

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

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32 **subterranean**

33 **Abstract**

34 Global climate changes during the Cenozoic (65.5 - 0 Ma) caused major biological range  
35 shifts and extinctions. In Northern Europe, for example, a pattern of few endemics and the  
36 dominance of wide-ranging species is thought to have been determined by the Pleistocene  
37 (2.59 – 0.01 Ma) glaciations. This study, in contrast, reveals an ancient subsurface fauna  
38 endemic to Britain and Ireland. Using a Bayesian phylogenetic approach we found that two  
39 species of stygobitic invertebrates (genus *Niphargus*) have not only survived the entire  
40 Pleistocene in refugia but have persisted for at least 19.5 million years. Other *Niphargus*  
41 species form distinct cryptic taxa that diverged from their nearest continental relative between  
42 5.6 and 1.0 Ma. The study also reveals an unusual biogeographical pattern in the *Niphargus*  
43 genus. It originated in Northwest Europe ~88 Ma and underwent a gradual range expansion.  
44 Phylogenetic diversity and species age are highest in Northwest Europe suggesting resilience  
45 to extreme climate change, and strongly contrasting the patterns seen in surface fauna.  
46 However, species diversity is highest in Southeast Europe indicating that once the genus  
47 spread to these areas (~ 25 Ma), geomorphological and climatic conditions enabled much  
48 higher diversification. Our study highlights that groundwater ecosystems provide an  
49 important contribution to biodiversity and offer insight into the interactions between  
50 biological and climatic processes.

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52

53 **INTRODUCTION**

54 Global climate has changed significantly throughout the Cenozoic (65.5- 0 Ma) with glacial  
55 cycles during the Miocene, Pliocene and Pleistocene (Louwye *et al.* 2008; Zachos *et al.*  
56 2001a; Zachos *et al.* 2008). Precipitation also fluctuated from extended arid (13.2 - 11.5 Ma)  
57 to very wet conditions (10.2-9.8 Ma; Bohme *et al.* 2008). Fauna, for example ectothermic  
58 vertebrates and freshwater Crustacea, experienced major range shifts or extinctions, and  
59 ecosystems were dramatically modified (Bohme 2003, Klaus and Grosse 2010). During the  
60 Pleistocene glaciations (2.59 - 0.01 Ma), large areas of the northern hemisphere were covered  
61 by glaciers or permafrost and were uninhabitable (reviewed in Provan & Bennett 2008), with  
62 particularly marked biogeographic impact in northern Europe.

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

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

## 103 MATERIAL AND METHODS

### 104 *Sampling*

105 A modified Cvetkov net sampler, notenboom sampler, or baited traps were used to collect  
106 samples from boreholes, springs and wells. 454 *Niphargus* specimens were preserved in  
107 >70% ethanol (Figure 1, Table S1), comprising samples from 63 populations (222  
108 individuals) in Britain and Ireland including five of the six species present. We were unable  
109 to obtain sufficient samples for DNA extraction of the rare *N. wexfordensis*. Additionally,  
110 224 individuals from 47 populations and 5 species were collected from Belgium, the  
111 Netherlands, Germany and France (Figure 1, Table S1) including all species known to co-  
112 occur in Britain and continental Europe (*N. aquilex*, *N. fontanus*, *N. kochianus*). Furthermore,  
113 samples were obtained from two species which occur in the vicinity of Britain located in  
114 France, but for which no DNA sequence data existed (*N. pachypus*, 1 population, 2  
115 individuals; *N. forelli*, 2 populations, 4 individuals). Samples from published data sets (see  
116 below) covered largely the central and south-eastern part of the distribution and included data  
117 of 185 populations from 74 described species (Figure 1, Table S1).

### 118 **De novo sequencing and data sets for phylogenetic analysis**

119 Genetic variation of *Niphargus* was assessed at two mitochondrial genes, cytochrome oxidase  
120 subunit I (COI) and 16s rRNA (16S) and the nuclear small subunit 28s rRNA (28S; for  
121 details see Supplementary materials). Our analysis combined these new DNA sequence data  
122 with all published *Niphargus* sequence data for 28S, COI and 16S available on GenBank on  
123 June 1st, 2012 (Fišer *et al.* 2008; Flot 2010; Flot *et al.* 2010; Hänfling *et al.* 2008; Hartke *et*  
124 *al.* 2011; Lefébure *et al.* 2006; Lefébure *et al.* 2007; Trontelj *et al.* 2009). Also included were  
125 published sequence data of the mitochondrial 12s rRNA region (12S) and the large subunit  
126 18s rRNA (18S) for the taxa covered in the combined data set. In total, data were included  
127 from 78 described species, and several putative cryptic species, from 170 locations across the  
128 genus' European range (Figure 1, Tables S1, S3). This included eight of the nine species (the  
129 ninth, *N. boulangei*, was too rare) that occur within 200 km of Britain (Table S1). The  
130 combined data set provided phylogeographic information (more than 10 populations) for 8 of  
131 the 78 described species (Table S2, *N. aquilex*, *N. fontanus*, *N. glenniei*, *N. kochianus*, *N.*  
132 *irlandicus*, *N. rhenorhodanensis*, *N. virei*, *N. schellenbergi*). A further 11 species were  
133 covered by more than 1 specimen from 1 - 3 locations.

134 A total of 36 taxa from 9 amphipod families were used as outgroups to root the *Niphargus*  
135 phylogeny, and provide calibration points for a molecular dating analysis (see Table S4). The  
136 outgroup taxa include previously identified sister groups to Niphargidae (Englisch *et al.*  
137 2003, Fiser *et al.* 2008) and representatives of clades from a dated phylogeny of gammarid  
138 amphipods (Hou *et al.* 2011). We used the genes 28S, COI, 18S and elongation factor 1 alpha  
139 (EF-1a). The alignment of COI and EF-1a sequences was carried out using MUSCLE (Edgar  
140 2004) in combination with MEGA version 5.05 (Tamura *et al.* 2011). Ribosomal genes were  
141 aligned with the software MAFFT version 6 (Kato *et al.* 2002) using the alignment  
142 strategies Q-INS-i or E-INS-i.

