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Peripatric origin of the only cave-restricted stonefly species known (Insecta: Plecoptera)

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Abstract

Cave animals have evolved specialised morphological and behavioural adaptations to a dark and stable environment. When their ancestors were surface species living in continental climates, and thus adapted to annual changes from extreme cold to extreme warm periods, it is highly intriguing how adaptation to cave stable temperature took place. The phylogenetic history of the cave animal can thus help to understand the kind of adaptation process undergone in such a different environment. Here we perform a mitochondrial DNA-based phylogeny for the only Palearctic cave-restricted plecopteran species (*Protonemura gevi*), whose geographic range is limited to a single cave in the south of the Iberian Peninsula, also including four other Iberian and Moroccan *Protonemura* species and two other species from different genera as outgroup taxa. Our results indicate that *P. gevi* shows a genetic distance with *P. culmenis* being an order of magnitude lower than those with the remaining *Protonemura* species. Using a relaxed molecular clock, phylogeny dating suggests that their common ancestor probably lived 1.4 million years ago, i.e. during Pleistocene. *P. culmenis* currently lives in the Pyrenees, so that it is conceivable that this species (or the common ancestor of both) migrated to a southern refuge during one of Pleistocene glaciations, and a few individuals remained isolated within the Siles cave managing to live in such new and different environmental conditions. We thus conclude that the peripatric origin of *P. gevi* was associated with Pleistocene glaciations, and that its adaptation to cave conditions was quite rapid.

Key words

Glaciations, mtDNA, peripatric, Plecoptera, *Protonemura gevi*, speciation.

1. Introduction

Cavernicolous animals generally exhibit a set of morphological traits resulting from strong environmental pressures and adaptation to this particular habitat, as well as losses of capabilities or structures superfluous in cave environments. This fact can obscure phylogenetic relationships among closely related cave and surface species (Porter 2007). Therefore, molecular phylogenetic studies are particularly useful to unveil evolutionary relationships among these organisms. Moreover, as pointed out by many authors, cave fauna, like that inhabiting remote islands, provides unique opportunities to study evolutionary mechanisms and historical factors related to bio-

geography and speciation processes (Poulson & White 1969; Porter 2007; Juan et al. 2010; Bauzà-Ribot et al. 2011).

In species with low dispersal capacity, where vicariance can be reasonably favoured over dispersal [although Theissinger et al. (2013) found that dispersion can be also important in particular stonefly species], biochemical and molecular research has greatly contributed, during the last two decades, i) to unveiling phylogenetic relationships among related taxa and ii) to describing biogeographical patterns including the timing of origin for the different phyletic lineages in the light of the most likely vicariant hypotheses (Caccone et al. 1997; Ketmaier et al. 2001, 2003; Fochetti et al. 2004, 2009; Juan et al. 2010).

Plecopterans most usually show low dispersal capacity (Fochetti & Tierno de Figueroa 2008), but biogeographical studies employing biochemical and molecular data are scarce and mainly focused on the Mediterranean area (Fochetti 1994; Fochetti et al. 1997, 2004, 2009, 2011). *Protonemura gevi* Tierno de Figueroa & López-Rodríguez, 2010 is the only known cave-restricted Palearctic plecopteran species with morphological and behavioural adaptations to cavernicolous life (Tierno de Figueroa & López-Rodríguez 2010a; López-Rodríguez & Tierno de Figueroa 2012). It is a seriously endangered species since it is known only from a range of about 300 square meters inside the Cueva de Siles (a cave in southern Spain), with small population size. In fact, this species has been catalogued as Critically Endangered in the Atlas and red book of endangered invertebrates of Spain (Tierno de Figueroa & López-Rodríguez 2010b). Samplings have been also carried out in a nearby stream outside the cave that the species inhabits (about 50 m downhill from the entrance of the cave), but *P. gevi* has been never collected. The species was neither detected by sampling in other nearby and remote cave systems (Grupo de Espeleología de Villacarrillo, GEV, pers. comm.).

