Niphargus* sequence data for 28S, COI and 16S available on GenBank on June 1st, 2012 (Fišer *et al.* 2008; Flot 2010; Flot *et al.* 2010; Hänfling *et al.* 2008; Hartke *et al.* 2011; Lefébure *et al.* 2006; Lefébure *et al.* 2007; Trontelj *et al.* 2009). Also included were published sequence data of the mitochondrial 12s rRNA region (12S) and the large subunit 18s rRNA (18S) for the taxa covered in the combined data set. In total, data were included from 78 described species, and several putative cryptic species, from 170 locations across the genus' European range (Figure 1, Tables S1, S3). This includedeight of the nine species (the ninth, *N. boulangei*, was too rare) that occur within 200 km of Britain (Table S1). The combined data set provided phylogeographic information (more than 10 populations) for 8 of the 78 described species (Table S2, *N. aquilex, N. fontanus, N. glenniei, N. kochianus, N. irlandicus, N. rhenorhodanensis, N. virei, N. schellenbergi*). A further 11 species were covered by more than 1 specimen from 1 - 3 locations.

A total of 36 taxa from 9 amphipod families were used as outgroups to root the *Niphargus* phylogeny, and provide calibration points for a molecular dating analysis (see Table S4), The outgroup taxa include previously identified sister groups to Niphargidae (Englisch *et al.* 2003, Fiser *et al.* 2008) and representatives of clades from a dated phylogeny of gammarid amphipods (Hou *et al.* 2011). We used the genes 28S, COI, 18S and elongation factor 1 alpha (EF-1a). The alignment of COI and EF-1a sequences was carried out using MUSCLE (Edgar 2004) in combination with MEGA version 5.05 (Tamura *et al.* 2011). Ribosomal genes were aligned with the software MAFFT version 6 (Katoh *et al.* 2002) using the alignment strategies Q-INS-i or E-INS-i.

Delineation of OTUs, multi-locus alignments and phylogenies

Cryptic diversity and taxonomic misclassification are common in *Niphargus*. We therefore used a DNA barcoding approach based on the two genes with the largest coverage (COI and 28S) to identify cryptic lineages within species and to delineate operational taxonomic units (OTUs) with independent evolutionary histories (for details see Supplementary materials). Many of these OTU's are likely to fulfil the criteria for separate species depending on the definition applied, but a discussion of species status is outside the scope of this paper. A multi-locus alignment was created using representatives of OTU's of *Niphargus* and selected outgroups. One representative of each OTU was chosen at random for inclusion in the supermatrix (Table S1). Amphipod outgroups included three representatives selected for each of the 4 *Gammarus* freshwater clades, 6 representatives of the marine *Gammarus* group, 3 representatives of the Baikalian Gammarids and all outgroups used in Hou *et al.* (2011), providing 13 time-calibrated nodes. For each gene, all sequences of the selected taxa were aligned. Phylogenetic analysis of the multi-gene matrix was carried out using Bayesian analysis as implemented in MrBayes v3.2 (Ronquist *et al.* 2012). Genes were used as partitions and model parameters between partitions were unlinked. Two independent Markov chain Monte-Carlo (MCMC) chains were run for 10,000,000 iterations each, sampling every 1,000 iterations. The first 25 % of each run was discarded as burnin with the remaining samples pooled and used to create a maximum clade credibility tree.

Molecular dating using a Bayesian analysis

BEAST (Bayesian Evolutionary Analysis Sampling Trees) version 1.7.4 (Drummond *et al.* 2012) was used to generate an ultrametric phylogeny and estimate the time of the most recent common ancestor (TMRCA) for each node using a Bayesian MCMC analysis. Tree topology was constrained to that obtained from the MrBayes phylogenetic analysis. Genes were used as partitions and substitution rates and clocks were unlinked in the analysis. An uncorrelated lognormal relaxed clock (Drummond *et al.* 2006) and a Yule speciation prior were used. A time calibrated phylogeny of the amphipod group Gammaridae (Hou *et al.* 2011) was used to provide 11 external calibration points (for details see Supplementary materials).

Ancestral longitude and latitude reconstructions

We used the Bayesian MCMC phylogenetic ancestral state reconstruction method introduced by Organ *et al.* (2007) to infer the geographical location of the MRCA for each node. The method was chosen because of its superior performance with phylogenetic trees that span millions of years (Montgomery *et al.* 2010). Similar methods have been used to infer ancestral longitudes and latitudes in a phylogenetic context (Bouckaert *et al.* 2012; Lemey *et al.* 2009). With exact geographical ranges mostly unknown it was not possible to calculate range centroids. The range size of most *Niphargus* is small, however, usually <100km in diameter. (Trontelj *et al.* 2009). The few taxa with a larger range such as *N. virei* and *N. rhenorhodanensis* consist of a number of cryptic taxa or distinct phylogeographic units with a much smaller range (Lefébure *et al.* 2006; Lefébure *et al.* 2007). This cryptic diversity is reflected in the OTUs used for the phylogenetic analysis. We therefore used the geographical coordinate of the individual chosen at random for the phylogenetic analysis as a proxy for the taxon's geographic location. We estimated a phylogenetic model of evolution for the *Niphargus* ingroup species where longitude and latitude were correlated using the computer program BayesTraits (Pagel *et al.* 2004). We ran the MCMC chain for one million iterations after apparent convergence sampling every 1,000 iterations from the chain and repeated the analysis multiple times. We also simultaneously estimated the phylogenetic signal parameter λ (Pagel 1999). The parameter λ varies between 0 and 1, where 1 is interpreted as having the traits covary and zero means that the traits evolve independently of the phylogenetic relationships among species. Repeated analyses produced almost identical results, thus we provide results from a single chain only.