### 143 **Delineation of OTUs, multi-locus alignments and phylogenies**

144 Cryptic diversity and taxonomic misclassification are common in *Niphargus*. We therefore  
145 used a DNA barcoding approach based on the two genes with the largest coverage (COI and  
146 28S) to identify cryptic lineages within species and to delineate operational taxonomic units  
147 (OTUs) with independent evolutionary histories (for details see Supplementary materials).  
148 Many of these OTU's are likely to fulfil the criteria for separate species depending on the  
149 definition applied, but a discussion of species status is outside the scope of this paper. A

150 multi-locus alignment was created using representatives of OTU's of *Niphargus* and selected  
151 outgroups. One representative of each OTU was chosen at random for inclusion in the  
152 supermatrix (Table S1). Amphipod outgroups included three representatives selected for each  
153 of the 4 *Gammarus* freshwater clades, 6 representatives of the marine *Gammarus* group, 3  
154 representatives of the Baikalian Gammarids and all outgroups used in Hou *et al.* (2011),  
155 providing 13 time-calibrated nodes. For each gene, all sequences of the selected taxa were  
156 aligned. Phylogenetic analysis of the multi-gene matrix was carried out using Bayesian  
157 analysis as implemented in MrBayes v3.2 (Ronquist *et al.* 2012). Genes were used as  
158 partitions and model parameters between partitions were unlinked. Two independent Markov  
159 chain Monte-Carlo (MCMC) chains were run for 10,000,000 iterations each, sampling every  
160 1,000 iterations. The first 25 % of each run was discarded as burnin with the remaining  
161 samples pooled and used to create a maximum clade credibility tree.

#### 162 ***Molecular dating using a Bayesian analysis***

163 BEAST (Bayesian Evolutionary Analysis Sampling Trees) version 1.7.4 (Drummond *et al.*  
164 2012) was used to generate an ultrametric phylogeny and estimate the time of the most recent  
165 common ancestor (TMRCA) for each node using a Bayesian MCMC analysis. Tree topology  
166 was constrained to that obtained from the MrBayes phylogenetic analysis. Genes were used  
167 as partitions and substitution rates and clocks were unlinked in the analysis. An uncorrelated  
168 lognormal relaxed clock (Drummond *et al.* 2006) and a Yule speciation prior were used. A  
169 time calibrated phylogeny of the amphipod group Gammaridae (Hou *et al.* 2011) was used to  
170 provide 11 external calibration points (for details see Supplementary materials).

#### 171 ***Ancestral longitude and latitude reconstructions***

172 We used the Bayesian MCMC phylogenetic ancestral state reconstruction method introduced  
173 by Organ *et al.* (2007) to infer the geographical location of the MRCA for each node. The  
174 method was chosen because of its superior performance with phylogenetic trees that span  
175 millions of years (Montgomery *et al.* 2010). Similar methods have been used to infer  
176 ancestral longitudes and latitudes in a phylogenetic context (Bouckaert *et al.* 2012; Lemey *et al.*  
177 2009). With exact geographical ranges mostly unknown it was not possible to calculate  
178 range centroids. The range size of most *Niphargus* is small, however, usually <100km in  
179 diameter. (Trontelj *et al.* 2009). The few taxa with a larger range such as *N. virei* and *N.*  
180 *rhenorhodanensis* consist of a number of cryptic taxa or distinct phylogeographic units with a

181 much smaller range (Lefébure *et al.* 2006; Lefébure *et al.* 2007). This cryptic diversity is  
182 reflected in the OTUs used for the phylogenetic analysis. We therefore used the geographical  
183 coordinate of the individual chosen at random for the phylogenetic analysis as a proxy for the  
184 taxon's geographic location. We estimated a phylogenetic model of evolution for the  
185 *Niphargus* ingroup species where longitude and latitude were correlated using the computer  
186 program BayesTraits (Pagel *et al.* 2004). We ran the MCMC chain for one million iterations  
187 after apparent convergence sampling every 1,000 iterations from the chain and repeated the  
188 analysis multiple times. We also simultaneously estimated the phylogenetic signal parameter  
189  $\lambda$  (Pagel 1999). The parameter  $\lambda$  varies between 0 and 1, where 1 is interpreted as having the  
190 traits covary and zero means that the traits evolve independently of the phylogenetic  
191 relationships among species. Repeated analyses produced almost identical results, thus we  
192 provide results from a single chain only.

### 193 ***Geographic variation in species diversity and diversification rates***

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

213

214 **RESULTS**215 *Data overview*

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

221 *Phylogenetic analysis*

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

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



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

256 Estimates of divergence times for all nodes separating British and Irish taxa from their  
257 nearest relatives are shown in Table 1. The ultrametric tree generated from the analysis is  
258 shown in Figure 4a. The Bayesian dating analysis and ancestral state reconstruction of the  
259 geographic origin of the MRCA for each node revealed that the MRCA of the two endemic  
260 British taxa was estimated to have lived in south-west England around 19.5 million years ago  
261 (95% HDP, 38.1 - 6.7Ma, Figure 4b, 4f, Table 1). Thus, *Niphargus* must have persisted in  
262 Britain and Ireland at least since the Miocene making it the oldest known fauna by at least  
263 two orders of magnitude. This common ancestor must have existed at a time when the British  
264 and Irish landmasses were joined. The two sister taxa may subsequently have become  
265 isolated during an Oligocene marine inundation of the Irish Sea Basin (Cope 1997).

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

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

### 279 ***Geographic variation in species diversity and diversification rates***

280 Investigation of the geographic variation in species diversity revealed that the number of  
281 *Niphargus* species varies greatly across different geographic areas from 1 species in Spain to  
282 136 in the Balkans (Figure S5). In contrast to phylogenetic diversity the species richness of  
283 the Western region is significantly lower than that of the Eastern region ( $P < 0.05$ ).  
284 Investigation of the geographic variation in diversification rates shows that the number of  
285 nodes along each root-to-tip path in the *Niphargus* species level phylogeny correlates  
286 significantly with distance towards the southeast (correlation coefficient [SD] = 0.18 [0.014],  
287 log Bayes Factor = 9.8). A log Bayes Factor value of between 6 and 10 provides strong  
288 support for the hypothesis tested. Net-diversification rate in *Niphargus* therefore increases in  
289 a south-easterly direction.

290

## 291 **DISCUSSION**

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

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

303 Furthermore, this ancient groundwater fauna has survived the extreme geological and climate  
304 changes that have occurred over the past 20 million years. Groundwater temperatures are

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

320 All the *Niphargus* lineages in Britain and Ireland have persisted throughout the multiple  
321 glaciations of the Quaternary. Our findings are congruent with those of Kornobis *et al.* (2010)  
322 who presented molecular evidence showing that the endemic subterranean amphipod  
323 *Crangonyx islandicus* has been present in Iceland for around 5 million years, surviving  
324 repeated glaciations. On the basis of the molecular analysis and the species distribution,  
325 Kornobis *et al.* (2010) suggest that *Crangonyx islandicus* may have survived in geothermally  
326 heated groundwaters associated with volcanic fissures. Our data demonstrate that some  
327 *Niphargus* populations have been resilient to climate changes that occur above ground in a  
328 region that is much less geothermally active. This suggests that groundwater ecosystems in  
329 general may have mechanisms that reduce the impacts of surface climate change, but our  
330 current understanding of these mechanisms is limited. During glaciations, groundwater taxa  
331 may have survived in caves or aquifers that were actively recharged by warm-based glaciers  
332 or pro-glacial rivers. Groundwater recharge from glaciers is well documented (Boulton *et al.*  
333 1995; Hutchinson & Thomasbetts 1990), and provides a source of oxygen and nutrients.  
334 However, these groundwaters would have been cooler than today and therefore surviving  
335 species must be resilient to these long term variations in groundwater temperatures.  
336 Geothermal heating may have maintained some groundwaters at higher temperatures during  
337 glacial periods. For example it has been suggested that areas of southwest England remained