The genus *Protonemura* is distributed over Europe, Asia and north Africa and includes 129 species currently classified into eight species groups, although most of the species cannot be included in those species groups (DeWalt et al. 2014). It is morphologically quite uniform, and characters of the male terminalia are the most important in the distinction of species and species groups. *Protonemura gevi* shows distinct morphological modifications, particularly in traits that can be considered as adaptations to cavernicolous life: adults have scarcely prominent compound eyes in comparison with other *Protonemura* species, relatively long antennae and reduced wings in both sexes (brachypterous); nymphs show scarce pilosity. These modifications make it difficult to establish relatedness with other species of the same genus. Thus, as pointed out in its description (Tierno de Figueroa & López-Rodríguez 2010a), morphologically, *P. gevi* is clearly different from the remaining *Protonemura* species, and cannot be included in currently recognized groups (Vinçon & Muranyi 2009). According to terminalia morphology, the most similar Iberian species is *P. culmenis* Zwick & Vinçon, 1993, which is a micro-endemic stenothermous species only known from a small part of the Eastern Pyrenees (Ariège in France and Andorra), at 2000–2700 m a.s.l. (Vinçon & Muranyi 2009). The female pregenital plate is similar in both species, but wider in *P. gevi*; the epiproct of *P. gevi* has a characteristic hump formed by the distal third of the ventral sclerite, not so prominent in *P. culmenis*; and the paraprocts of males of the two species differ mainly in the shape of the inner branch of the median lobes (finger-shaped in *P. culmenis*, apically enlarged in *P. gevi*) (Tierno de Figueroa & López-Rodríguez 2010a). These authors also pointed out that some similarities can be found in the shape of the male paraprocts and the female subgenital plate with the North African subgroup of *P. talboti* (Navás, 1929) of the *corsicana* group, but the absence of the apical tube-like projection in the epiproct of *P. gevi* indicates that this species does not belong to the *corsicana* group. Finally, morphological similarities were also found with *P. hassankifi* Aubert, 1964 from Iran.

The aim of the present study is to clarify the phylogenetic relationships of *P. gevi* with other species of its genus. For this purpose, we performed a molecular phylogenetic analysis for three mitochondrial genes in several species showing higher morphological resemblance with *P. gevi* and/or inhabiting the same region. The results provided useful information on the evolutionary origin of this unusual species, and allowed to understand the zoogeographic events that led to its current distribution.

2. Material and Methods

2.1. Taxon sampling and specimen collection

Collection data of the 10 *Protonemura* (ingroup) specimens employed in the present study are cited below. Wherever possible, specimens from the different species were collected at the location closest to *P. gevi*. All collections were performed with corresponding permits.

- *Protonemura alcazaba* (Aubert, 1954): Arroyo de San Blas, Sierra de Segura, SE Spain; 03.ii.2010, 1 individual; López-Rodríguez & Tierno de Figueroa leg. [50–100 m from *P. gevi* locality]
- *Protonemura alcazaba* (Aubert, 1954): Río Poqueira, Sierra Nevada, SE Spain; 12.x.2004, 1 individual; López-Rodríguez & Tierno de Figueroa leg. [160–170 km from *P. gevi* locality]
- *Protonemura culmenis* Zwick & Vinçon, 1993: Arroyo Coma Pedrosa, Pyrenees, Andorra; 07.vii.1986, 1 individual; Vinçon leg. [590–600 km from *P. gevi* locality]
- *Protonemura gevi* Tierno de Figueroa & López-Rodríguez, 2010: Cueva de Siles (source of Arroyo de San Blas), Sierra de Se-

Table 1. DNA sequence and properties of the primers used in this study. COI = subunit I of cytochrome oxidase, COII = subunit II of cytochrome oxidase.