Geographic variation in species diversity and diversification rates

To quantify geographic patterns in the distribution of species, we used the checklist of *Niphargus* species publically available at http://niphargus.info/ (Cene Fišer, unpublished) and created presence/absence data for 9 geographic regions in Europe based on the biogeographic areas for European freshwater fauna described in Illies (1978). Some regions were pooled to reduce the effect of uncertainty in geographic distribution (see Table S1). Species richness and species richness standardised for area (species/100,000 km^2) were calculated using area sizes from Hof *et al.* (2008). To test the hypothesis that species richness differed between the Western and the Eastern parts of the genus' distribution, biogeographic areas were grouped into West (Spain, British Isles, West Europe, Central Europe) or East (Italy, Balkans, Ponto-Danubian, Caucasus) and their mean species richness standardised for area compared using a Mann-Whitney U-test. We tested a geographic association of net-diversification rates accounting for shared ancestry as implied by our phylogeny. We implemented the 'simple test' described in Freckleton *et al.* (2008) to relate traits to net-speciation rate (as determined by root-to-tip node count) in a Bayesian analytical framework. In order to explicitly test a hypothesis of an increase in diversification rate towards the south-east, a spatial rotation was applied to the coordinates of the samples to produce axes aligned at 15 degrees from the original. The most north-westerly point within the dataset was used as a new origin for the x axis, and the distance between this origin and the other points along the axis was calculated to provide a measure of how far towards the southeast the each point lies.

RESULTS

Data overview

In all 43 OTUs were identified based on the COI phylogeny including 19 previously described cryptic lineages and 9 newly identified OTUs (Figure S1). Eighty nine additional taxa were identified based on 28S sequences (Figure S2), most corresponding to described or previously reported cryptic species (Table S1). In total 132 OTUs were identified using DNA barcoding.

Phylogenetic analysis

Results from multigene phylogenies revealed that the island endemics, *N. irlandicus* and *N. glenniei* are sister taxa with no close relative in Continental Europe (Figure 2b, S1, S2). The remaining taxa fall into eleven divergent lineages. These show strong geographical associations demonstrating poor dispersal within the genus even at large scales and over long geological time scales (Figure 2c). Phylogenetic diversity of *Niphargus* in Ireland and Britain is very high given the low species diversity, with the six species representing four different major lineages. This high phylogenetic diversity is apparent in other northern parts of the genus distribution. Nine lineages occur north-west of the Alps, with only three lineages south-east of the Alps. The overall pattern is a decrease in phylogenetic diversity from northwest to southeast Europe.

Outgroup rooting revealed that the split between the *N. irlandicus* / *N. glenniei* group and the remaining species represents the most basal node in the phylogenetic tree (Figure 2b). Our results also show that the three species which co-occur in Britain and Continental Europe (*N. aquilex*, *N. fontanus* and *N. kochianus*) are in fact phylogenetic clades comprising 7, 4 and 4 highly divergent lineages respectively which met our criteria for OTU's (Figure 3a-d) Each complex contains endemic British OTU's. (Table 1). Three cryptic *N. aquilex* OTU's occur in Britain, two of which (*N. aquilex A1 and B*) have not been found in continental Europe and have evolved independently. The two other non-endemic British taxa *N. kochianus and N. fontanus* are also represented by genetically distinct British lineages that diverged from their continental European counterparts after separate isolation events.

Whilst it is possible that there are additional OTUs not included in this analysis that are more closely related to the UK OTUs, this is unlikely because of the comprehensive sampling coverage in this study. Importantly we have (i) sampled 8 of the 9 species which occur in the vicinity of 200 km from the British coast line. The only unsampled species from this group (*N. boulangei)* is extremely rare and has only been described once from a single location; (ii) all taxa occurring in Britain and Ireland have been sampled on a phylogeographic scale covering most of their range; (iii) there has been extensive groundwater sampling in France and Belgium, for example during the recent large EU funded Pascalis project (Dole-Olivier *et al*. 2009). Therefore it is unlikely that there are additional undescribed *Niphargus* species in the countries adjacent to the UK; (iv) long distance colonisation is extremely unlikely and any additional undescribed species in more distant areas are unlikely to impact on the conclusions of this study. Furthermore, our findings are strengthened by the fact that we found a consistent pattern across all taxa.