338 permafrost free in the last glaciation due to a high heat-flux (Hutchinson & Thomasbetts  
339 1990) and there are small geothermal heat anomalies (~2 to 6°C) within 100 m of the surface  
340 in southern and eastern England (Busby *et al.* 2011). However, there is little relation between  
341 modern day distributions of *Niphargus* in the British Isles and geothermally heated waters.  
342 For example, *Niphargus* are not recorded in Derbyshire in Northern England where there are  
343 extensive geothermal springs and suitable geological habitats for invertebrates, and are  
344 present in areas of southern England where there is no evidence of geothermal warming of  
345 groundwater. Given the poor dispersal capabilities of *Niphargus* it therefore seems unlikely  
346 that geothermal heating of groundwater was the only factor enabling their survival during  
347 glacial periods. A geothermal gradient of about 1°C per 20 to 40 m (Anderson 2005) results  
348 in warmer waters at depth, which may have provided some protection against cold  
349 groundwaters if *Niphargids* were able to migrate to warmer, deeper waters. However,  
350 permeability and fracturing generally decrease substantially with depth (Jiang *et al.* 2010;  
351 Williams A *et al.* 2006), resulting in limited groundwater circulation and low oxygen, and  
352 therefore the deep groundwater environment (> 100 m) may not always provide a suitable  
353 habitat for invertebrates. Overall it seems probable that surviving *Niphargus* species have  
354 some mechanism of adapting to changing groundwater temperatures. Modern day occurrence  
355 of groundwater crustacea in sub-glacial refugia has been documented in Castleguard Cave,  
356 Canada, ca. 500 km north of the glacial limit, where groundwater temperatures are around  
357 2°C (Holsinger *et al.* 1983) and in lava caves beneath ice in Iceland (Kornobis *et al.* 2010).  
358 Nevertheless, other evidence indicates that the Pleistocene had a considerable negative  
359 impact on the distribution and survival of *Niphargus*. The British *Niphargus* species (Figure  
360 4) and *N. virei* in France (Foulquier *et al.* 2008) are largely found to the south of the  
361 maximum extent of the Anglian and Devensian glaciers, and species diversity in  
362 northwestern Europe is relatively low, suggesting that some populations were eradicated  
363 during glacial or periglacial conditions.

#### 364 ***Geographic origin of Niphargus and spread during the Cenozoic***

365 The geographic distribution of MRCAs for nodes of different ages showed a second,  
366 unexpected pattern (Figure 4). The origin of *Niphargus* is in northwestern Europe with the  
367 MRCA of all *Niphargus* in what is now central France in the late Cretaceous (88Ma), when  
368 Europe consisted of a number of islands (Hay *et al.* 1999; Rogl 1999). The genus therefore  
369 predates (and must have survived) the Cretaceous-Palaeogene mass extinctions of 65 Ma  
370 possibly facilitated by a subterranean life-style. The schematic maps in Figures 4c-4f depict

371 some of the major palaeogeographical changes that occurred between 100 and 25 Ma,  
372 although there were smaller scale fluctuations in sea level and uplift superimposed on these  
373 broad patterns (Jarvis *et al.* 2002; Voigt *et al.* 2006). The ancestor of *Niphargus* probably  
374 colonised a central island (Figure 4c) which was subsequently further inundated by the  
375 Tethys Sea (Figure 4d). From there the ancestral locations move with decreasing node age  
376 towards the southeast. During the Eocene the retreating Tethys Sea provided the opportunity  
377 for *Niphargus* to spread in emerging freshwater aquifers (Figure 4e). This is consistent with  
378 palaeogeographic models but is contrary to a previous hypothesis, which suggested that the  
379 enhanced species diversity in the northern parts of the Balkan Peninsula indicated an origin in  
380 southeast Europe (Karaman & Ruffo 1986). Our phylogenetically controlled analysis of  
381 diversification rates shows an increase in diversification in a south-easterly direction thereby  
382 providing an alternative explanation for the enhanced species diversity in the Balkans. The  
383 timing of this diversification (around 25 Ma) coincides with the closing of the Tethys Sea that  
384 had previously separated the Balkans and Central Europe (Hrbek & Meyer 2003; Rogl 1999)  
385 and provided an opportunity for further dispersion towards the south-east (Figure 4f).  
386 Available niche space in the geomorphologically complex Balkans may have enabled the  
387 high diversification rate; a mechanism which has also been suggested to explain  
388 diversification in other fauna (Hrbek & Meyer 2003).

### 389 **Conclusions**

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

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

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

421

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432

433 **Author contributions** B.H., G.S.P. A.L.R., and L.M., conceived the study; B.H., C.V., T.M.  
434 and A.L.R conducted analyses; C.E.M., B.H. and S.M., carried out laboratory work and  
435 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