Gene	Primer Name	Sequence	Size (nt)	AT (ºC)	Source
12S rDNA	12SAI	AAACTAGGATTAGATACCCTATTAT	~350	50	SIMON et al. (1994)
12S rDNA	12SBI	AAGAGCGACGGGCGATGTGT			
COI	plecoi1F	ATCTGCCGGAATTGCCCATGC	~400	55	This study
COI	plecoi2R	CCAATGAACCAAAGCTTCC			
COII	COII_F_Leu	TCTAATATGGCAGATTAGTGC	~700	52	WHITING et al. (2002)
COII	COII_R_Lys	GAGACCAGTACTTGCTTTCAGTCATC			

Table 2. Acronyms and accession numbers of the DNA sequences obtained in this study.

		Accession number		
Specimen (Location)	**Acronym**	**12S**	**COI**	**COII**
P. alcazaba (S. Segura)	PALC_1	KF881053	KF881064	KF881075
P. alcazaba (S. Cazorla)	PALC_3	KF881055	KF881066	KF881077
P. culmenis (Pyrenees)	PCUL	KF881056	KF881067	KF881078
P. gevi (S. Segura 2009 ind. 1)	PGEV_09_1	KF881057	KF881068	KF881079
P. gevi (S. Segura 2009 ind. 2)	PGEV_09_2	KF881058	KF881069	KF881080
P. gevi (S. Segura 2010 ind. 1)	PGEV_10_1	KF881059	KF881070	KF881081
P. gevi (S. Segura 2010 ind. 2)	PGEV_10_2	KF881060	KF881071	KF881082
P. gevi (S. Segura 2010 ind. 3)	PGEV_10_3	KF881061	KF881072	KF881083
P. meyeri (S. Nevada)	PMEY	KF881062	KF881073	KF881084
P. talboti (Atlas)	PTAL	KF881063	KF881074	KF881085

gura, SE Spain; 22.x.2009, 2 individuals; 03.vi.2010, 3 individuals; López-Rodríguez & Tierno de Figueroa leg.

- *Protonemura meyeri* (Pictet, 1842): Río Poqueira, Sierra Nevada, SE Spain; 15.iii.2005. 1 individual. López-Rodríguez & Tierno de Figueroa leg. [160–170 km from *P. gevi* locality]
- *Protonemura talboti* (Navás, 1929): Atlas, Morocco; 30.iii.1988; 1 individual; Sánchez-Ortega leg. [450–750 km from *P. gevi* locality]

Selected sequences of *Amphinemura nigritta* (Provancher, 1876) and *Leuctra fusca* (Linnaeus, 1758) from GenBank were used as outgroup taxa (see 'Sequence and phylogenetic analyses').

2.2. DNA extraction, amplification and sequencing

Materials for molecular analysis were stored in absolute ethanol at room temperature until usage. Total genomic DNA was extracted from each specimen by crushing it in liquid nitrogen using the GenElute Mammalian Genomic DNA miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA). We amplified the fragments of mitochondrial genes, namely 12S rDNA, and subunits I and II of cytochrome oxidase (COI and COII), which have been frequently used in other molecular studies in stoneflies (TERRY 2003; FOCHETTI et al. 2009).

PCR reactions were performed in a total volumen of 25 µL that was composed of 1 × PCR buffer, 2 mM $MgCl_2$, 200 uM dNTPs, 0.4 pmol/L of each primer, 10 ng DNA and 1U of Taq polymerase (MBL002). In addition, we added 1 µg of bovine serum albumine (BSA) to PCR reactions for species with degraded DNA (FULTON & STILLER 2012). Amplifications were performed in an Eppendorf thermocycler ep Gradient under the following protocol: an initial denaturation step for 5 min at 95ºC, 30 s at 94ºC, 30 s at annealing temperature, 30 s at 72ºC, during 30 cycles, and a final extension of 7 min at 72ºC. The annealing temperature and the size of the amplicon for each primer are shown in Table 1. Primers for the COI fragment were designed from a longest fragment of ~1300 nt obtained from *P. gevi*, by PCR with the C1-J-1763 and TL2-N-3014 universal primers (LUNT et al. 1996) and 46ºC of annealing temperature. PCR products were sequenced by the capillarity Sanger method in Macrogen Inc., with their respective primers.