Bayesian dating analysis and ancestral longitude and latitude reconstructions of MRCAs

Estimates of divergence times for all nodes separating British and Irish taxa from their nearest relatives are shown in Table 1. The ultrametric tree generated from the analysis is shown in Figure 4a. The Bayesian dating analysis and ancestral state reconstruction of the geographic origin of the MRCA for each node revealed that the MRCA of the two endemic British taxa was estimated to have lived in south-west England around 19.5 million years ago (95% HDP, 38.1 - 6.7Ma, Figure 4b, 4f, Table 1). Thus, *Niphargus* must have persisted in Britain and Ireland at least since the Miocene making it the oldest known fauna by at least two orders of magnitude. This common ancestor must have existed at a time when the British and Irish landmasses were joined. The two sister taxa may subsequently have become isolated during an Oligocene marine inundation of the Irish Sea Basin (Cope 1997).

Three cryptic *N. aquilex* taxa occur in Britain, two of which (*N. aquilex A1 and B*) have not been found in continental Europe and have evolved independently for 1.0 and 5.6 Ma respectively (Table 1). The two other non-endemic British taxa *N. kochianus and N. fontanus* are also represented by genetically distinct British lineages that diverged from their continental European counterparts after separate isolation events 2.9 and 0.8 Ma respectively. Collectively these data suggest that almost the entire *Niphargus* fauna of Britain and Ireland is comprised of endemic lineages of Miocene or late-Pliocene to mid-Pleistocene origin. Final isolation of these taxa from continental populations may have resulted from the formation of the English Channel at 0.45 Ma (Gupta *et al.* 2007).

The geographic distribution of MRCAs for nodes of different ages identified central France in northwestern Europe as the origin of the *Niphargus* genus in the late Cretaceous (88 Ma). From there the ancestral locations move with decreasing node age towards the southeast (Figure 4b).

Geographic variation in species diversity and diversification rates

Investigation of the geographic variation in species diversity revealed that the number of *Niphargus* species varies greatly across different geographic areas from 1 species in Spain to 136 in the Balkans (Figure S5). In contrast to phylogenetic diversity the species richness of the Western region is significantly lower than that of the Eastern region ($P < 0.05$). Investigation of the geographic variation in diversification rates shows that the number of nodes along each root-to-tip path in the *Niphargus* species level phylogeny correlates significantly with distance towards the southeast (correlation coefficient [SD] = 0.18 [0.014], log Bayes Factor = 9.8). A log Bayes Factor value of between 6 and 10 provides strong support for the hypothesis tested. Net-diversification rate in *Niphargus* therefore increases in a south-easterly direction.

DISCUSSION

Phylogenetic evidence for long-term persistence of Niphargus in NW Europe

Paleontological and genetic evidence suggests that the majority of surface fauna that currently live in Britain and Ireland originated from late Pleistocene/Holocene dispersal from Continental Europe (Hewitt 2004; Wheeler 1977; Yalden 1982). Endemic fauna are therefore rare (Pimm *et al.* 1995), and are restricted to a few surface invertebrate and vertebrate sub-species (e.g. the Irish hare, Reid 2011); and the Shelly freshwater whitefish, Kottelat & Freyhof 2007; and the avian Scottish crossbill, which is sometimes considered a species, Summers *et al.* 2007, see Table S7 for more examples). Critically these fauna have only been present for a few tens of thousands of years. In contrast our data indicate that groundwater contains by far the oldest endemic fauna, which have persisted for millions of years and represent a significant contribution to biodiversity.

Furthermore, this ancient groundwater fauna has survived the extreme geological and climate changes that have occurred over the past 20 million years. Groundwater temperatures are influenced by air temperature (Figuera *et al.* 2011) and can range from 0 and 6°C in glacial and periglacial climates (Parsons 1970; Williams 1970) to > 25° C in areas with warm climates (Eberhard *et al.* 2009; Weyhenmeyer *et al.* 2000). Niphargids must therefore have survived a wide range of groundwater temperature conditions as climate changed between glacial and warm conditions. However, temperature and chemistry change much more slowly in groundwater than surface waters, and hence groundwaters are buffered from temperature extremes and rapid hydrological and biological change (MacDonald *et al.* 2012), and the relative stability of the subsurface environment may explain the persistence of groundwater invertebrates through changing climates. *N. glenniei* and *N. irlandicus* persisted in NW Europe throughout the Miocene surviving both glacial and extreme wet periods (Zachos *et al.* 2001a; Zachos *et al.* 2001b) which were associated with range shifts and local extinctions in other fauna (Zachos *et al.* 2001a; Zachos *et al.* 2008). Together with *N. aquilex B* they also persisted in Britain throughout the Pliocene when temperatures and sea levels were higher than today (Dwyer & Chandler 2009), and groundwaters would have been substantially warmer than they are now.