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

438

439

#### 440 **References**

- 441 Anderson MP (2005) Heat as a Ground Water Tracer. *Groundwater* **43**, 951-968.
- 442 Bohme M (2003) The Miocene Climatic Optimum: evidence from ectothermic vertebrates of Central  
443 Europe. *Palaeogeography Palaeoclimatology Palaeoecology* **195**, 389-401.
- 444 Bohme M, Ilg A, Winklhofer M (2008) Late Miocene "washhouse" climate in Europe. *Earth and  
445 Planetary Science Letters* **275**, 393-401.
- 446 Bouckaert R, Lemey P, Dunn M, *et al.* (2012) Mapping the Origins and Expansion of the Indo-  
447 European Language Family. *Science* **337**, 957-960.
- 448 Boulton GS, Caban PE, Vangijssel K (1995) Groundwater-flow beneath ice sheets. 1. Large-scale  
449 patterns. *Quaternary Science Reviews* **14**, 545-562.
- 450 Busby J, Kingdon A, Williams J (2011) The measured shallow temperature field in Britain. *Quarterly  
451 Journal of Engineering Geology and Hydrogeology* **44**, 373-387.
- 452 Chapin FS, Zavaleta ES, Eviner VT, *et al.* (2000) Consequences of changing biodiversity. *Nature* **405**,  
453 234-242.
- 454 Cope JCW (1997) The Mesozoic and Tertiary history of the Irish Sea. In: *Petroleum Geology of the  
455 Irish Sea and Adjacent Areas* (eds. Meadows NS, Trueblood SP, Hardman M, Cowan G), pp.  
456 47-59.
- 457 Dole-Olivier M, Castellarini JF, Coineau N, Galassi DMP, Martin P, Mori N, Valdecasas A, Gibert J  
458 (2009) Towards an optimal sampling strategy to assess groundwater biodiversity:  
459 comparison across six European regions. *Freshwater Biology* **54**, 777-796
- 460 Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with  
461 confidence. *Plos Biology* **4**, 699-710.
- 462 Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian Phylogenetics with BEAUti and the  
463 BEAST 1.7. *Molecular Biology and Evolution* **29**, 1969-1973.
- 464 Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing  
465 chemosynthesis. *Nature Reviews Microbiology* **6**, 725-740.
- 466 Dwyer GS, Chandler MA (2009) Mid-Pliocene sea level and continental ice volume based on coupled  
467 benthic Mg/Ca palaeotemperatures and oxygen isotopes. *Philosophical Transactions of the  
468 Royal Society a-Mathematical Physical and Engineering Sciences* **367**, 157-168.
- 469 Eberhard SM, Halse SA, Williams MR, *et al.* (2009) Exploring the relationship between sampling  
470 efficiency and short-range endemism for groundwater fauna in the Pilbara region, Western  
471 Australia. *Freshwater Biology* **54**, 885-901.
- 472 Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput.  
473 *Nucleic Acids Research* **32**, 1792-1797.
- 474 Englisch U, Coleman CO, Wagele JW (2003) First observations on the phylogeny of the families  
475 Gammaridae, Crangonyctidae, Melitidae, Niphargidae, Megaluropidae and Oedicerotidae  
476 (Amphipoda, Crustacea), using small subunit rDNA gene sequences. *Journal of Natural  
477 History* **37**, 2461-2486.
- 478 Figuera S, Livingstone DM, Hoehn E, Kipfer R (2011) Regime shift in groundwater temperature  
479 triggered by Arctic Oscillation. *Geophysical Research Letters* **38**, L23401.
- 480 Fišer C, Sket B, Trontelj P (2008) A phylogenetic perspective on 160 years of troubled taxonomy of  
481 Niphargus (Crustacea: Amphipoda). *Zoologica Scripta* **37**, 665-680.

- 482 Flot J (2010) Toward a molecular taxonomy of the amphipod genus *Niphargus*: examples of use of  
483 DNA sequences for species identification. *Bull Soc Sci Nat Ouest Fr* **32**, 62-68.
- 484 Flot JF, Worheide G, Dattagupta S (2010) Unsuspected diversity of *Niphargus* amphipods in the  
485 chemoautotrophic cave ecosystem of Frasassi, central Italy. *Bmc Evolutionary Biology* **10**.
- 486 Foulquier A, Malard F, Lefebure T, Gibert J, Douady CJ (2008) The imprint of Quaternary glaciers on  
487 the present-day distribution of the obligate groundwater amphipod *Niphargus virei*  
488 (*Niphargidae*). *Journal of Biogeography* **35**, 552-564.
- 489 Freckleton RP, Phillimore AB, Pagel M (2008) Relating traits to diversification: A simple test.  
490 *American Naturalist* **172**, 102-115.
- 491 Galassi DMP, Stoch F, Fiasca B, Di Lorenzo T, Gattone E (2009) Groundwater biodiversity patterns in  
492 the Lessinian Massif of northern Italy. *Freshwater Biology* **54**, 830-847.
- 493 Gibert J, Stanford JA, Dole-Olivier M-J, Ward JV (1994) Basic attributes of groundwater ecosystems  
494 and prospects for research. In: Gibert J, Danielopol DL & Stanford JA (eds) *Groundwater*  
495 *Ecology*. Academic Press, San Diego, CA, 8-42.
- 496 Gupta S, Collier JS, Palmer-Felgate A, Potter G (2007) Catastrophic flooding origin of shelf valley  
497 systems in the English Channel. *Nature* **448**, 342-U345.
- 498 Hänfling B, Douerelo-Soler I, Knight L, Proudlove G (2008) Molecular studies on the *Niphargus*  
499 *kochianus* group (Crustacea: Amphipoda: Niphargidae) in Great Britain and Ireland. *Cave and*  
500 *Karst Science* **35**, 35-40.
- 501 Hartke TR, Fišer C, Hohagen J, et al. (2011) Morphological and molecular analyses of closely related  
502 species in the stygobiontic genus *Niphargus* (Amphipoda). *Journal of Crustacean Biology* **31**,  
503 701-709.
- 504 Hay WH, DeConto RM, et al. (1999) Alternative global Cretaceous paleogeography In: *Evolution of*  
505 *the Cretaceous Ocean-Climate System* Pages (eds. Barrera E, Johnson CC), pp. 1-47.  
506 Geological Society of America, Boulder, Colorado.
- 507 Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical*  
508 *Transactions of the Royal Society of London Series B-Biological Sciences* **359**, 183-195.
- 509 Hof C, Brandle M, Brandl R (2008) Latitudinal variation of diversity in European freshwater animals is  
510 not concordant across habitat types. *Global Ecology and Biogeography* **17**, 539-546.
- 511 Holsinger JR, Mort JS, Recklies AD (1983) The subterranean crustacean fauna of Castleguard Cave,  
512 Columbia icefields, Alberta, Canada, and its zoogeographic significance. *Arctic and Alpine*  
513 *Research* **15**, 543-549.
- 514 Hou ZE, Sket B, Fišer C, Li SQ (2011) Eocene habitat shift from saline to freshwater promoted  
515 Tethyan amphipod diversification. *Proceedings of the National Academy of Sciences of the*  
516 *United States of America* **108**, 14533-14538.
- 517 Hrbek T, Meyer A (2003) Closing of the Tethys Sea and the phylogeny of Eurasian killifishes  
518 (Cyprinodontiformes: Cyprinodontidae). *Journal of Evolutionary Biology* **16**, 17-36.
- 519 Hutchinson JN, Thomasbetts A (1990) Extent of permafrost in southern Britain in relation to  
520 geothermal flux. *Quarterly Journal of Engineering Geology* **23**, 387-390.
- 521 Illies J (1978) *Limnofauna Europaea, 2nd edn*. Gustav Fischer Verlag, Stuttgart.
- 522 Jarvis I, Mabrouk A, Moody RTJ, de Cabrera S (2002) Late Cretaceous (Campanian) carbon isotope  
523 events, sea-level change and correlation of the Tethyan and Boreal realms. *Palaeogeography*  
524 *Palaeoclimatology Palaeoecology* **188**, 215-248.
- 525 Jiang X-W, Wang X-S, Wan L (2010) Semi-empirical equations for the systematic decrease in  
526 permeability with depth in porous and fractured media. *Hydrogeology Journal* **18**, 839-  
527 850.
- 528 Karaman GS, Ruffo S (1986) Amphipoda: *Niphargus* group. In: *Stygofauna mundi* (ed. Botosaneanu  
529 L), pp. 514-534. E.J. Brill, Leiden.
- 530 Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence  
531 alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059-3066.