2.3. Sequence and phylogenetic analyses

Electropherograms were reviewed and sequences edited by using the software Geneious v4.8 (DRUMMOND et al. 2009). For the ingroup, each haplotype found (regarding the concatenated sequences of the three genes) was

included in the analysis and represents a terminal node in the resulting trees (only *P. gevi* individuals 1 and 2 from 2009 had identical sequences, see Figs. 1, 2). The DNA sequences used for ingroup taxa in this study were sent to the GenBank database with the accession numbers indicated in Tab. 2. As outgroup taxa, we chose DNA sequences from the GenBank for *Amphinemura nigritta* (12S: EF623346; COII: EF623045) and *Leuctra fusca* (12S: FM212941; COI: FM213089).

We aligned the DNA sequences obtained for each fragment, separately, by using MAFFT v7.123b (Katoh & Toh 2008) with automatic election of parameters. Incongruence between different types of sequences was tested using incongruence length difference (ILD, Farris et al. 1995) test implemented in PAUP* v4.0b (Swofford 2003). We used DAMBE v5.3.9 (Xia & Xie 2001) for testing whether sequence information was saturated. Informative sites from the three resulting alignments were extracted by means of Gblocks server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana 2002). This alignment was concatenated with Geneious v4.8 (Drummond et al. 2010) for subsequent analysis. The matrices of p-distances between species were calculated with the MEGA v5.05 (Tamura et al. 2011) software with pairwise deletion of gaps.

We estimated phylogenetic trees with four different methods considering haplotypes for the concatenated sequences. For Bayesian Inference (BI) the tree was built using MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003). The concatenated alignment was partitioned into the three regions with the most probable substitution model for each region using MrModeltest v2.3 (Nylander 2004) and Akaike information criterion (Akaike 1974). The analysis lasted 4 million MCMC generations with sampling frequency of 100 generations, removing the first 25% of trees as burn-in, and checking trace files with Tracer v1.5 (Rambaut & Drummond 2007) in order to test convergence of the two independent Bayesian MCMC runs. Maximum Likelihood (ML) tree was estimated using PhyML v3.0 (Guindon et al. 2010) assuming a GTR model and with default options. We calculated the Neighbor-Joining (NJ) tree applying the Tamura 3-parameters model in the MEGA5 software (Tamura et al. 2011). Maximum Parsimony (MP) trees were built with MEGA5 (Tamura et al. 2011), using the Close Neighbor Interchange (CNI) on Random Trees option with 10 initial trees and generating 13 trees equally most parsimonious and getting a consensus tree. We performed 1000 bootstrap replicates to estimate branch supports for MP, NJ and MP. In order to preserve the information on all three genes in the ingroup, in spite of lacking some of them for the outgroups, we considered the absent information in the latter (likewise gaps) as missing data for BI and ML analyses, and used the pairwise deletion option for the NJ tree and the partial deletion option with 95% cutoff for the MP method.

2.4. Divergence rates and time estimation

To test the existence of a molecular clock for these sequences (i.e. the uniformity of the evolutionary rate among different lineages), we performed a likelihood-ratio test (Huelsenbeck & Rannala 1997) using PAUP 4.0b and LRT calculator of jModeltest v0.2 (Posada 2008). A dated phylogenetic tree was obtained by means of BEAST v1.7.0 (Drummond et al. 2012) using, as prior, the substitution rate recognized as universal molecular clock for insects, i.e. 0.0115 substitutions per lineage per million year (Brower 1994), applying a Lognormal relaxed clock.