All the *Niphargus* lineages in Britain and Ireland have persisted throughout the multiple glaciations of the Quaternary. Our findings are congruent with those of Kornobis *et al.* (2010) who presented molecular evidence showing that the endemic subterranean amphipod *Crangonyx islandicus* has been present in Iceland for around 5 million years, surviving repeated glaciations. On the basis of the molecular analysis and the species distribution, Kornobis et al. (2010) suggest that *Crangonyx islandicus* may have survived in geothermally heated groundwaters associated with volcanic fissures. Our data demonstrate that some *Niphargus* populations have been resilient to climate changes that occur above ground in a region that is much less geothermally active. This suggests that groundwater ecosystems in general may have mechanisms that reduce the impacts of surface climate change, but our current understanding of these mechanisms is limited. During glaciations, groundwater taxa may have survived in caves or aquifers that were actively recharged by warm-based glaciers or pro-glacial rivers. Groundwater recharge from glaciers is well documented (Boulton *et al.* 1995; Hutchinson & Thomasbetts 1990), and provides a source of oxygen and nutrients. However, these groundwaters would have been cooler than today and therefore surviving species must be resilient to these long term variations in groundwater temperatures. Geothermal heating may have maintained some groundwaters at higher temperatures during glacial periods. For example it has been suggested that areas of southwest England remained permafrost free in the last glaciation due to a high heat-flux (Hutchinson & Thomasbetts 1990) and there are small geothermal heat anomalies (~2 to 6°C) within 100 m of the surface in southern and eastern England (Busby *et al.* 2011). However, there is little relation between modern day distributions of *Niphargus* in the British Isles and geothermally heated waters. For example, *Niphargus* are not recorded in Derbyshire in Northern England where there are extensive geothermal springs and suitable geological habitats for invertebrates, and are present in areas of southern England where there is no evidence of geothermal warming of groundwater. Given the poor dispersal capabilities of *Niphargus* it therefore seems unlikely that geothermal heating of groundwater was the only factor enabling their survival during glacial periods. A geothermal gradient of about 1°C per 20 to 40 m (Anderson 2005) results in warmer waters at depth, which may have provided some protection against cold groundwaters if *Niphargids* were able to migrate to warmer, deeper waters. However, permeability and fracturing generally decrease substantially with depth (Jiang *et al.* 2010; Williams A *et al.* 2006), resulting in limited groundwater circulation and low oxygen, and therefore the deep groundwater environment (> 100 m) may not always provide a suitable habitat for invertebrates. Overall it seems probable that surviving *Niphargus* species have some mechanism of adapting to changing groundwater temperatures. Modern day occurrence of groundwater crustacea in sub-glacial refugia has been documented in Castleguard Cave, Canada, ca. 500 km north of the glacial limit, where groundwater temperatures are around 2°C (Holsinger *et al.* 1983) and in lava caves beneath ice in Iceland (Kornobis *et al.* 2010). Nevertheless, other evidence indicates that the Pleistocene had a considerable negative impact on the distribution and survival of *Niphargus*. The British *Niphargus* species (Figure 4) and *N. virei* in France (Foulquier *et al.* 2008) are largely found to the south of the maximum extent of the Anglian and Devensian glaciers, and species diversity in northwestern Europe is relatively low, suggesting that some populations were eradicated during glacial or periglacial conditions.

Geographic origin of Niphargus and spread during the Cenozoic

The geographic distribution of MRCAs for nodes of different ages showed a second, unexpected pattern (Figure 4). The origin of *Niphargus* is in northwestern Europe with the MRCA of all *Niphargus* in what is now central France in the late Cretaceous (88Ma), when Europe consisted of a number of islands (Hay *et al.* 1999; Rogl 1999). The genus therefore predates (and must have survived) the Cretaceous-Palaeogene mass extinctions of 65 Ma possibly facilitated by a subterranean life-style. The schematic maps in Figures 4c-4f depict some of the major palaeogeographical changes that occurred between 100 and 25 Ma, although there were smaller scale fluctuations in sea level and uplift superimposed on these broad patterns (Jarvis *et al.* 2002; Voigt *et al.* 2006). The ancestor of *Niphargus* probably colonised a central island (Figure 4c) which was subsequently further inundated by the Tethys Sea (Figure 4d). From there the ancestral locations move with decreasing node age towards the southeast. During the Eocene the retreating Tethys Sea provided the opportunity for *Niphargus* to spread in emerging freshwater aquifers (Figure 4e). This is consistent with palaeogeographic models but is contrary to a previous hypothesis, which suggested that the enhanced species diversity in the northern parts of the Balkan Peninsula indicated an origin in southeast Europe (Karaman & Ruffo 1986). Our phylogenetically controlled analysis of diversification rates shows an increase in diversification in a south-easterly direction thereby providing an alternative explanation for the enhanced species diversity in the Balkans. The timing of this diversification (around 25 Ma) coincides with the closing of the Tethys Sea that had previously separated the Balkans and Central Europe (Hrbek & Meyer 2003; Rogl 1999) and provided an opportunity for further dispersion towards the south-east (Figure 4f). Available niche space in the geomorphologically complex Balkans may have enabled the high diversification rate; a mechanism which has also been suggested to explain diversification in other fauna (Hrbek & Meyer 2003).

