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Genetic diversity and species richness patterns in Baetidae (Ephemeroptera) in the Montseny Mountain range (North-East Iberian Peninsula)

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ABSTRACT

Genetic diversity and species richness patterns in Baetidae (Ephemeroptera) in the Montseny Mountain range (North-East Iberian Peninsula)

This study aimed to describe patterns of diversity of Baetidae (Ephemeroptera) at the community and population levels within the Montseny Mountain range (North-East Iberian Peninsula). We studied both the distribution of 4 species of baetids in 20 sites among three catchments along the altitudinal gradient (350-1700 masl); and the genetic diversity of the mtDNA cytochrome c oxidase subunit I (*cox1*) gene of the two common species *Baetis alpinus* and *Baetis rhodani*. We found a gradual replacement of the dominant species along the altitudinal gradient. *Baetis alpinus* inhabited sites at high-altitudes, and this species was replaced by *B. rhodani* when the altitude decreased. *Baetis melanonyx* and *Alainites muticus* attained low abundance at all river sections, and no clear altitudinal trend appeared. Our hypothesis at the population level was that genetic structuring is associated with geographic distance and limited by drainage boundaries among the three studied catchments because of the short-time dispersion of adults. Unexpectedly, analyses of molecular variance (AMOVA) and isolation-by-distance (IBD) showed genetic diversity was unstructured by distance for both species, which may be explained by the relatively short spatial scale studied and small topographic barriers among the three catchments. The Generalized Mixed Yule-Coalescent (GMYC) model showed that *B. rhodani* had two differentiated genetic lineages that co-occurred in all sites. Overall, diversity of baetids was structured at the community level along the altitudinal gradient, whereas it was unstructured at the population level within the Montseny Mountain range.

Key words: Aquatic insects, cryptic species complex, genetic diversity, GMYC model, morphospecies, species assemblage, stream ecology.

RESUMEN

Diversidad genética y patrones de riqueza de especies de los Baetidae (Ephemeroptera) en la montaña del Montseny (Nord-Este de la Península Ibérica)

*Este estudio pretende describir los patrones de diversidad de las especies (tanto a nivel de comunidad como de población) de los Baetidae (Ephemeroptera) presentes en la montaña del Montseny (Noreste de la Península Ibérica). Se estudiaron conjuntamente la distribución de 4 morfoespecies de baétidos en 20 puntos de muestreo en tres cuencas a lo largo del gradiente altitudinal (350-1700 msnm) y la diversidad genética del gen mitocondrial Citocromo Oxidasa Subunidad I (*cox1*) de las dos especies comunes* B. alpinus *y* B. rhodani. *Se encontró una sustitución gradual de las morfoespecies dominantes a lo largo del gradiente altitudinal,* B. alpinus *se localizó en puntos de muestreo de mayor altitud y fue reemplazada por* B. rhodani *en puntos de muestreo de menor altitud.* Baetis melanonyx *y* Alainites muticus *se encontraron con baja*

abundancia a lo largo de todo el gradiente altitudinal y no apareció ningún patrón claro. Nuestra hipótesis a nivel de población era una estructuración genética asociada a la distancia geográfica y limitada por las montañas entre las tres cuencas estudiadas debido al efímero vuelo de los adultos. Sorprendentemente, los análisis genéticos de varianza molecular (AMOVA) y aislamiento por distancia (IBD) mostraron que la diversidad genética no estaba estructurada por la distancia en ninguna de las dos especies, y esto podría ser explicado por la escala espacial pequeña del trabajo y la poca altitud de las barreras topográficas entre las tres cuencas. El modelo Generalized Mixed Yule-Coalescent (GMYC) mostró que B. rhodani *tenía dos linajes genéticos diferenciados que coexistían en todos los puntos de muestreo. En conjunto, la diversidad de baétidos a nivel de comunidad estaba estructurada a lo largo del gradiente altitudinal, mientras que no se encontró ninguna estructura a nivel de población en la montaña del Montseny.*

Palabras clave: *Insectos acuáticos, complejos de especies crípticas, diversidad genética, modelo GMYC, morfoespecies, ensamblaje de especies, ecología de ríos.*

INTRODUCTION

Studies on the diversity patterns of freshwater macroinvertebrates at both community and genetic levels and at multiple spatial scales have contributed greatly to our understanding of the distribution and structure of freshwater diversity. One of the most important factors that determine freshwater diversity is the spatial hierarchical organisation of streams (Frissell *et al.*, 1986), which has structured diversity at multiple temporal scales from species diversification to population genetic structure (Múrria *et al.*, 2013). At the regional scale, the dispersion of aquatic organisms seems to be limited by drainage boundaries, which reduces connectivity between riverine invertebrate communities, thereby increasing β-diversity among catchments (Malmqvist, 2002). Limited connectivity also leads to some degree of intraspecific genetic differentiation across catchments for most freshwater species (Hughes *et al.*, 2009). The topographical barriers among catchments are higher in headwaters than in mid-order or lowland river sections, thus β- and γ-diversity are reduced from higher to lower reaches of rivers at both the species and genetic levels (Finn *et al.*, 2011; Múrria *et al.*, 2013). At the catchment scale, the longitudinal gradient of physical, chemical, and biological parameters from headwaters to the river mouth along river zonation (i.e., discharge, temperature, sediments) have driven the aquatic species distribution (Vannote *et al.*, 1980, Ward, 1998) and species diversification within lineages (Múrria *et al.*, 2012). At the local scale, hydraulics and variable flow forces produce habitat differentiations (i.e., riffles versus pools) that have promoted ecological niche segregation among species, with each micro-habitat harbouring different species (Statzner *et al.*, 1988; Statzner, 2008). Therefore, the spatial hierarchical organisation of streams constrains the evolutionary, demographic and ecological processes of freshwater organisms and contributes in varying degrees to determine diversity at the local scale and the links between the local diversity and the regional diversity pool (Ward & Tockner, 2001; Clarke *et al.*, 2008; Finn *et al*., 2011; Múrria *et al*., 2013).