3. Results and discussion

Mutational saturation test did not show saturation for any of the three fragments. The ILD test did not indicate significant incongruence between data sets, so it allowed us to concatenate all alignments. We thus obtained a 1518 nt alignment (360 nt from 12S, 419 nt from COI and 739 nt from COII), from which we selected the most informative sites with Gblocks.

All trees built by different methods indicated that *P. culmenis* is the closest relative of *P. gevi* (Fig. 1). The genetic distance between these two species was one order of magnitude lower than those shown with the remaining species analysed (Tab. 3: 0.010 versus 0.068 for the 2nd-smallest distance involving *P. gevi*). Therefore, no close relationship was found between *P. gevi* and *P. meyeri* (0.100 distance to *P. gevi*) or *P. alcazaba* (0.111), the only species of the *Protonemura* genus being present, like *P. gevi*, in the South of the Iberian Peninsula, or with the north African *P. talboti* (0.068) from the *corsicana* group. The distance between *P. gevi* and *P. alcazaba* is particularly interesting, as *P. alcazaba* inhabits a stream 50–100 m downhill from the cave where *P. gevi* occurs.

The closest relationship between *P. gevi* and *P. culmenis* shown by molecular data is consistent with the fact that the epigean species being morphologically most similar to *P. gevi* is *P. culmenis* (Tierno de Figueroa & López-Rodríguez 2010a). On this basis, *P. gevi*, in spite of its morphological peculiarities derived from adaptation to cave life, should be included, with *P. culmenis*, in the group of *P. tuberculata* (Despax, 1929). Nevertheless, the validity of this group has recently been questioned (Vinçon & Muranyi 2009), so that many species previously included in it have been relocated to other groups, but the other elements, including the closest relatives to *P. gevi*, i.e. two endemisms from the Pyrenees (*P. culmenis* and *P. tuberculata*) and another endemism from the Caucasus (*P. alticola* Zhiltzova, 1958), could not be included in those other groups.

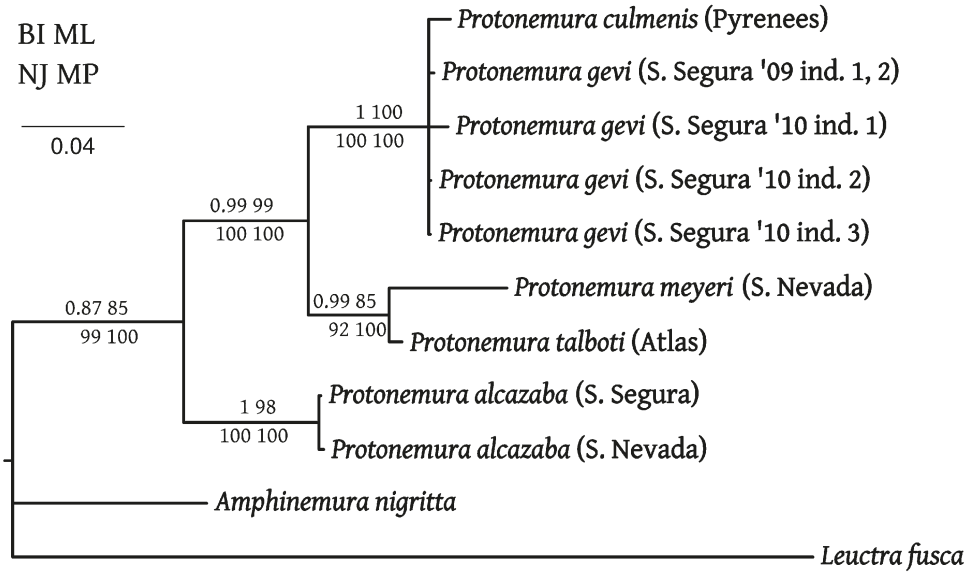

Fig. 1. Phylogenetic relationships among the studied species. Tree built with the Bayesian inference (BI) method showing branch supports estimated by BI posterior probability, and showing also the bootstrap values (%) for Maximum Likelihood (ML), Neighbour-Joining (NJ) and Maximum Parsimony (MP).