- 532 Klaus S, Gross M (2010) Synopsis of the fossil freshwater crabs of Europe (Brachyura: Potamoidea:  
533 Potamidae). *Neues Jahrbuch Fur Geologie Und Palaontologie-Abhandlungen* **256**, 39-59.
- 534 Kornobis E, Palsson S, Kristjansson BK, Svavarsson J (2010) Molecular evidence of the survival of  
535 subterranean amphipods (Arthropoda) during Ice Age underneath glaciers in Iceland.  
536 *Molecular Ecology* **19**, 2516-2530.
- 537 Kottelat M, Freyhof J (2007) *Handbook of European freshwater fishes*. Publications Kottelat.
- 538 Lefébure T, Douady CJ, Gouy M, *et al.* (2006) Phylogeography of a subterranean amphipod reveals  
539 cryptic diversity and dynamic evolution in extreme environments. *Molecular Ecology* **15**,  
540 1797-1806.
- 541 Lefébure T, Douady CJ, Malard F, Gibert J (2007) Testing dispersal and cryptic diversity in a widely  
542 distributed groundwater amphipod (*Niphargus rhenorhodanensis*). *Molecular Phylogenetics  
543 and Evolution* **42**, 676-686.
- 544 Lemey P, Rambaut A, Drummond AJ, Suchard MA (2009) Bayesian Phylogeography Finds Its Roots.  
545 *Plos Computational Biology* **5**.
- 546 Lopez-Garcia P, Philippe H, Gail F, Moreira D (2003) Autochthonous eukaryotic diversity in  
547 hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge.  
548 *Proceedings of the National Academy of Sciences of the United States of America* **100**, 697-  
549 702.
- 550 Louwye S, Foubert A, Mertens K, Van Rooij D, Party IES (2008) Integrated stratigraphy and  
551 palaeoecology of the lower and Middle Miocene of the Porcupine Basin. *Geological  
552 Magazine* **145**, 321-344.
- 553 MacDonald AM, Bonsor HC, Ó Dochartaigh BE, Taylor RG (2012) Quantitative maps of groundwater  
554 resources in Africa. *Environmental Research Letters* **7**, 024009.
- 555 Martin P, De Broyer C, Fiers F, *et al.* (2009) Biodiversity of Belgian groundwater fauna in relation to  
556 environmental conditions. *Freshwater Biology* **54**, 814-829.
- 557 Montgomery SH, Capellini I, Barton RA, Mundy NI (2010) Reconstructing the ups and downs of  
558 primate brain evolution: implications for adaptive hypotheses and *Homo floresiensis*. *Bmc  
559 Biology* **8**.
- 560 Organ CL, Shedlock AM, Meade A, Pagel M, Edwards SV (2007) Origin of avian genome size and  
561 structure in non-avian dinosaurs. *Nature* **446**, 180-184.
- 562 Pagel M (1999) Inferring the historical patterns of biological evolution. *Nature* **401**, 877-884.
- 563 Pagel M, Meade A, Barker D (2004) Bayesian estimation of ancestral character states on phylogenies.  
564 *Systematic Biology* **53**, 673-684.
- 565 Parsons ML (1970) Groundwater Thermal Regime in a glacial complex. *Water Resources Research* **6**,  
566 1701-1720.
- 567 Pimm SL, Russell GJ, Gittleman JL, Brooks TM (1995) The future of biodiversity. *Science* **269**, 347-350.
- 568 Provan J, Bennett KD (2008) Phylogeographic insights into cryptic glacial refugia. *Trends in Ecology &  
569 Evolution* **23**, 564-571.
- 570 Reid N (2011) European hare (*Lepus europaeus*) invasion ecology: implication for the conservation of  
571 the endemic Irish hare (*Lepus timidus hibernicus*). *Biological Invasions* **13**, 559-569.
- 572 Robertson AL, Smith JWN, Johns T, Proudlove GS (2009) The distribution and diversity of stygobites  
573 in Great Britain: an analysis to inform groundwater management. *Quarterly Journal of  
574 Engineering Geology and Hydrogeology* **42**, 359-368.
- 575 Rogl F (1999) Mediterranean and Paratethys. Facts and hypotheses of an Oligocene to Miocene  
576 paleogeography (short overview). *Geologica Carpathica* **50**, 339-349.
- 577 Ronquist F, Teslenko M, van der Mark P, *et al.* (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic  
578 Inference and Model Choice Across a Large Model Space. *Systematic Biology* **61**, 539-542.
- 579 Summers RW, Dawson RJG, Phillips RE (2007) Assortative mating and patterns of inheritance  
580 indicate that the three crossbill taxa in Scotland are species. *Journal of Avian Biology* **38**,  
581 153-162.

- 582 Tamura K, Peterson D, Peterson N, *et al.* (2011) MEGA5: Molecular Evolutionary Genetics Analysis  
583 Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods.  
584 *Molecular Biology and Evolution* **28**, 2731-2739.
- 585 Trontelj P, Blejec A, Fišer C (2012) Ecomorphological convergence of cave communities. *Evolution* **66**,  
586 3852-3865.
- 587 Trontelj P, Douady CJ, Fišer C, *et al.* (2009) A molecular test for cryptic diversity in ground water:  
588 how large are the ranges of macro-stygobionts? *Freshwater Biology* **54**, 727-744.
- 589 Vainola R, Witt JDS, Grabowski M, *et al.* (2008) Global diversity of amphipods (Amphipoda;  
590 Crustacea) in freshwater. *Hydrobiologia* **595**, 241-255.
- 591 Van Dover CL, German CR, Speer KG, Parson LM, Vrijenhoek RC (2002) Marine biology - Evolution  
592 and biogeography of deep-sea vent and seep invertebrates. *Science* **295**, 1253-1257.
- 593 Voigt S, Gale AS, Voigt T (2006) Sea-level change, carbon cycling and palaeoclimate during the Late  
594 Cenomanian of northwest Europe; an integrated palaeoenvironmental analysis. *Cretaceous*  
595 *Research* **27**, 836-858.
- 596 Weyhenmeyer CE, Burns SJ, Waber HN, *et al.* (2000) Cool Glacial Temperatures and Changes in  
597 Moisture Source Recorded in Oman Groundwaters. *Science* **287**, 842-845.
- 598 Wheeler A (1977) The origin and distribution of the freshwater fishes of the British Isles. *Journal of*  
599 *Biogeography* **4**, 1-24.
- 600 Williams A, Bloomfield J, Griffiths K, A B (2006) Characterising the vertical variations in hydraulic  
601 conductivity within the Chalk aquifer. *Journal of Hydrology* **330**, 53-62.
- 602 Williams JR (1970) Ground water in the permafrost regions of Alaska, p. 83. U.S. Geological Survey,  
603 Professional Paper 696.
- 604 Yalden DW (1982) When did the mammal fauna of the British Isles arrive. *Mammal Review* **12**, 1-57.
- 605 Zachos J, Pagani M, Sloan L, Thomas E, Billups K (2001a) Trends, rhythms, and aberrations in global  
606 climate 65 Ma to present. *Science* **292**, 686-693.
- 607 Zachos JC, Dickens GR, Zeebe RE (2008) An early Cenozoic perspective on greenhouse warming and  
608 carbon-cycle dynamics. *Nature* **451**, 279-283.
- 609 Zachos JC, Shackleton NJ, Revenaugh JS, Palike H, Flower BP (2001b) Climate response to orbital  
610 forcing across the Oligocene-Miocene boundary. *Science* **292**, 274-278.

