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Cryptic diversity and population structure at small scales: the freshwater snail *Ancylus* (Planorbidae, Pulmonata) in the Montseny mountain range

Jan N. Macher*, Martina Weiss, Arne J. Beermann and Florian Leese

Aquatic Ecosystem Research, University of Duisburg-Essen, Universitätsstraße 5, 45141 Essen, Germany

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Abstract – Anthropogenic impacts like intensified land use and climate change are severe threats to freshwater biodiversity and effective biodiversity monitoring is therefore one of the most urgent tasks. This is, however, often hampered by the lack of knowledge regarding the number and ecology of species. Molecular tools have shown many freshwater taxa to comprise morphologically cryptic species, which often occur in sympatry on a small geographic scale. Here, we studied the freshwater snail *Ancylus fluviatilis* (Müller, 1774) species complex in the Iberian Montseny Mountains. We hypothesized that (1) several species of *A. fluviatilis* sensu lato occur in the Montseny and (2) the different species seldom co-occur in syntopy due to different ecological demands or interspecific competition. We barcoded 180 specimens from 36 sites in the Montseny for the cytochrome c oxidase subunit I (COI) barcoding gene and molecularly identified two *Ancylus* species. These species seldom occurred in syntopy and a species distribution modelling approach showed differing bioclimatic preferences of the species. One species occurs mainly in cooler, higher altitude streams while the second species occurs in lower-altitude areas with higher temperatures. Tests of population structure showed that both species possibly do not disperse well in the study area and that populations within species might be adapted to certain bioclimatic conditions in different regions of the Montseny. Our results highlight the need to incorporate molecular techniques into routine monitoring programmes.

Key words: Cryptic species complex / barcoding / species distribution modelling / freshwater invertebrates

Introduction

Anthropogenic impacts like intensified land use and climate change are severe threats to biodiversity (Vörösmarty *et al.*, 2010; Steffen *et al.*, 2015). Therefore, monitoring the ongoing loss of biodiversity is highly important and many countries worldwide have established programmes to do so. However, the overall loss of biodiversity can only be monitored when accurate knowledge on the number, ecology, distribution and genetic diversity of species is available. For many areas and ecosystems, such information often either does not exist or is inaccurate: in recent years, the use of molecular methods has shown that the number of species is underestimated in many taxa (*e.g.* Amato *et al.*, 2007; Pfenninger and Schwenk, 2007; Adams *et al.*, 2014). This is especially true for freshwater ecosystems, which harbour a large number of morphologically indistinguishable or cryptic animal species (*e.g.* Pauls *et al.*, 2010; Weigand *et al.*, 2011; Weiss *et al.*, 2014). The ecology of most cryptic species is, however, rarely known since it has been studied only for relatively few taxa (*e.g.* Ortells *et al.*, 2003; Rissler and Apodaca, 2007; Lagrue *et al.*, 2014; Fišer *et al.*, 2015). This lack of knowledge poses a risk, since monitoring programmes and biodiversity assessments can come to inaccurate conclusions if species with different ecologies are treated as being identical regarding their ecological demands and thus, their suitability to indicate ecosystem health (*e.g.* Macher *et al.*, 2016). Also, extinction events and loss of biodiversity can go unnoticed. In this regard, using molecular methods to study freshwater species in mountain ranges is especially promising since many mountain ranges have been shown to harbour a large number of cryptic freshwater species (*e.g.* Pauls *et al.*, 2009; Katouzian *et al.*, 2016; Mamos *et al.*, 2016). Further, mountain ranges comprise many different habitats due to their topographic and climatic complexity, often leading to different species communities occurring within a small geographic area (Finn and Poff, 2005; Múrria *et al.*, 2014; Cauvy-Fraunié *et al.*, 2015). Topography and climate

*Corresponding author: jan.macher@uni-due.de

form natural barriers to dispersal and many taxa occurring in mountain ranges show phenotypic and genetic adaptation to the highly differing conditions along the altitudinal gradient (Liebherr, 1986; Bonin *et al.*, 2006; Keller *et al.*, 2013; Watanabe *et al.*, 2014), ultimately leading to mountain ranges being centres of high biodiversity. Using molecular methods to study species in such environments can help understand species diversity, ecology and their possible genetic adaptation to different habitats. Furthermore, it can make it possible to infer the potential loss of species and genetic diversity when the environment changes.