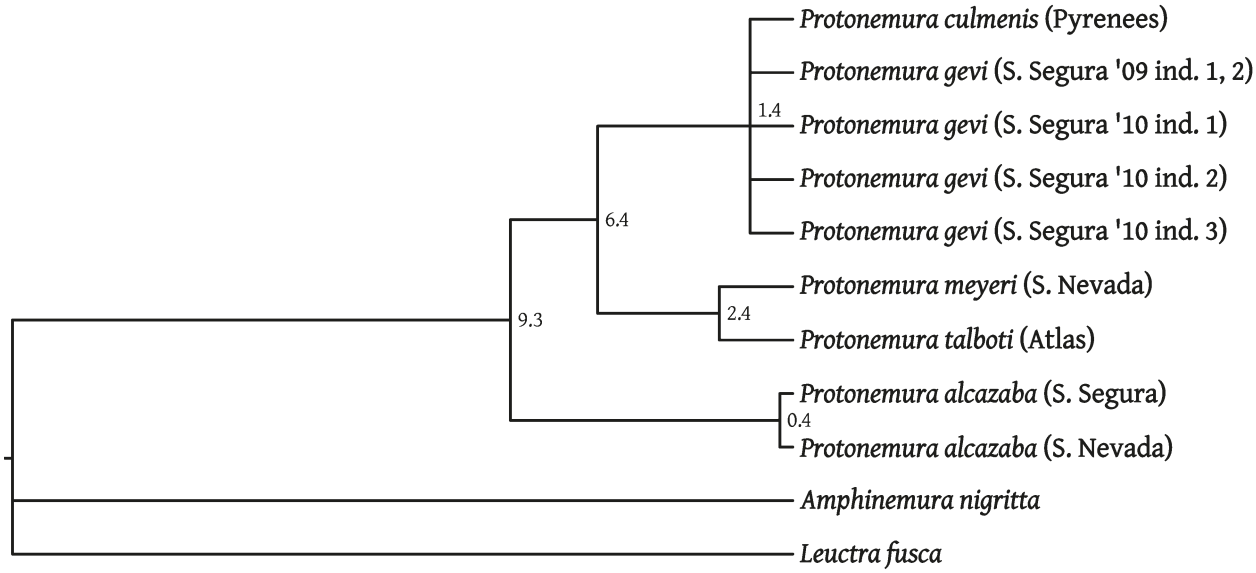

Fig. 2. Ultrametric tree showing the age of each node (Mya).

The molecular clock null hypothesis was rejected by the likelihood-ratio test (LRT = 2 [L0 – L1] = 24.40488, df = 9, p = 0.0037) suggesting the need of applying methods allowing different rates among lineages to estimate the age of each node (relaxed clock). The tree provided by BEAST (Fig. 2) showed that *P. culmenis* and *P. gevi* shared a common ancestor about 1.4 Mya, i.e. during the earlier Pleistocene (Calabrian stage). The drastic climatic changes that occurred since the end of the Tertiary, and especially during Pleistocene glaciations, profoundly impacted insect distributions (Zwick 2009). The Pleistocene spanned from about 2.6 million years to about 12000 years ago, and it was characterized by an overall pattern of cooling and warming (glacial-interglacial cycles). Three types of biota response are conceivable: i) some species were able to "float" with their optimal habitat and shifted across latitude and altitude, ii) other species remained where they were and adapted to altered local environments, and iii) still other species underwent range reduction and eventual extinction (Lomolino et al. 2010). Plecoptera, as very stenoecious organisms usually adapted to cold, well-oxygenated waters (Fochetti & Tierno de Figueroa 2008), gave rise to many relict taxa in high mountain areas all along southern Europe, as a result of glaciations that favoured isolation and speciation processes (Fochetti & Tierno de Figueroa 2006). The

Table 3. P-distance matrix calculated with MEGA5.