Baetidae (Ephemeroptera) have a worldwide distribution and high species diversity. Their aquatic larvae are collector-gatherers and facultative scrapers, while short-life flying adults disperse and complete their life cycle outside the water (Merritt *et al.*, 2008). The life cycle of larval "downstream" drift and adult "upstream" short-time flight is comparable for several species of baetids and occurs on a scale of approximately 2 km in each direction and is limited by the geographical distance to a stream-scale (Monaghan *et al.*, 2002). However, local conditions and wind intensity, frequency and direction may increase this distance (Monaghan *et al.*, 2005a). Environmental gradients at the large spatial scale determine the distribution and geographical replacement of individual

baetid species. For example, the widespread *Baetis alpinus* (Pictet, 1843) is adapted to high oxygen concentrations and low temperatures and is mainly distributed at high altitudes across Europe (Monaghan *et al.*, 2002, Finn *et al.*, 2013, Finn *et al.*, 2014). In contrast, the widespread and abundant *Baetis rhodani* (Pictet, 1983) is more tolerant of warmer and low oxygen water and occurs in mid-order and lowlands throughout the West Paleartic area (Gattolliat & Sartori, 2008).

The established patterns of species distribution of Baetidae may change, because molecular studies showed evidence of distinct genetic lineages within the already recognised morphological species. For example, DNA-based studies of *B. rhodani* showed a considerable amount of variation within the mtDNA gene cytochrome c oxidase subunit I (*cox1*) that clustered in several differentiated lineages across Europe (Williams *et al.*, 2006, Lucentini *et al.*, 2011). For instance, there are three widely distributed haplogroups across the British Islands and Europe (I, III, and VII), whereas one haplogroup was restricted to distribution in Denmark-France-Italy (VI) and three haplogroups were only located in Switzerland-Italy (II, IV, and V) (Williams *et al.*, 2006, Lucentini *et al.*, 2011). Similarly, *B. alpinus* in the Iberian Peninsula (Finn *et al.*, 2013; Finn *et al.*, 2014) and *B. vernus* in Finland (Ståhls & Savolainen, 2008) showed high genetic divergence, suggesting also the existence of cryptic molecular speciation. Nevertheless, little is known of the distribution patterns of such cryptic species (i.e., coexistence in a site or species replacement along gradients) and their evolutionary history (Williams *et al.*, 2006; Lucentini *et al.*, 2011).

Genetic studies of baetids at the population level showed variable results associated mainly with river zonation. For example, the genus *Baetis* showed weak genetic substructuring for mid-order and lowland species (Schmidt *et al.*, 1995; Alp *et al.*, 2012,). In contrast high-altitude species showed limited gene flow and genetic