611

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

613

614 Tables:

615

616 Table 1. A) Estimates of the time of the most recent common ancestor (TMRCA) between  
 617 British and Irish *Niphargus* taxa and their closest relatives based on a BEAST analysis. The  
 618 prior used and mean and median estimates in millions of years (Ma) are given, including the  
 619 upper and lower bounds of the highest posterior density (HPD) intervals. B) Details of the  
 620 external calibration points estimated from a subset of representative data from Hou *et al.*  
 621 (2011) are given.

622

623

A) Node	prior	Mean (Ma)	Median (Ma)	95% HPD lower	95% HPD upper
<i>N. aquilex</i> E/F	Tree prior	6.69	6.22	2.15	12.32
<i>N. aquilex</i> (A1,A2)/B	Tree prior	5.93	5.57	2.02	10.35
<i>N. aquilex</i> A1/A2	Tree prior	1.06	0.95	0.23	2.12
<i>N. fontanus</i> A1/A2	Tree prior	0.89	0.77	0.17	1.90
<i>N. irlandicus</i> / <i>N. glenniei</i>	Tree prior	21.05	19.48	6.74	38.09
<i>N. kochianus</i> A/(B,C)	Tree prior	3.06	2.89	1.26	5.21
<i>Niphargus</i> root	Uniform [45-558]	88.16	87.14	65.38	113.94
<b>B) External calibration points from [4]</b>					
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