	L. fusca	*A. nigritta*	*P. alcazaba*	*P. culmenis*	*P. gevi*	*P. meyeri*
A. nigritta	0.145					
P. alcazaba	0.183	0.144				
P. culmenis	0.194	0.145	0.118			
P. gevi	0.194	0.136	0.111	0.010		
P. meyeri	0.183	0.134	0.105	0.105	0.100	
P. talboti	0.194	0.129	0.102	0.072	0.068	0.043

same was true for many cave organisms, at least in some cases (see Bellés 1991 for a discussion on this topic).

Considering the date of separation of the *P. culmenis* and *P. gevi* lineages, ca. 1.4 Mya (based on an average molecular evolutionary rate for insects), it can be hypothesized that glacial-interglacial cycles played an important role in the speciation process giving rise to *P. gevi*. A conceivable scenario is the expansion of a Pyrenean, cold-adapted species *P. culmenis* (or, more correctly, its ancestor) towards southern Iberian locations during a glacial period, with subsequent extinction in most colonized areas during the subsequent interglacial period. This could have occurred in several consecutive glacial-interglacial periods. But, in one of them, some individuals could have colonized the Siles cave and thus escaped the impact of subsequent interglacial periods in situ. These specimens remained isolated in an environmental spot (a cave) within a fragmented habitat, such as the southern Iberian Peninsula during the interglacial period.

Considering the absence of epigean close relatives of *P. gevi* in the same area – *P. alcazaba* is the only species living close to the Cueva de Siles cave but it is genetically much more distant from *P. gevi* than *P. culmenis* – the colonization of the hypogean environment by the ancestor of *P. gevi* is consistent with the "climatic-relict" model (Howarth 1973; Holsinger 2000). Due to intra-specific variation of gene sequences, the divergence between haplotypes of a locus that are later sorted into species-specific lineages can be older than the speciation events concerned (Maddison 1997). Therefore, the origin of *P. gevi* is most likely more recent than the 1.4 Ma indicated by the mtDNA phylogeny. The coalescence time is affected by both effective population size (N_e) and speciation time, so that larger N_e and shorter speciation time will tend to maximize incongruence between the gene and species trees. Epigean species in the *Protonemura* genus usually show large N_e, so that the origin of *P. gevi* could actually be more recent than 1.4 Ma. The extremely localized geographical distribution of *P. gevi*, with only one population known, fits perfectly to a case of peripatric speciation based on founder effect (Mayr 1954).

Even considering that the scarce molecular information in stoneflies, including the *Protonemura* genus, shows a molecular evolutionary rate considerably lower than that of many insects (Fochetti et al. 1997, 2004, 2009), the isolation event would have happened during the Pleistocene period, supporting the hypothesis of glacial-interglacial environmental changes. A relatively young origin, also associated with climatic events in the Pleistocene, has been suggested for some other plecopteran taxa (within the *Isoperla* and *Protonemura* genera), as well as for other species of invertebrates such as earthworms, subterranean aquatic isopods, cave beetles and land snails (in Fochetti et al. 2009), from Corsica and Sardinia (Fochetti et al. 1997).

Our present results also show how isolation in a very different environment can lead to rapid morphological differentiation, as a consequence of adaptation, without the need for large genetic differences. This resembles the well known result of isolation on islands. For instance, morphological evolution appears to be accelerated among island mammals by rapid adaptation to the new environment following isolation (Millien 2006). In salamanders, rapid evolution of viviparity has been reported in islands but, in this case, rapid genetic differentiation was also observed (Velo-Antón et al. 2012). Island species are often so different that it is difficult to identify their ancestors (Poulakakis et al. 2002). In the case of *P. gevi*, only the molecular phylogenetic analysis and the extremely low genetic distance with *P. culmenis* allowed inferring the precise ancestry of the former, a singular species being the only cave-restricted plecopteran hitherto known.

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