Conclusions

This study reveals the presence of an ancient endemic groundwater fauna in the British Isles, where endemism is otherwise rare. The unusually high levels of endemism in groundwater fauna in northern latitudes identified by the study highlights the need to recognise this unique ecosystem and its ancient organisms' contribution to our understanding of climatic and palaeogeographic controls on global biodiversity. The extent to which *Niphargus* may be resilient to recent anthropogenic perturbations of groundwater ecosystems is unknown. However, the small ranges of these taxa shown in this study and others (Foulquier *et al.* 2008; Holsinger *et al.* 1983), and their smaller clutch sizes, delayed maturity, slower growth and lower population numbers compared to epigean relatives (Gibert et al. 1994), suggest that despite their ancient resilience, the European *Niphargus* fauna could now be vulnerable. Conservation policy measures to protect groundwater ecosystems in Europe lag far behind countries such as Australia. *N. glenniei* has been designated as a UK Biodiversity Action Plan (BAP) species but other *Niphargus* species have no such recognition and current European groundwater monitoring programmes do not consider groundwater ecosystems.

The study also reveals an unusual biogeographical pattern within the *Niphargus* genus. The oldest and most phylogenetically diverse species occur in northern Europe where endemism is low in surface fauna, which are dominated by large range species and post-glacial colonisers. In contrast the species diversity is highest in Southern Europe indicating that once the genus dispersed to these areas, climatic and geomorphological conditions enabled a much higher diversification rate than has occurred in Northern Europe.

These groundwater organisms provide an unusual opportunity to improve our understanding of biological processes such as speciation, adaptation and convergence, and as narrow range endemics they allow further exploration of island biogeographical processes. Furthermore, our discovery that these groundwater species are the oldest known inhabitants of Britain and Ireland, persisting through millions of years of changing climate may cast significant light on one of the major challenges facing the scientific community today; that of predicting the resilience of ecosystems to climate change (Chapin *et al.* 2000). Our findings show that groundwater fauna (or their habitats) are likely to have a highly variable response to the extinguishing effects of climate change. A more detailed knowledge of the mechanisms behind this variation could help us to understand the likely impacts of the current anthropogenically induced challenges to the biosphere.

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Author contributions B.H., G.S.P. A.L.R., and L.M., conceived the study; B.H., C.V., T.M. and A.L.R conducted analyses; C.E.M., B.H. and S.M., carried out laboratory work and assembled the data; C.E.M., S.M, L.R.F.D.K., L.M., A.L.R., J.A., J.S.G.D and K.E collected

the samples and contributed data; B.H., L.M., A.L.R., G.S.P. and C.E.M. wrote the paper; all authors commented on the final draft.

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Supplementary information is available in the online version of the paper.

Tables:

Table 1. A) Estimates of the time of the most recent common ancestor (TMRCA) between British and Irish *Niphargus* taxa and their closest relatives based on a BEAST analysis. The prior used and mean and median estimates in millions of years (Ma) are given, including the upper and lower bounds of the highest posterior density (HPD) intervals. B) Details of the external calibration points estimated from a subset of representative data from Hou *et al.* (2011) are given.

A)	**Node**	**prior**	**Mean (Ma)**	**Median (Ma)**	**95% HPD lower**	**95% HPD upper**
	N. aquilex E/F	Tree prior	6.69	6.22	2.15	12.32
	N. aquilex (A1,A2)/B	Tree prior	5.93	5.57	2.02	10.35
	N. aquilex A1/A2	Tree prior	1.06	0.95	0.23	2.12
	N. fontanus A1/A2	Tree prior	0.89	0.77	0.17	1.90
	N. irlandicus/*N. glenniei*	Tree prior	21.05	19.48	6.74	38.09
	N. kochianus A/(B,C)	Tree prior	3.06	2.89	1.26	5.21
	Niphargus root	Uniform [45-558]	88.16	87.14	65.38	113.94
B)	**External calibration points from [4]**					
	node 1	Normal [5.0; 1]	5.89	5.87	4.26	7.53
	node 2	Normal [30.0; 1]	29.75	29.76	27.84	31.70
	node 3	Normal [44.9; 8]	47.62	47.49	35.83	59.31
	node a	Normal [80.3; 15]	78.52	78.19	64.87	93.26
	node b	Normal [61.3; 9]	59.34	59.22	51.20	67.86
	node c	Normal [42.8; 6]	44.85	44.78	39.83	50.22
	node d	Normal [36; 6]	32.24	32.28	26.50	37.89
	node f	Normal [25.6; 5]	21.67	21.79	14.14	28.93
	node g	Normal [28.2; 5]	23.16	23.15	16.91	29.86
	node h	Normal [32.9; 5]	33.17	33.30	25.29	40.41
	node i	Normal [36.6; 5]	32.61	32.71	26.48	38.71