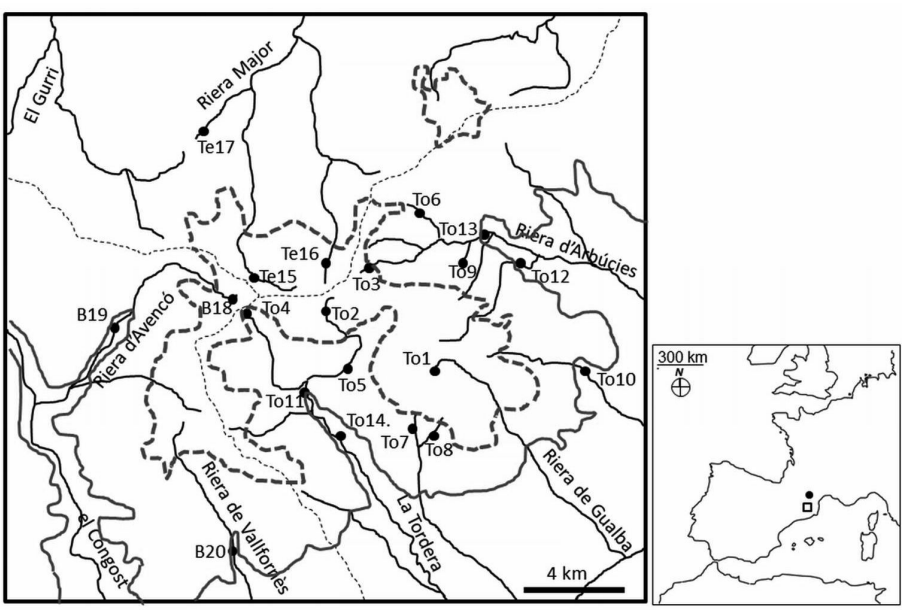

Figure 1. Location of the Montseny Mountain range (square) and sites sampled in Valira, Pyrenees (black dot) in western Europe. Geographical distribution of streams and rivers in the Montseny Mountain range, North-East Iberian Peninsula. Sampled sites are indicated by black circles. Fine dotted lines indicate drainage boundaries, the thick dotted line is an altitude of 1000 masl, and the thick line is an altitude of 500 masl. *Localización de la montaña del Montseny Mountain (cuadro) y el punto de muestreo en el río Valira, Pirineos (punto negro) en el oeste de Europa. Distribución geográfica de los arroyos y ríos de la montaña del Montseny, Noreste de la Península Ibérica. Los puntos de muestreo están indicados con círculos negros. La línea de puntos gris indica los límites de cada cuenca, la línea de puntos gris es la topográfica de 1000 m. s.n.m., la línea continua gris es la topográfica de 500 m.s.n.m.*

diversity structured among adjacent valleys (Monaghan *et al*., 2002; Peckarsky *et al.*, 2005; Finn *et al*., 2013). These contradictory results reveal the effects of the interacting factors determining genetic diversity, such as topographical barriers among and within catchments, the geographical scale of the study and differences in species traits.

Baetids represent an interesting group for biogeographical studies because of the short time of their adult stage and their limited ability to disperse. Our main objective was to describe the diversity patterns of baetids at both species and population levels within a single mountain range (Montseny, North-East Iberian Peninsula). For this reason, baetid species assemblages were assessed together with the genetic structure of *cox1* gene for two common species, the headwater *B. alpinus* and mid-order and lowland *B. rhodani*. We hypothesised a replacement of species along the altitudinal gradient due to the high altitudinal gradient within the Montseny Mountain range (350-1700 masl). At the population level, it is expected a higher gene flow among catchments for lowland species than for high-altitude species (Finn *et al.*, 2011; Múrria *et al.*, 2013). However, based on the small topographical barriers among headwater reaches, which are in close vicinity in this rounded mountain range, we expected a higher genetic structure for *B. rhodani* than *B. alpinus*. In addition, the genetic analysis may contribute to the assessment of the presence of differentiated genetic lineages within the studied species.

MATERIALS AND METHODS

Study sites

The Montseny Mountain range (North-East of the Iberian Peninsula) is located in the Mediterranean climate area (2°16′-2°33′E, 41°42′-41°52′N) and shows an altitudinal gradient that ranges from 350 to 1700 masl within only a few kilometres. Streams of this mountain range flow into three main catchments discharging directly to the sea (Tordera, Besòs, and Ter). These catchments are characterised by different catchment area size, total discharge inside the Montseny Mountain range (Ter > Besòs > Tordera) and river flow seasonality with variable temporal patterns in the discharge regime (http://ecobill.diba.cat/, Fig. 1). In May and early June of 2007, baetid specimens were collected from 20 pristine localities distributed in a small area of 20 km × 20 km throughout the three catchments, encompassing the entire range of environmental heterogeneity, geographic distribution and altitudinal gradient (Fig. 1, Table 1). The high altitude sites of the three catchments were in close vicinity, as shown Fig. 1, Table 1. Sites were grouped into three different altitudinal zone categories: high-altitude (> 1000 m, 7 sites), mid-altitude (1000 m-500 m, 6 sites), and low-altitude (< 500 m, 7 sites). The shortest aerial distance between sites was assigned on a map scale of 1:24000 (available at http://www.icc.cat). A few specimens of *B. alpinus* from the Valira del Nord stream, Pyrenees, Andorra (Fig. 1, Table 1), located 100 km north of the Montseny Mountain range, were sequenced to increase the genetic variability and resolution for running the species delimitation analysis.

Morphological species identification

Aquatic macroinvertebrates were collected from all in-stream habitats at each site using a kick net (250 μm mesh size) and similar kicking-effort between sites (approximately 30 minutes/site). The larvae of baetids were sorted in the field and directly preserved in absolute ethanol. In the laboratory, organic material of head capsules and abdomens were digested in 10 % potassium hydroxide at 85 °C, rinsed in distilled water, and dehydrated in ethanol (successively 70 % and 96 %). After digestion, each part was mounted on a slide with Euparal® solution to examine the mouthparts, legs, and abdomens with a light microscope. All individuals were identified at the taxonomic species level using several taxonomical keys (Müller-Liebenau, 1969; Belfiore, 1983; Puig, 1983a; Elliot *et al.*, 1988). The thorax of each animal was preserved in absolute ethanol and kept frozen for molecular analysis.

Analysis of the structure of morphospecies assemblages

The abundance of the four recorded species per site and similarity of species assemblages among sites were used to assess the structure of the species assemblages along the altitudinal gradient and geographical distance. First, the differences in species composition among the three altitudinal zone categories were examined. The differences among these categories were assessed by one-way Kruskal-Wallis tests due to the non-parametric distribution of the abundance of species. The similarity between species assemblages was measured by the Sørensen similarity index (Koleff *et al.*, 2003). A Mantel test was conducted to separately test the decays of similarity of the species assemblages considering altitudinal differences and geographical distance among the sites. All statistical analyses were run using "vegan" (Oksanen *et al.*, 2011) and "stats" libraries implemented in the R package (R Development Core Team, 2011).