Here, we analyzed diversity and spatial distribution patterns in a common European stream invertebrate taxon, the freshwater limpet *Ancylus fluviatilis* (Müller, 1774) sensu lato, in the Montseny mountain range on the Iberian Peninsula. The Montseny is part of the Catalan pre-coastal range (North East Iberian Peninsula). It is located at the intersection of the warm and arid climate of the Mediterranean lowlands and the cooler and more precipitation-rich climate of the mountainous region reaching to the Pyrenees (Thuiller *et al.*, 2003). There are three main catchments within this area, all of which are characterized by steep altitudinal gradients ranging from less than 500 to 1706 m above sea level (masl) within approximately 10 kms and thus, comprising highly variable climatic conditions (Peñuelas and Boada, 2003; Jump *et al.*, 2007). We chose to study the widespread hololimnic freshwater limpet *A. fluviatilis sensu lato*, because it is known to comprise several cryptic species (Hubendick, 1970; Pfenninger *et al.*, 2003; Albrecht *et al.*, 2006). Of those species, Clade 1 and Clade 4 (Pfenninger *et al.*, 2003) potentially co-occur in the North East Iberian Peninsula, but have never been found in the area studied here. On a European scale, Pfenninger *et al.* (2003) found that the different *A. fluviatilis* clades differ significantly in their ecological demands: while Clade 1 prefers cooler areas with precipitation-rich summers, Clade 4 occurs mainly in arid, generally hotter areas. Both climatic conditions can be found in the Montseny, making it an ideal area for studying the number and distribution of species within the *A. fluviatilis* species complex. *A. fluviatilis sensu stricto* is able to disperse over longer distances (Cordellier and Pfenninger, 2008), *e.g.* by passive transport via waterbirds and other organisms (Rees, 1965), a phenomenon commonly found in other snails and freshwater molluscs (Rees, 1965; Boag, 1986; Van Leeuwen *et al.*, 2012). The current distribution of species in the *A. fluviatilis* species complex is thus expected to be limited by ecological demands (Cordellier and Pfenninger, 2008).

In this study, we expected (1) to find morphologically cryptic species of the *A. fluviatilis* species complex in the Montseny mountain range, and (2) that different *A. fluviatilis* species seldom co-occur in syntopy due to different ecological demands or interspecific competition.

To test these hypotheses, we firstly analyzed the partial mitochondrial cytochrome c oxidase subunit 1 gene (COI) to determine the number and distribution of *A. fluviatilis* species found in the Montseny. Secondly, we used a modelling approach based on bioclimatic variables to identify variables that might help to explain the occurrence of species. In addition, we performed population genetic analyses to find possible intraspecific partitioning of genetic variation.

Materials and methods

Sampling

Sampling was performed in the Montseny mountain range (located on the North East Iberian Peninsula, Fig. 1a) and the direct surrounding area in September 2013. A total of 44 sites were checked for the presence of *A. fluviatilis*, which was found in 36 of these sites (see Table A1 for coordinates). The three main catchments (Tordera, Besòs, Ter) and an altitudinal gradient from 120 to 1295 masl (Fig. 1b) were covered by the sampling. *A. fluviatilis* specimens were collected by hand picking specimens from stones in the streams. All specimens were immediately stored in 70% ethanol, later transferred to 96% ethanol and stored at 4 °C until further analysis.

A sampling permit for protected areas (Parque Natural del Montseny) was obtained from the park management prior to sampling.