624

625

626 Figure captions:

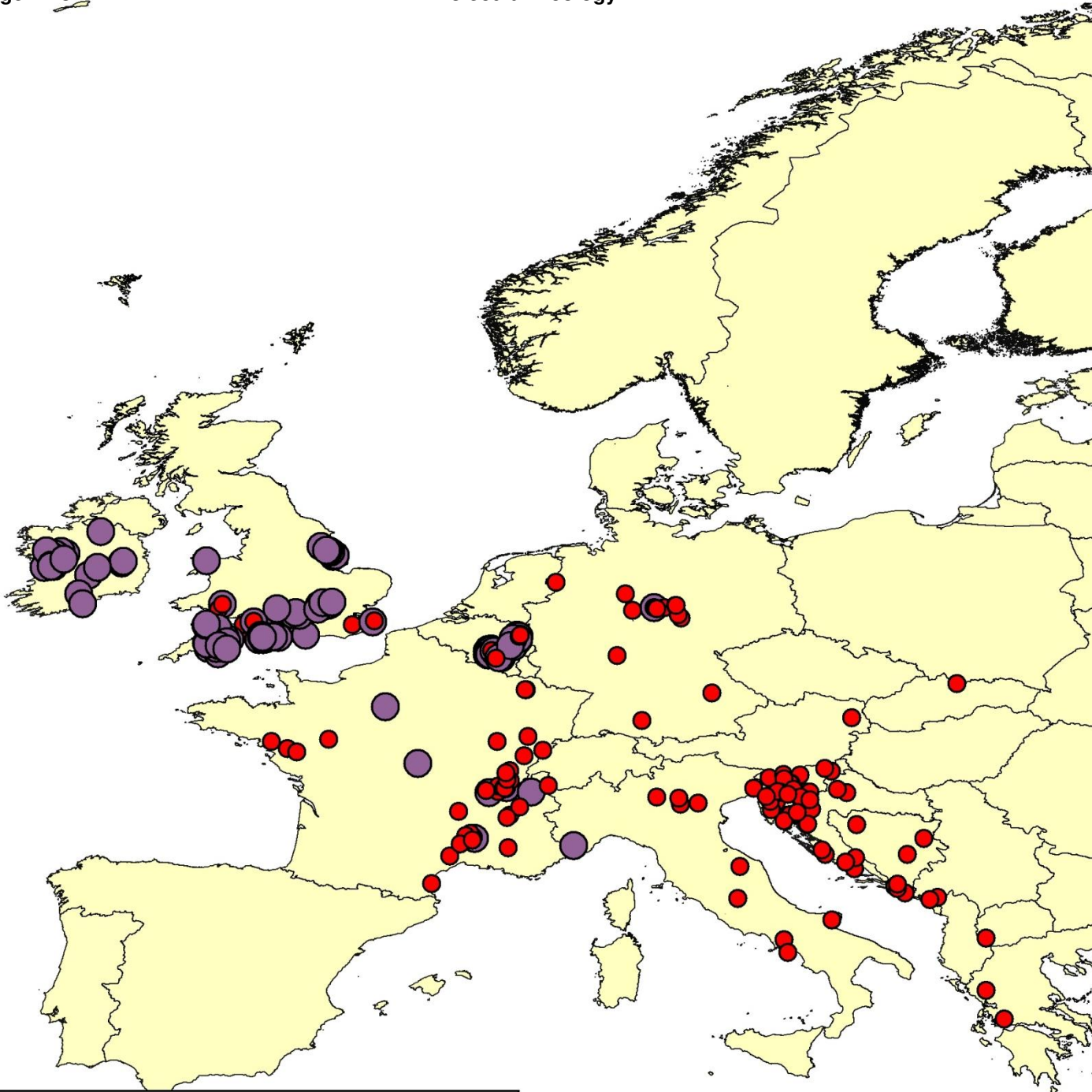
627

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

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

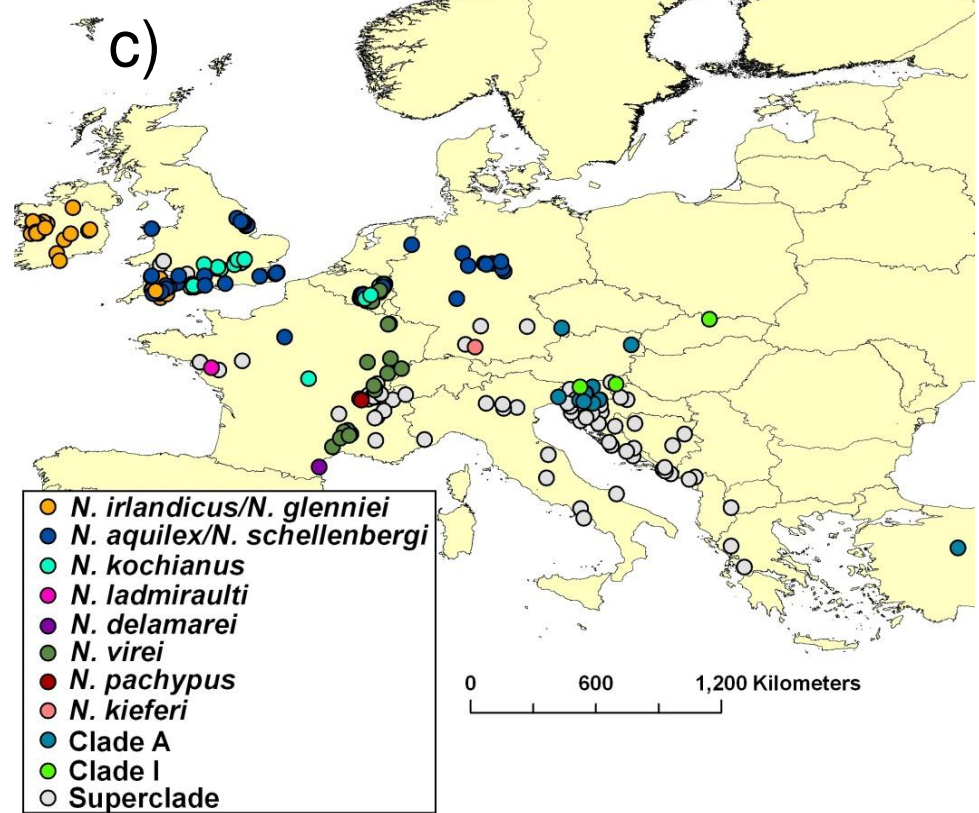
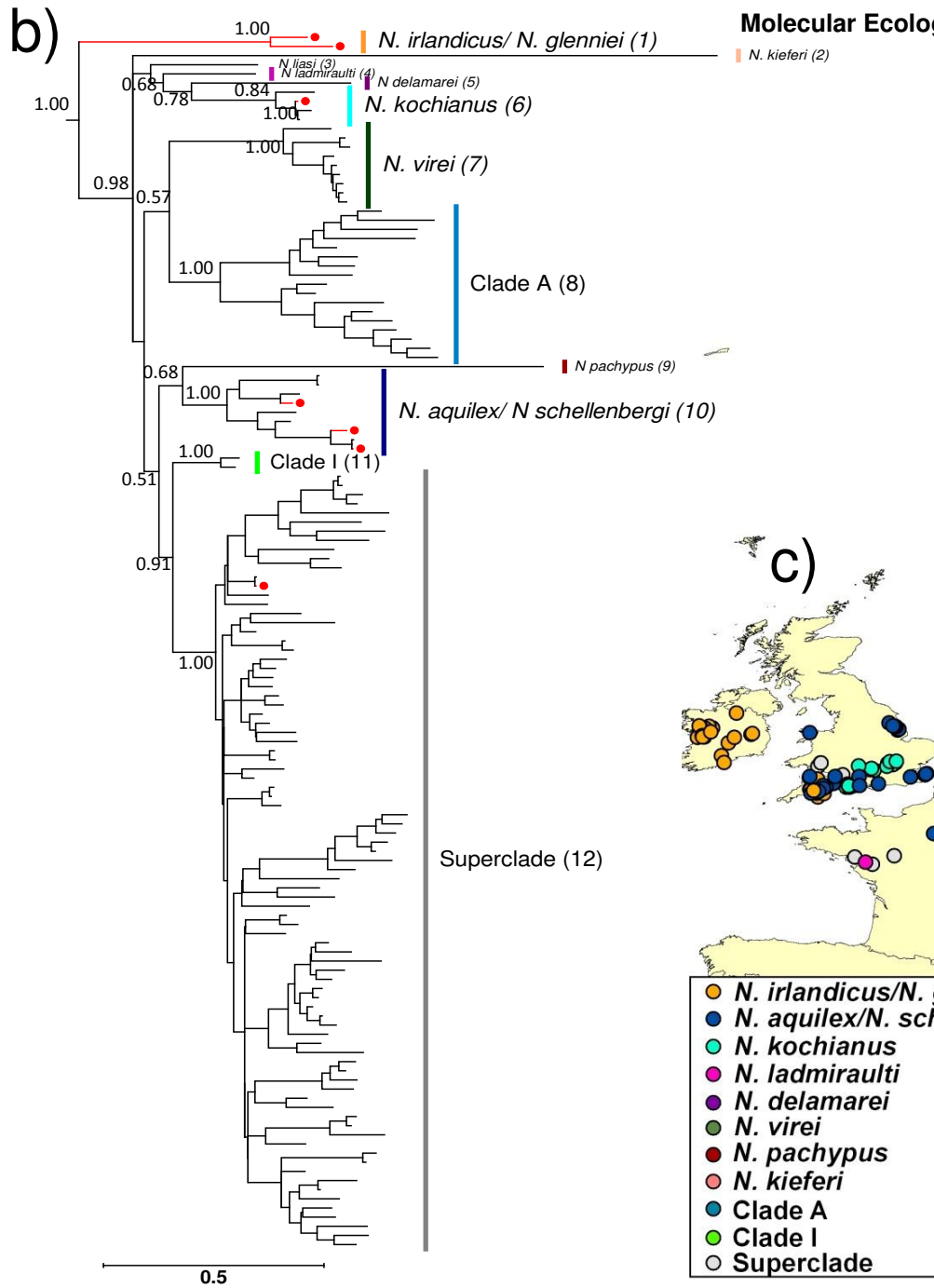
637 Figure 3: Geographic distribution of British and Irish OTU's and European sister taxa. The  
638 green and pink lines represent the maximum extent of the glacial ice sheets during the  
639 Devensian and Anglian glacial periods respectively. Small black dots are sites of known  
640 distribution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