DNA extraction, amplification, and sequencing

The DNA extraction of *B. alpinus* and *B. rhodani* was performed using standard phenol:chloroform protocol. Primers LCO-1490 and HCO-2198 (Folmer *et al.*, 1994) were used to amplify a

Table 1. Stream, catchment, location and species composition of sites sampled in the Montseny Mountain range together with catchment and site location for the site located in Valira stream (Pyrenees, Andorra). *Balp = Baetis alpinus*; *Brho = Baetis rhodani*, *Bmel = Baetis melanonyx*; *Amut = Alainites muticus*. *Río, cuenca, localización y composición de especies de los puntos de muestreo en la montaña del Montseny, y cuenca y localización del punto de muestreo en el río Valira (Pirineos, Andorra).*

							Species composition				
Site	Site name	Catchment	Sub-catchment	X_UTM	Y_UTM	Altitude (m asl)	*Balp*	*Brho*	*Bmel*	*Amut*	total
To1	Riera de Passavets	Tordera	Tordera	454522	4625774	1206	4	0	6	0	10
To2	Sant Marçal	Tordera	Tordera	451891	4628243	1060	9	0	0	0	9
To3	El Rigrós	Tordera	Riera d'Arbúcies	451254	4628661	1055	59	0	0	1	60
To4	Riera de la Bessa	Tordera	Tordera	447666	4627303	1013	45	0	0	3	48
To5	Tordera	Tordera	Tordera	450869	4626238	733	4	10	2	0	16
To6	Sot de Lliors	Tordera	Riera d'Arbúcies	452364	4630590	715	5	3	0	7	15
To7	Riera de Ciuret a	Tordera	Tordera	452865	4622880	708	0	11	1	0	12
To8	Riera de Ciuret b	Tordera	Tordera	453396	4622657	696	7	3	0	7	17
To9	Riera d'Arbúcies b	Tordera	Riera d'Arbúcies	455195	4630228	550	6	16	7	0	29
To10	Riera de Riells	Tordera	Riera d'Arbúcies	459754	4625967	489	0	0	0	12	12
To11	La Llavina	Tordera	Tordera	448218	4624971	460	13	14	5	0	32
To12	Riera de les Truites	Tordera	Riera d'Arbúcies	457210	4629644	430	0	21	0	0	21
To13	Riera d'Arbúcies a	Tordera	Riera d'Arbúcies	455722	4630825	425	3	36	2	0	41
To14	Piscines Montseny	Tordera	Tordera	449246	4623563	397	0	16	0	0	16
Te15	Torrent de Rentadors	Ter	Riera Major	446684	4629051	1293	3	19	0	10	32
Te16	Torrent de Collpregon	Ter	Riera Major	449564	4629282	1242	7	0	0	0	7
Te17	Riera de l'Erola	Ter	Riera Major	447278	4631967	800	0	53	0	0	53
B18	Collformic	Besòs	Congost	445899	4628321	1125	33	1	0	0	34
B19	Riera de Vallcàrquera	Besòs	Congost	440309	4625916	448	0	35	0	11	46
B20	Riera de Cànoves	Besòs	Mogent	448979	4617125	420	0	38	0	0	38
Valira	Andorra	Ebre	Valira del Nord	532357	3712300	1802					

fragment of the mtDNA gene *cox1*. When these primers failed for some individuals of *B. rhodani*, we used the primers C1-J-1718 and C1-N-2191 (Simon *et al.*, 1994). The Polymerase Chain Reaction (PCR) was carried out in 25 µl with 1 µl of templated DNA; 1 µl of dNTPs; 1 µl of each primer (10 µM); 2 µl of $MgCl_2$, 13.9 µl of ddH_2O; 5 µl of buffer; and 0.1 µl of Taq Polymerasa. After 5 minutes at 94 °C, the PCR mixture was subjected to 35 cycles of 1 minute at 42 °C, 40 seconds at 94 °C, and 1 minute at 72 °C, followed by a final extension step of 5 minutes at 72 °C. The PCR products were purified and sequenced using Big Dye v.3.1 Terminator technology (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 automated sequencer (Applied Biosystems). The intraspecific variability of the *cox1* gene is commonly used to determine gene flow among populations and to infer evolutionary histories of species, despite the inherent limitations of the mitochondrial *cox1* gene in some genetic studies because the *cox1* is a single, maternally inherited, haplotype marker (Avise, 2009). In addition, *cox1* is the most used molecular marker in freshwater biology studies at the population level for assessing genetic structure (Pauls *et al.*, 2014).

Molecular species delimitation

The Generalized Mixed Yule-Coalescent (GMYC) model (Fujisawa & Barraclough, 2013) was used to distinguish differentiated genetic lineages within *B. rhodani* and *B. alpinus* based on molecular criteria. This model tested for a change in branching rates at the species-population boundary and divided them into either inter-specific ("diversification") or intraspecific ("coalescent"). Each "diversification" branch was delimited and considered an "independently evolving" mtDNA cluster. The unique *cox1* haplotypes for an edited fragment of 578 bp for *B. alpinus* and 469 bp for *B. rhodani* located in Montseny Mountain range plus one *cox1* sequences for each haplogroup in Williams *et al.* (2006) and the out-group *B. vernus* (see GenBank accession numbers in Fig. 4) were used to perform the GMYC analysis. A maximum likelihood phylogeny was obtained with RAxML v.7.0.4 .(Stamatakis, 2006) under a GTR + Γ substitution model. The resulting topology was made ultrametric using the penalised likelihood as implemented in r8s v. 1.7 (Sanderson, 2003) with the optimal smoothing parameter selected by cross-validation. The GMYC analysis was conducted using the "Splits" package from the R package (Ezard *et al.*, 2009) with the "single threshold" option. The resulting independent evolutionary entities were considered separately in the population genetic analysis. Pairwise genetic distance (% of net nucleotide substitution per site) for haplogroups of *B. rhodani* was measured using DnaSP v5 (Librado & Rozas, 2009).