DNA extraction, amplification and sequencing

DNA was extracted from muscle tissue of 180 specimens (5 per site, 36 sampling sites) using a salt extraction protocol (Weiss & Leese, 2016; modified from Sunnucks and Hales, 1996) (Overview of the samples: Table A1). A 658 bp-fragment of the barcoding gene COI was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). The polymerase chain reaction (PCR) mix was prepared using the following protocol: 1 × PCR buffer, 0.2 mM dNTPs, 1 µL of DNA template, 0.025 U.µL^{-1} Hotmaster Taq (5 PRIME GmbH, Hilden, Germany) and 0.5 µM of each primer. The mix was filled up to 25 µL with sterile H_2O and placed in a thermocycler for amplification. PCR settings for the COI amplification were: initial denaturation at 94 °C for 2 min; 36 cycles of denaturation at 94 °C for 20 s, annealing at 46 °C for 30 s, extension at 65 °C for 60 s; final extension at 65 °C for 5 min. Further, 9 µL of the PCR product were purified enzymatically with 10 U of Exonuclease I and 1 U Shrimp Alkaline Phosphatase (Thermo Fisher Scientific, Waltham) by incubating at 37 °C for 25 min and a denaturation step at 80 °C for 15 min. Bidirectional sequencing was performed on an ABI 3730 sequencer by GATC Biotech (Constance, Germany).

Species delimitation

Raw reads were assembled and edited using Geneious 6.0.5 (Biomatters). The MAFFT plugin (v. 7.017, Katoh