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



● New data from this study  
● Previously published data

0 500 1,000 Kilometers



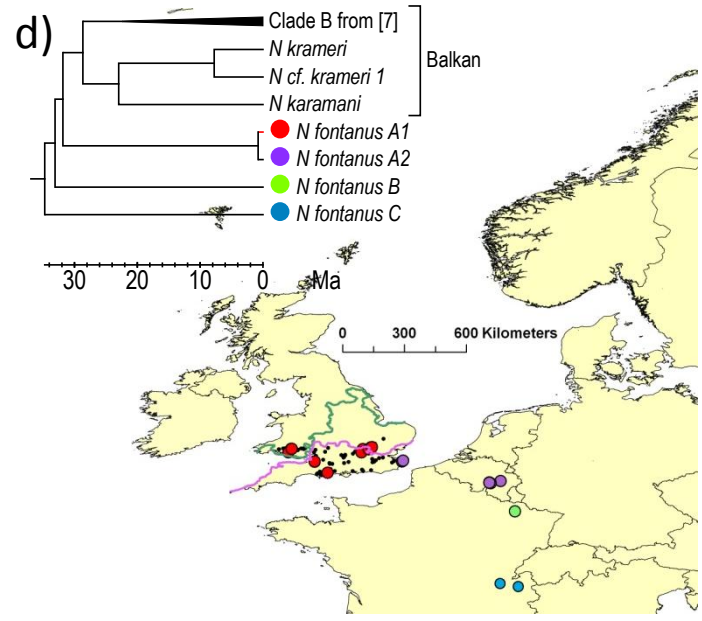
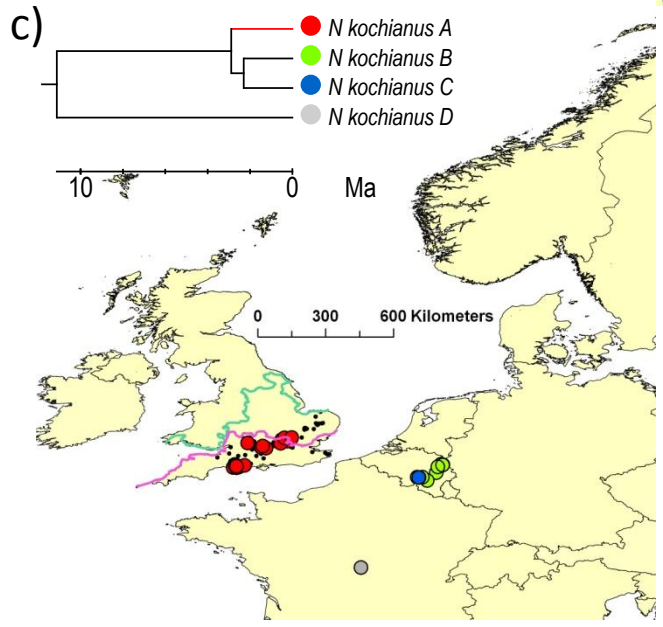
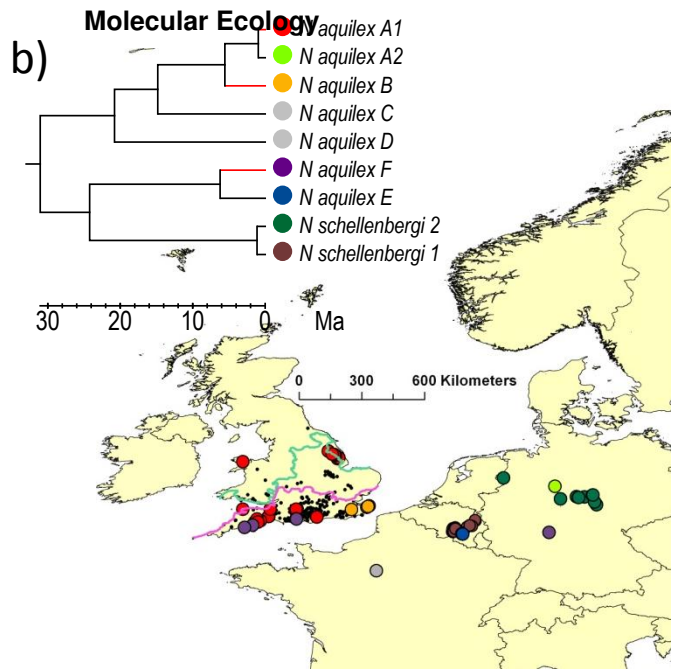
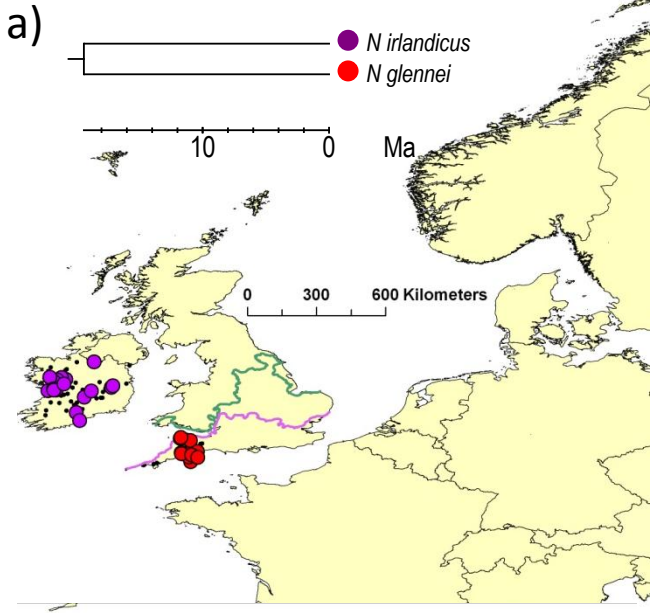


Table 1. A) Estimates of the time of the most recent common ancestor (TMRCA) between British and Irish *Niphargus* taxa and their closest relatives based on a BEAST analysis. The prior used and mean and median estimates in millions of years (Ma) are given, including the upper and lower bounds of the 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
<i>N. aquilex</i> E/F	Tree prior	6.69	6.22	2.15	12.32
<i>N. aquilex</i> (A1,A2)/B	Tree prior	5.93	5.57	2.02	10.35
<i>N. aquilex</i> A1/A2	Tree prior	1.06	0.95	0.23	2.12
<i>N. fontanus</i> A1/A2	Tree prior	0.89	0.77	0.17	1.90
<i>N. irlandicus/N. glenniei</i>	Tree prior	21.05	19.48	6.74	38.09
<i>N. kochianus</i> A/(B,C)	Tree prior	3.06	2.89	1.26	5.21
<i>Niphargus</i> root	Uniform [45-558]	88.16	87.14	65.38	113.94
<b>B) External calibration points from [4]</b>					
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