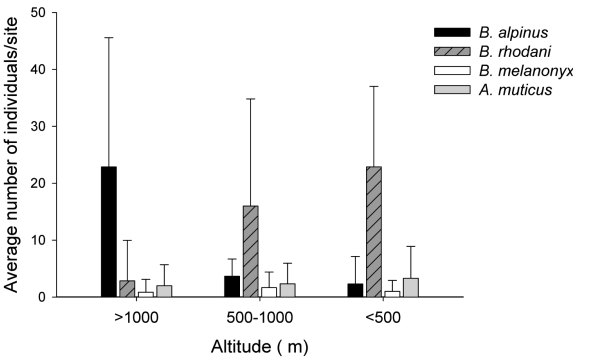

Figure 2. Mean and standard deviation of individuals per site for each morphological Baetidae species, grouped by three altitudinal categories: > 1000 m, 500 m-1000 m, and < 500 m. *Media y desviación estándar del número de individuos por punto de muestreo para cada especie morfológica de baétidos agrupados en tres categorías de altitud: > 1000 m, 500-1000 m y < 500 m.*

Analysis of intraspecific genetic structure

The analysis of the population genetic structure to infer dispersion and gene flow among populations was carried out using only GMYC entities that had more than 5 individuals sequenced in average per population (Table 2). The genetic diversity for each GMYC entity was calculated by polymorphic sites and nucleotide diversity π (i.e., the average number of nucleotide differences per site between two sequences) and its variance (Nei, 1987) using DnaSP v5 (Librado & Rozas, 2009). In order to test genetic isolation-by-distance (IBD), genetic divergence

D_{xy} between populations (i.e., the average number of nucleotide substitutions per site between populations, Nei, 1987) and the geographical distances between populations were correlated by a Mantel test using the "vegan" library (Oksanen *et al*., 2011) implemented in the R package (R Development Core Team, 2011). The molecular variance (AMOVA) was used to assess the level of gene flow among populations and to test at what spatial scale genetic variability was structured. The AMOVA was carried out by estimating Φ_{ST} using 10 000 random permutations and tested at three spatial hierarchic levels: among catchments; among populations in each catchment; and within populations. These analyses were conducted using ARLEQUIN v.3.5 (Excoffier *et al.*, 2005).

RESULTS

A total of 548 individuals of *Baetis* and *Alainites* were collected from 20 localities belonging to four different morphospecies: *B. rhodani* (276 individuals); *B. alpinus* (198 individuals); *B. melanonyx* (Pictet, 1843) (23 individuals); and *A. muticus* (Waltz & MacCafferty, 1994) (51 individuals) (Table 1). *Baetis alpinus* was mainly collected at high-altitude sites ($\chi^2 = 8.12$, $p = 0.02$). *Baetis rhodani* became the predominant morphospecies at low-altitude sites ($\chi^2 = 7.94$, $p = 0.02$) (Fig. 2). *Baetis melanonyx* and *A. muticus* exhibited low abundance and were evenly distributed across altitude categories ($\chi^2 = 1.45$, $p = 0.48$ and $\chi^2 = 0.04$, $p = 0.98$, respectively). All morphospecies were located

Table 2. Haplotype composition of the sampled sites for *B. alpinus* and *B. rhodani* haplogroup 1 (*B. rhodani1*) and haplogroup 2 (*B. rhodani2*). Sample size (Ind), number of individuals for each unique haplotype, and nucleotide diversity (π) in each of the populations are indicated. *Composición de los haplotipos de los sitios muestreados de* B. alpinus *y* B. rhodani *haplogrupo 1 (*B. rhodani1*) y haplogroup 2 (*B. rhodani2*). Se indica el tamaño de muestra (Ind), número de individuos para cada haplotipo único y diversidad nucleotidica (π) en cada población.*

Species	site	Ind	Haplotypes (ind)	Nucleotide diversity (π)
B. alpinus	Te16	5	1(1), 2(4)	0.00173
	B18	4	1(2), 2(2)	0.00173
	To2	6	1(6)	—
	To3	4	1(4)	—
	To4	6	1(2), 2(4)	0.00173
	Total	25		0.00173 ± 0.00087
	Valira	9	1(2), 3(6), 4(1)	0.00346
B. rhodani 1	Te15	5	1(3), 2(1), 4(1)	0.00711
	Te17	10	1(2), 4(8)	0.0064
	To11	6	1(3), 3(2), 4(1)	0.00569
	To13	6	1(2), 4(3), 6(1)	0.00995
	B19	7	1(2), 5(5)	0.01279
	B20	6	3(1), 4(5)	0.00213
	Total	40		0.00967
B. rhodani 2	Te15	3	7(3)	—
	Te17	2	7(2)	—
	To11	1	10(1)	—
	To13	4	7(2), 8(1), 10(1)	0.01421
	B19	2	9(1), 10(1)	0.0064
	B20	1	7(1)	—
	Total	13		0.01173