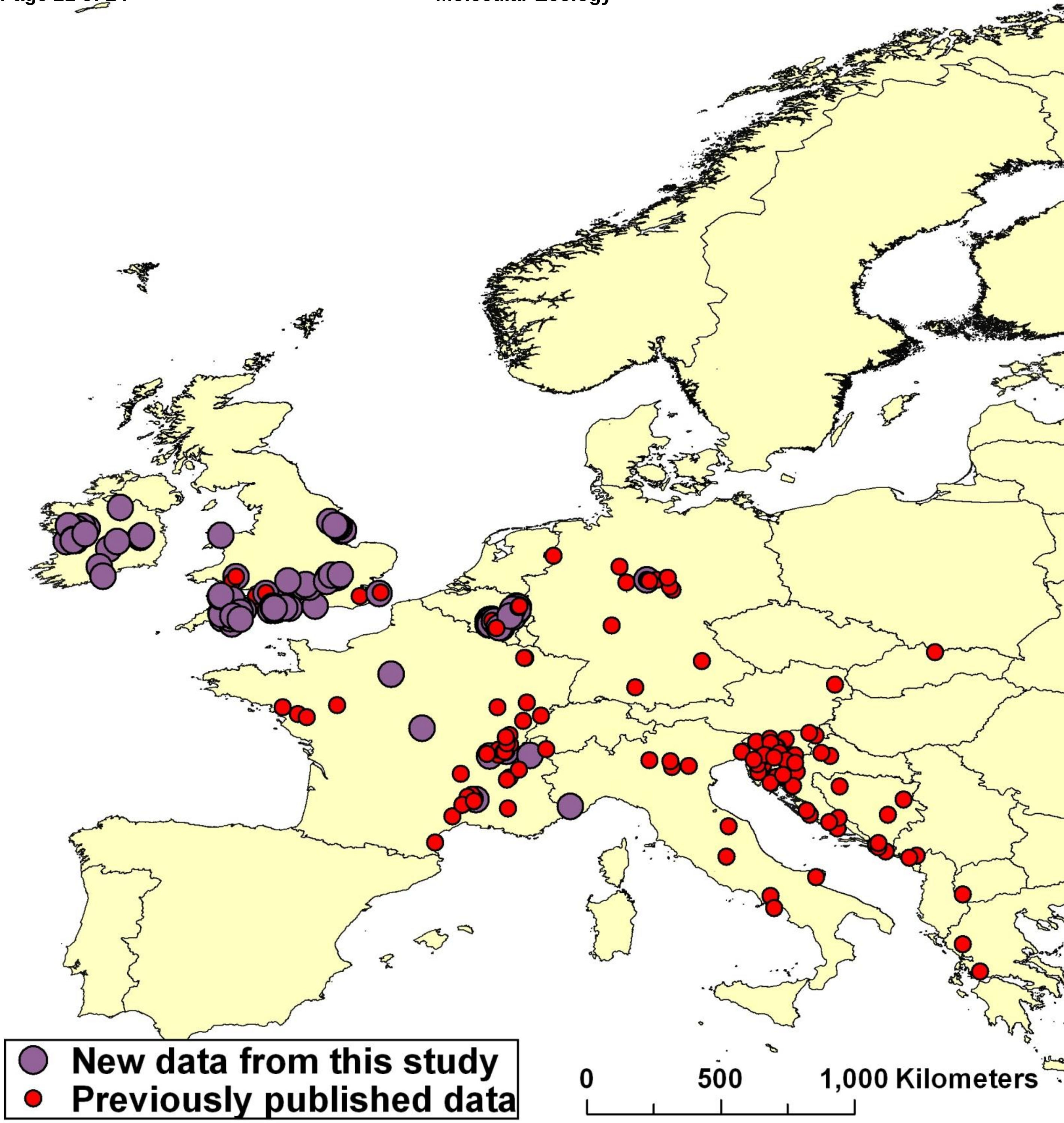
Figure captions:

Figure 1: Distribution of sampling locations from this study and published data included in the analysis

Figure 2: Image of the ancient British endemic *Niphargus glenniei*, photo credit Chris Proctor (a); multi-gene phylogeny of *Niphargus* based on a Bayesian analysis (outgroup not shown), posterior probabilities (PP) > 0.5 of nodes above the clade level are show above branches. See Fig. 3 for PP of nodes within important clades. British and Irish taxa are marked with a red circle and branches leading to them are highlighted red; number in brackets refer to clade numbers in Fig. 3(b) and geographic distribution of major phylogenetic lineages; the exact location of the *N. liasi* sample is not known, but the species occurs in France (c).

Figure 3: Geographic distribution of British and Irish OTU's and European sister taxa. The green and pink lines represent the maximum extent of the glacial ice sheets during the Devensian and Anglian glacial periods respectively. Small black dots are sites of known distribution for each group; coloured dots represent sampled populations for each OTU. Partial ultrametric phylogenies from the BEAST analysis for each species complex are shown above maps. *N irlandicus/ N glenniei group* (a); *N. aquilex/ N. schellenbergi group* (b); *N. kochianus* (c), *N fontanus* (d).

Figure 4: Time calibrated phylogeny of *Niphargus* generated with BEAST (outgroup not shown), black dots indicate nodes with a posterior probability (PP) > 0.5; British and Irish OTUs are marked in red; clade numbers refer to clade numbers in Fig. 2(a); geographic location of the common ancestor for each node with a PP > 0.5 based on Bayesian model based ancestral state reconstruction; Cricle sizes are proportional to the age of nodes (b); schematic maps depicting some of the major palaeogeographical changes that occurred in Europe between 100 and 25 Ma; modified from Ron Blakey, NAU Geology (http://jan.ucc.nau.edu/rcb7/): 100 Ma, circle indicates putative location of *Niphargus* ancestor (c), 75Ma isolation of *Niphargus* on a central European island and within the Tethys Sea; the question mark indicates the possibility that the *N. glenniei/ N. irlandicus* lineage became first isolated during this time on a north-western European island (d) 50Ma spread of *Niphargus* across Central Europe (e) 25Ma spread of *Niphargus* to the Balkan and Italian Penisulas, circle indicates the location of the common ancestor of *N. irlandicus* and *N. glenniei* (f).