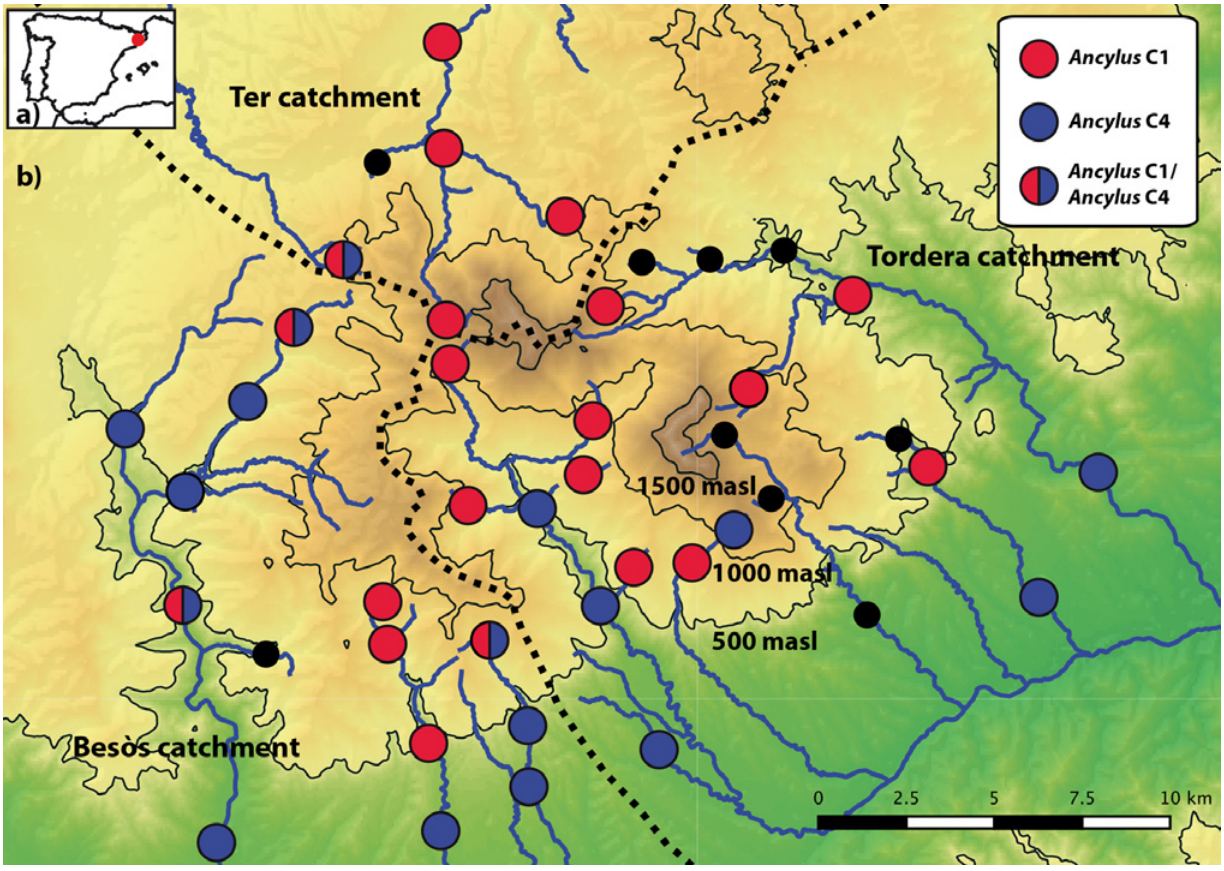

Fig. 1. (a) Location of the Montseny mountain range on the Iberian Peninsula (red circle); (b) map showing the occurrence of *Ancylus fluviatilis* Clade 1 and Clade 4 in the Montseny. Red dots indicate presence of *A.fluviatilis* C1, blue dots presence of *A. fluviatilis* C4. Mixed blue and red dots indicate the presence of both species at one sampling site. Black dots indicate absence of *Ancylus fluviatilis*. Catchment boundaries are shown as dashed black lines. Rivers are shown as blue lines.

and Standley, 2013) in Geneious was used to compute a multiple sequence alignment (automatic algorithm selection, default settings). The final length of the cropped alignments was 655 bp. The alignment was translated into amino acids using translation table 5 (invertebrate mitochondrial codon usage table) to make sure that no stop codons were present. The best model of evolution for further analyses of the data was selected with jModeltest 2.1.2 (Darriba *et al.*, 2012) (default settings). Fabox (Villesen, 2007) was used to collapse sequences into haplotypes. PopART (v.1, Leigh and Bryant, 2015) was used to create statistical parsimony haplotype networks (Clement *et al.*, 2000) with a 95% connection limit.

Two approaches were used to test for the presence of cryptic species in *Ancylus fluviatilis sensu lato*: first, the tree-based Generalized Mixed Yule Coalescent (GMYC) approach (Pons *et al.*, 2006) and second, the automated distance-based barcode gap determination approach (ABGD, Puillandre *et al.*, 2012). An ultrametric tree for all unique COI haplotypes was calculated for the GMYC analyses using BEAST v.1.8.0 (Drummond *et al.*, 2012). BEAST was run for 10 million MCMC generations, sampling every 100th tree and using both standard coalescent and the GTR + G sequence evolution model. Tracer v.1.6 (Rambaut *et al.*, 2013) was used to test for effective sampling size (ESS) and convergence of parameters. TreeAnnotator v.1.8 (Rambaut and Drummond, 2013) was used to generate a linearized consensus tree, discarding the first 3000 trees as burn-in. R v. 3.1.1 (R Core Team, 2015) was used for analysis of the resulting tree with 'SPLITS' (Species Limit by Threshold Statistics) (Ezard *et al.*, 2009) with the single threshold model to test for the presence of multiple species within the dataset. The second approach used for species delimitation was ABGD. Default settings were used, with Pmax = 0.1 and the K2P-model of distance correction (Kimura, 1980), as this is the common approach in DNA barcoding studies. Once the number of genetic clades within the dataset was determined, specimens from each group were blasted against the Barcode of Life database (Ratnasingham and Hebert, 2007) to verify species assignment. The *A. fluviatilis* sequences from Pfenninger *et al.* (2003, accession numbers AY350509 – AY350525) were downloaded and aligned with the sequences generated in this study to verify assignment of sequences to one of the known cryptic species. Alignments for each species were created with Geneious and networks were computed with popArt as described above. QGIS (v 2.8, available from www.qgis.org) was used to create distribution maps.