in all catchments, except for *B. melanonyx* that was present only along the Tordera stream. The sampled sites were mono-specific (7 sites), di-specific (6 sites) or tri-specifics (7 sites), and no sites contained the four species. Species co-occurrence, irrespective to species identity, was common. The distance decay of similarity of morphospecies assemblages was significant when considering only altitudinal differences among the sites ($r = 0.31$, $p < 0.01$), whereas it was decoupled along geographical distance ($r = 0.017$, $p = 0.47$) (Fig. 3).

The 53 individuals sequenced for *B. rhodani* resulted in 10 unique *cox1* haplotypes (GenBank accession numbers KM098089-KM098098). The GMYC analysis clustered these haplotypes in two independent evolutionary lineages: haplogroup 1 and haplogroup 2 (likelihood of null model: −23.64, maximum likelihood of GMYC model: −15.24, likelihood ratio test: $p = 0.0008$) (Fig. 4). These two GMYC entities for the morphospecies *B. rhodani* had high genetic divergences between them ($D_{xy} = 0.129$ with a total 54 polymorphic sites) and co-occurred in all sites in the Montseny Mountain range. The 40 individuals sequenced from the morphospecies *B. rhodani* haplogroup 1 resulted in six unique haplotypes that differed in 10 segregating sites. The 13 individuals sequenced from the *B. rhodani* haplogroup 2 resulted in 4 unique haplotypes with 11 segregating sites. Sequences of the *B. rhodani* haplogroup 1 clustered with the haplogroup VII described by Williams *et al*. (2006), whereas sequences of *B. rhodani* haplogroup 2 clustered with haplogroup III. These two haplogroups were closely related in our phylogeny (Fig. 4). The 25 individuals sequenced for *B. alpinus* in the Montseny Mountain range resulted in two haplotypes and 1 segregating site. The 9 specimens from Valira resulted in 3 haplotypes, and one of these was also located in the Montseny Mountain range (GenBank accession numbers KM098085-KM098088). The GMYC analysis clustered together these four unique haplotypes. Table 2 shows haplotype composition and nucleotide diversity (π) per site.

Baetis rhodani haplogroup 1 and *B. alpinus* showed similar intraspecific genetic structure characterised by significant differences within populations and among populations within a catchment and non-significant structure among the three catchments (Table 3). The IBD for both GMYC entities were non-significant (*B. rhodani* haplogroup 1: $r = 0.25$, $p = 0.26$; *B. alpinus*: $r = 0.12$, $p = 0.52$), indicating that each haplotype can be everywhere at the studied spatial scale.

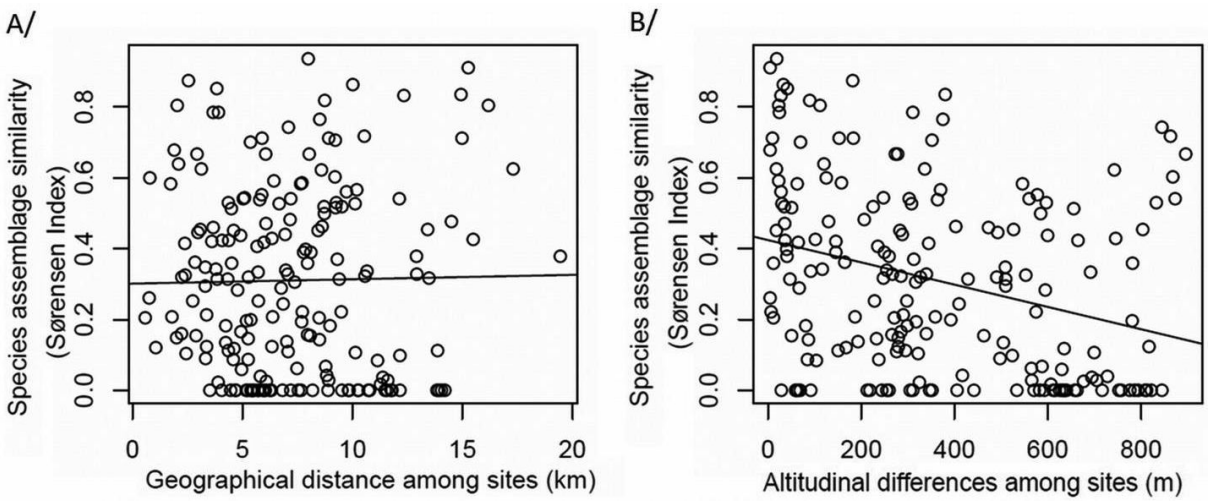

Figure 3. Distance decay of similarity (Sørensen index) for all pairwise comparisons of species assemblages of *Baetis* in the Montseny Mountain range plotted against (A) geographic distance (Mantel test, $r = 0.017$, $p = 0.47$) and (B) altitude difference (Mantel test, $r = 0.31$, $p = 0.001$). *Decaimiento de la similitud (índice de Sørensen) de todos los pares de comparaciones de ensamblajes de especies del género* Baetis *en la montaña del Montseny con (A) la distancia geográfica (Mantel test,* r = *0.017,* p = *0.47) y (B) la diferencia de altitud (Mantel test,* r = *0.31,* p = *0.001).*