● New data from this study
● Previously published data
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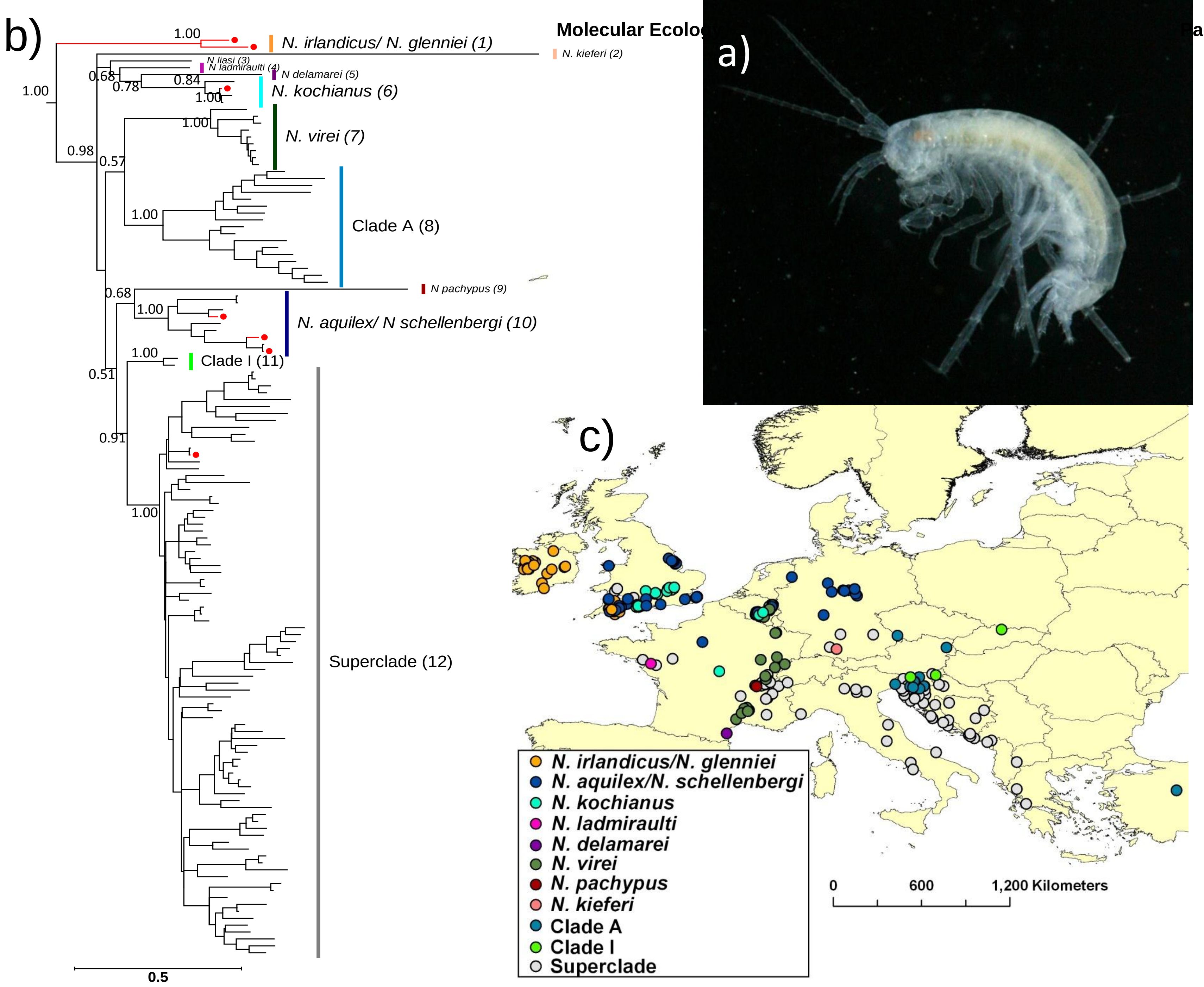
b)
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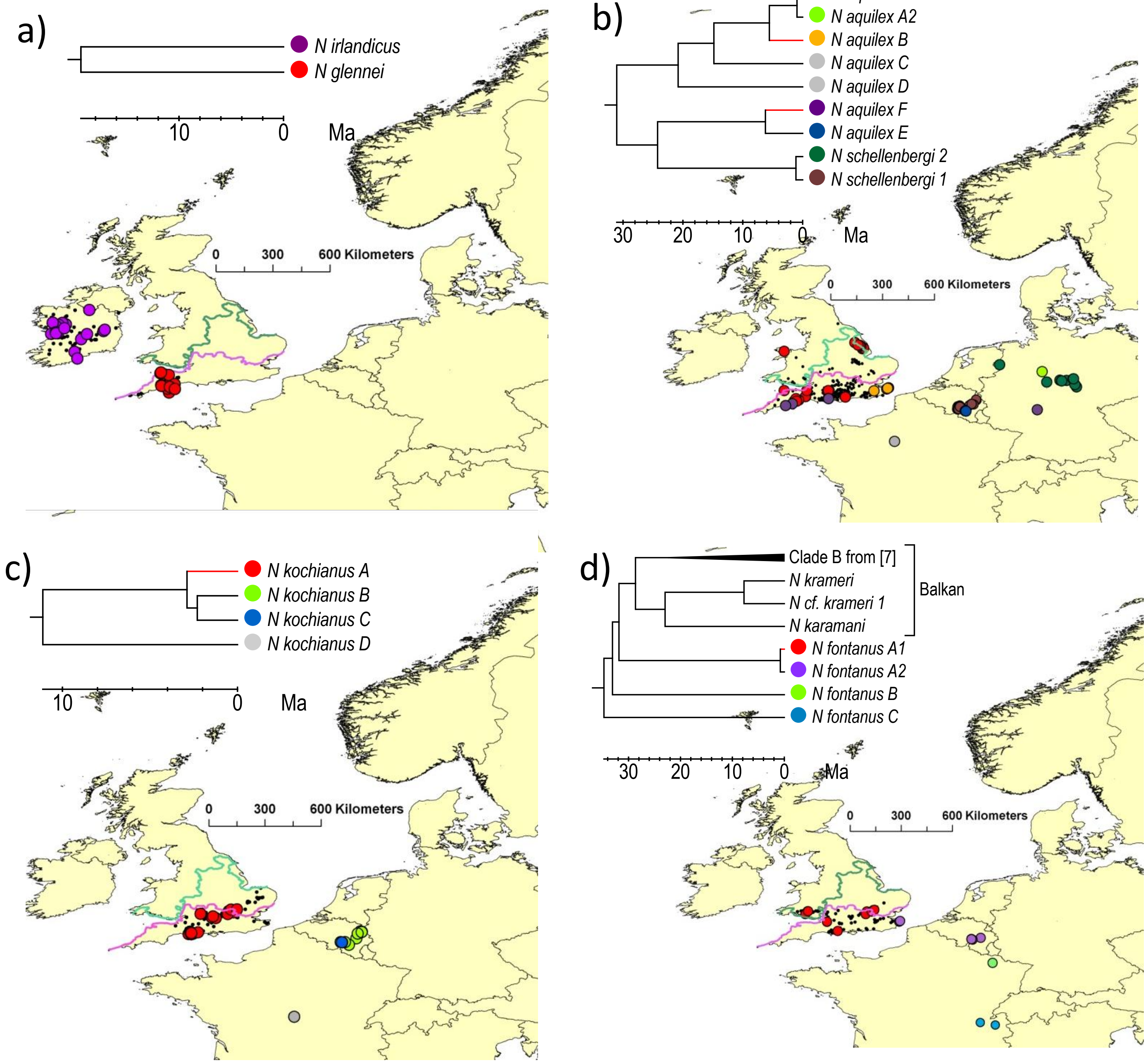
N. irlandicus/ N. glenniei (1)
N. kieferi (2)
N liasi (3)
N ladmiraulti (4)
N delamarei (5)
0.68
0.84
0.78
N. kochianus (6)
1.00
1.00
1.00
N. virei (7)
0.98
0.57
1.00
Clade A (8)
N pachypus (9)
0.68
1.00
N. aquilex/ N schellenbergi (10)
1.00
Clade I (11)
0.51
0.91
1.00
Superclade (12)
0.5
a)
c)
N. irlandicus/N. glenniei
N. aquilex/N. schellenbergi
N. kochianus
N. ladmiraulti
N. delamarei
N. virei
N. pachypus
N. kieferi
Clade A
Clade I
Superclade
0
600
1,200 Kilometers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N irlandicus
N glennei
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Ma
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b)
N aquilex A1
N aquilex A2
N aquilex B
N aquilex C
N aquilex D
N aquilex F
N aquilex E
N schellenbergi 2
N schellenbergi 1
30
20
10
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0 300 600 Kilometers
c)
N kochianus A
N kochianus B
N kochianus C
N kochianus D
10
0
Ma
0 300 600 Kilometers
d)
Clade B from [7]
N krameri
N cf. krameri 1
N karamani
Balkan
N fontanus A1
N fontanus A2
N fontanus B
N fontanus C
30
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Table 1. A) Estimates of the time of the most recent common ancestor (TMRCA) between British and Irish *Niphargus* taxa and their closest relatives based on a BEAST analysis. The prior used and mean and median estimates in millions of years (Ma) are given, including the upper and lower bounds of the highest posterior density (HPD) intervals. B) Details of the external calibration points estimated from a subset of representative data from Hou *et al.* (2011) are given.

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	node b	Normal [61.3; 9]	59.34	59.22	51.20	67.86
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