Bioclimatic variables analyses

The bioclimatic preferences of species were modelled using a maximum entropy method in MaxEnt 3.3.3e (Phillips and Dudík, 2008), which has been shown to work well with small sample sizes (Pearson *et al.*, 2007). The region modelled was part of the North East Iberian Peninsula (area between coordinates 42°18′N, 1°48′E, 41°06′N, 3°00′E; WGS84). A total of 19 climate layers in the 30 arc-seconds grid were obtained from WorldClim (Hijmans *et al.*, 2005) and resampled to a cell size of 800×800 m^2. WorldClim datasets are based upon standard meteorological precipitation and temperature measurements, which are transformed into bioclimatic variables (Hijmans *et al.*, 2005). These datasets are commonly used as predictor variables in species distribution modelling. To avoid using highly non-independent variables in the analyses and thus omit overfitting of models, a Spearman's rank correlation tests was performed across all pairs of variables using ENMtools (Warren *et al.*, 2010) and R (R Core Team 2015). The correlation coefficient values used as thresholds beyond which values were treated as independent were 0.7, 0.8 and 0.9. All species presence points were used to build the model; 25% of the presence points were retained for training the model. All models were run 10 times with random partitioning of training and validation points. The accuracy of all computed models was evaluated with the area under receiver operation characteristic curve, which was also used to choose models for use in further analyses (Boubli and De, 2009). Range overlap and bioclimatic niche overlap were computed by using Schoener's D statistics as implemented in ENMtools. The values range from 0 (meaning no bioclimatic niche overlap) to 1 (identical range and bioclimatic niche, respectively).

Geographic partitioning of genetic variation

Φ_{ST} values as an indicator of population subdivision were calculated separately for all species found in the *A. fluviatilis* species complex using Arlequin software (v. 3.11, Excoffier *et al.*, 2005). Φ_{ST} was chosen since it takes population history (number of mutations between haplotypes) into account. For analyses of population differentiation between altitude zones, populations of all species were classed in three groups: < 500, 500–1000 and > 1000 masl as in Múrria *et al.* (2014). For analyses of population differentiation between catchments, populations were classed as belonging to one of the three catchments (Tordera, Besòs, Ter) and Φ_{ST} values between groups were calculated. The Bayesian Clustering software GENELAND (v.4.0.5 as implemented in R; Guillot *et al.*, 2005) was used to further analyse population structure in the found *A. fluviatilis* species. Fabox was used to extract variable sites from alignments of the found species and PGDSpider (Excoffier and Lischer, 2010) was used to convert these alignments files into the GENELAND format. The settings used for running GENELAND were: five independent runs with a maximum of 10 populations, 300 nuclei, 10 million iterations, thinning interval of 10 000, resulting in 1000 retained trees. The first 200 trees were discarded as burn-in.

Results

Molecular species delimitation

Ancylus was found in 36 out of 44 sampled sites (Fig. 1b, see Table A1 for coordinates). A total of 180 specimens were analysed for the COI barcoding gene. The 655 bp alignment had 54 (9.2%) variable sites and a GC content of 29.6%. The null model of a single species was rejected both with the GMYC (likelihood ratio for single threshold model: 31.68, $P < 0.001$) and the ABGD approach (Pmax 0.1%). Both ABGD and GMYC suggested the presence of two groups in *A. fluviatilis sensu lato*. Blast searches against the Barcode of Life database assigned all sequences of both molecularly identified clades to either *A. fluviatilis* Clade 1 or *A. fluviatilis* Clade 4, both submitted by Pfenninger *et al.* (2003). Alignment of the generated sequences with those obtained from Genbank clustered 102 specimens with *A. fluviatilis* Clade 1, while 78 sequences clustered with *A. fluviatilis* Clade 4. Both clades were defined by Pfenninger *et al.* (2003). Clade 1 corresponds to *A. fluviatilis sensu stricto*, while Clade 4 is a yet undescribed species with circum-Mediterranean distribution (Pfenninger *et al.*, 2003). The maximum genetic distance between specimens clustering with *A. fluviatilis* Clade 1 was found to be 0.5%. The minimum distance of *A. fluviatilis* specimens from the Montseny to specimens of *A. fluviatilis* Clade 1 from Pfenninger *et al.* (2003) was 0.3%, while the maximum distance was 3.1%. The minimum genetic distance between specimens of *A. fluviatilis* Clade 4 from the Montseny to those from Pfenninger *et al.* (2003) was 2.4%, while the maximum distance was 2.7%. The maximum genetic distance between the two clades found in the Montseny was 7.8% (all uncorrected pairwise distances). The identified molecular clades are referred to as *Ancylus* C1 and *Ancylus* C4.