DISCUSSION

The distance decays of similarity of species assemblages of baetids in the Montseny Mountain range were higher along the altitudinal gradient than the geographical distance, which indicated the relevance of the altitude on patterns of species distribution. As expected, the dominant *B. alpinus* preferred reaches at the highest altitudes and gradually was replaced by *B. rhodani* along the altitudinal gradient. In contrast, the less abundant *B. melanonyx* and *A. muticus* had less habitat preferences along river sections, which suggested a more neutral-geographical based distribution than niche-based distribution for these two species. This result

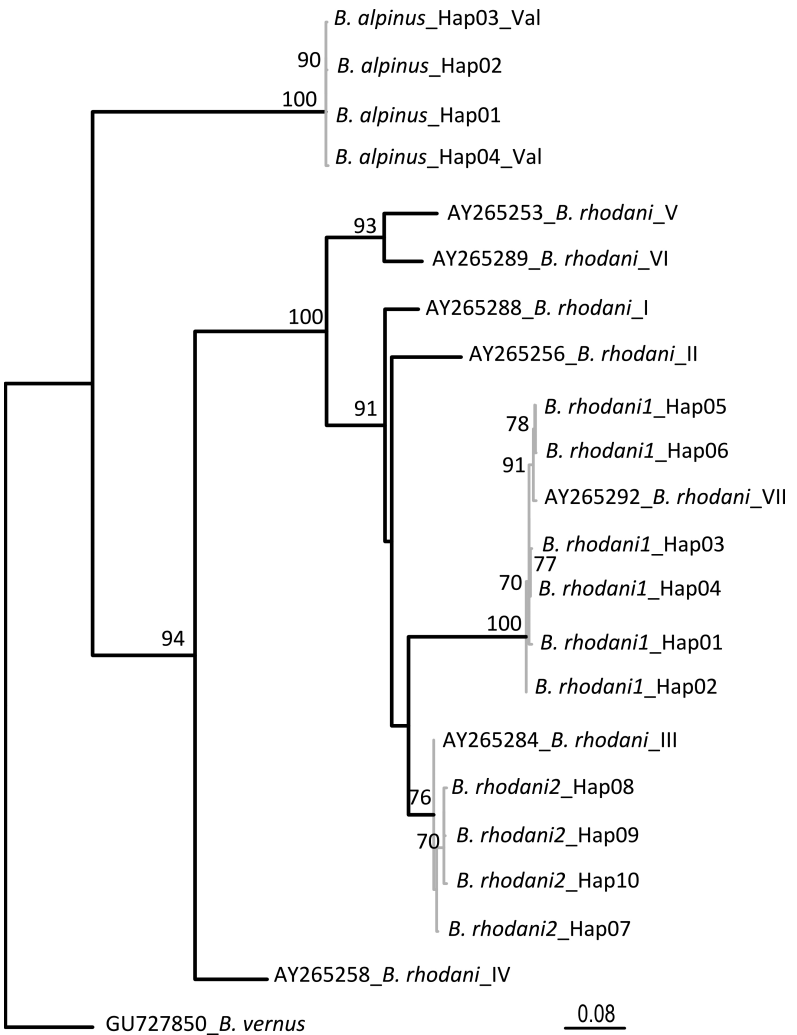

Figure 4. Phylogenetic relationship among *B. alpinus* and *B. rhodani* located in the Montseny Mountain range and Valira River, Pyrenees (Val) based on the Maximum Likelihood tree of unique *cox1* haplotypes. *Baetis vernus* was used as the out-group, and one *cox1* sequence for each *B. rhodani* haplogroup in Williams *et al*. (2006) was included (GenBank accession number indicated). Branch support is indicated as the maximum likelihood bootstrap (> 70). Gray phylogenetic clades are allocated to the coalescent by the GMYC model that corresponds to GMYC entities. *Relación filogenética utilizando un árbol de máxima verosimilitud entre los haplotipos únicos del gen* cox1 *de* B. alpinus *y* B. rhodani *encontrados en la montaña del Montseny y el río Valira, Pirineos (Val). Además fueron usados* B. vernus *como grupo externo y una secuencia de* cox1 *para cada grupo de haplotipos del trabajo de Williams* et al. *(2006). Se indica el suporte de cada rama del árbol con valores de bootstrap > 60. En rojo se marcan los clados filogenéticos que fueron diferenciados por coalescencia por el modelo GMYC que corresponden a entidades GMYC.*

is not surprising because environmental factors related to altitudinal gradients (e.g., oxygen concentration, temperature, flow velocity) have driven species diversification of freshwater invertebrates (Múrria *et al.*, 2012) and are among the most influential factors affecting the distribution of freshwater macroinvertebrate communities, including baetids (Vannote *et al*., 1980;, Alba-Tercedor, 1984; Puig, 1984b; Minshall, 1988; Alba-Tercedor *et al.*, 1991; Monaghan *et al.*, 2005b). The new contribution of this study to such well known patterns is the gradual replacement of the two dominant species and their co-occurrence in 8 of the 20 sampled sites, which discards a pattern caused by a clear border between their distributions as expected by habitat segregation (Ogitani *et al.*, 2011). The weak decay of similarity of species assemblages along the geographical distance was unexpected because the geographical distance was an important factor for structuring freshwater communities (Astorga *et al.*, 2012; Bonada *et al.*, 2012) and is particularly relevant for baetids, given the short period of time aerial dispersion. However, the dispersion of the studied species seemed to exceed the geographical range of our study, hence individuals of *B. melanonyx* and *A. muticus* arrived and established in all streams within the Montseny Mountain range. Individuals of *B. alpinus* and *B. rhodani* can establish if they can pass the environmental filters related to altitude.

The GMYC model provided evidence of two independent evolutionary lineages within *B. rhodani* for the gene *cox1*. These results confirmed the previous findings that morphology alone does not discover the true diversity hidden within *B. rhodani* characterised by a very distinct molecular differentiation (Williams *et al.*, 2006; Lucentini *et al.*, 2011). This prompts the need to reassess the morphological characters based on a neotype recently designed by Gattolliat & Sartori (2008). The evolutionary history of this species complex is unknown, so the co-occurrence of two GMYC entities in all sites in the Montseny Mountain range provides valuable data for future studies. A study that assessed the evolutionary history of three coexisting cryptic species of *B. rhodani* found reinforced genetic differentiation by both phylogenetic over-dispersion (i.e., coexisting species had different ecological niches to reduce competitive exclusion) and the temporal turnover of adult emergence during a year (Lucentini *et al*., 2011). In contrast, our phylogeny indicated that the haplogroups III and VII were phylogenetically closely related. These two haplogroups are widely distributed across Europe. Haplogroup III was previously recorded in Norway, Sweden, Denmark, Switzerland, and the UK. Haplogroup VII was located in Denmark, Ireland, Germany, Spain, Switzerland, and the UK. Therefore, further work is needed to elucidate the evolutionary history of *B. rhodani* cryptic species complex based on ecological niches, temporal segregation at the local scale and the geographical distribution of each distinct genetic lineage.

Table 3. Results of the hierarchical analysis of molecular variance (AMOVA) of haplotype divergence (Φ_{ST}) for *Baetis alpinus* and *Baetis rhodani* haplotype 1. *$p < 0.05$, **$p < 0.001$. *Resultados del análisis jerárquico de la varianza molecular (AMOVA) de la divergencia de los haplotipos (Φ_{ST}) para* Baetis alpinus *y* Baetis rhodani *haplotipo 1.* *p < *0.05,* **p < *0.001.*

B. alpinus	d.f.	Variance components	Percentage variation	Fixation indexs
Among catchments	2	−0.02	−9.25	$F_{CT} = -0.09$
Among populations within catchments	2	0.13	49.31	$F_{SC} = 0.45$*
Within populations	20	0.15	59.94	$F_{ST} = 0.4$*
B. rhodani1	d.f.	Variance components	Percentage variation	Fixation indexs
Among catchments	2	−0.12	−8.54	$F_{CT} = -0.08$
Among populations within catchments	3	0.55	39.7	$F_{SC} = 0.36$*
Within populations	35	0.95	68.84	$F_{ST} = 0.31$**

The intraspecific genetic structure indicated that adults of *B. alpinus* and *B. rhodani* dispersed across drainage boundaries within the Montseny Mountain range and most likely their dispersion exceeded the studied spatial scale. However, individual dispersion was limited and each species was restricted by altitude. For the headwater *B. alpinus*, the detected gene flow among the catchments may be explained by the short aerial distances between sites (max. distance 4 Km) and also by the low-altitude of the drainage boundaries. Similarly, connectivity among the midstream and lowland sites may explain the high gene flow detected for *B. rhodani*. This result was partially in contradiction with the expected patterns of higher dispersal limitation by topographic barriers for high-altitude than mid- and low-altitude species (Finn *et al.*, 2011; Múrria *et al.*, 2013). However, the small spatial scale of our study and the geographical distribution of catchments in very close proximity to one another, with the absence of a predominant wind direction, can explain the detected pattern. Other population genetic studies of baetids found the genetic structure within and among populations in a catchment were unrelated to the geographical distance (Schmidt *et al.*, 1995; Bunn & Hughes, 1997; Monaghan *et al.*, 2002; Peckarsky *et al.*, 2005). This finding is in agreement with the patterns observed in the present study. This pattern was attributed to "patchy recruitment" when local populations were genetically stochastic as a result of few matings (Schmidt *et al.*, 1995; Bunn & Hughes, 1997).

In conclusion, results at the species and population levels revealed different portions of the patterns of baetid diversity within the Montseny Mountain range. The altitudinal gradient has driven the replacement of the morphospecies of baetids, whereas this gradient and the associated topographic barriers among catchments appeared to be unrelated to the genetic population structure. Moreover, the studied spatial scale may hide the general patterns of baetid diversity detectable at larger spatial scales. Differentiated genetic lineages within *B. rhodani* co-occurred at local sites, hence their distribution was unrelated to habitat segregation. Overall, the patterns at different organisational levels revealed different portions of the diversity, associating habitat heterogeneity and biological traits with intraspecific genetic structure, lineage diversification and the processes of species assembly.

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