Bioclimatic characterization

For the modelling approach based on the 19 bioclimatic variables obtained from WorldClim, 3, 6 and 10 variables were retained after Spearman's Rank Correlation tests with thresholds of 0.7, 0.8 and 0.9, respectively. The 6 variable model with a Spearman's Rank Correlation threshold of 0.8 resulted in good area under the receiver operating characteristic curve (AUC) values for both species (*Ancylus* C1: 0.96, *Ancylus* C4: 0.89), thus this model was chosen for all further analyses to mediate between lower variable correlation and higher model fitting (see Table A2 for all AUC values and variables). The best explaining bioclimatic variables for the occurrence of *Ancylus* C1 were the variables bio7 ("Temperature

Annual Range") and bio19 ("Precipitation of Coldest Quarter"). Occurrence of *Ancylus* C4 was best predicted by the variables bio7 ("Temperature Annual Range") and bio15 ("Precipitation Seasonality") (Table A2). Bioclimatic niche overlap for *Ancylus* C1 and *Ancylus* C4 was 0.643, the range overlap computed for occurrence likelihoods of > 50% was 0.728 (Table A3).

Geographic partitioning of genetic variation within *Ancylus* species

Both *Ancylus* C1 and *Ancylus* C4 were found in all three studied catchments of the Montseny. *Ancylus* C1 was found at 22 sampling sites and *Ancylus* C4 at 17 sampling sites. Both species occurred in syntopy at 4 sampling sites (11.43%; < 500 masl zone: 1 site, 500–1000 masl zone: 2 sites, > 1000 masl zone: 1 site; Fig. 2b). *Ancylus* C1 was found more often at higher altitude sites (332–1295 masl, median 665 masl) than *Ancylus* C4, which was more often found at lower altitude sites (120–1172 masl, median 440 masl).

Ancylus C1 showed significant population differentiation between the Tordera and Besòs (Φ_{ST}: 0.322, $P = 0.00001$) and the Tordera and Ter catchment (Φ_{ST}: 0.167, $P = 0.0001$) (Table A4). The most common haplotype (HC1_1) was found at 14 sites and in all three catchments (Tordera: 5 sites, Besòs: 4 sites, Ter: 5 sites) (Fig. 2a). HC1_2 was found at 5 sites, of which 4 are located in the Tordera catchment and 1 in the Ter catchment. HC1_3 was found at 9 sites and all three catchments (Tordera: 6 sites, Besòs: 2 sites, Ter: 1 site). Haplotypes C1_4, C1_5, C1_6, C1_7 and C1_8 were found in a maximum of two specimens each and at single sampling sites only. Significant population differentiation in *Ancylus* C1 was also found between the altitude zones < 500 and > 1000 masl (Φ_{ST}: 0.343, $P = 0.00001$) and between 500–1000 and > 1000 masl (Φ_{ST}: 0.274, $P = 0.0001$) (Table A4). GENELAND found three geographically defined groups in *Ancylus* C1. Group 1 contains the sampling sites dominated by HC1_2, mainly lying above 1000 masl (4 out of 5 sites). Group 2 contains sampling sites mainly dominated by HC1_1 (Populations in all altitude zones, but mainly (9 sites) in the 500–1000 masl zone). Group 3 contains sampling sites mainly dominated by HC1_3 (500–1000 masl zone: 5 sites; < 500 masl zones: 3 sites) (Fig. 2a). A maximum of three substitutions were found between haplotypes of *Ancylus* C1 (Fig. 2b).

Ancylus C4 showed significant population differentiation between the Tordera and Besòs (Φ_{ST}: 0.408, $P = 0.0001$) and the Besòs and Ter catchments (Φ_{ST}: 0.876, $P = 0.00001$), respectively. The most common haplotype (HC4_3) was found at 13 sampling sites (Besòs catchment: 9 sites, Tordera catchment: 4 sites). The second most common haplotype (HC4_1) was found in all three catchments (Tordera catchment: 4 sites, Ter catchment: 1 sites, Besòs catchment: 1 site). The haplotypes C4_2, C4_4 and C4_5 were found at single sites and in single specimens only (Fig. 2c). In *Ancylus* C4, significant population differentiation was found between populations in the < 500 masl and the 500–1000 masl zone (Φ_{ST}: 0.335, $P = 0.009$) as well as between the < 500 and > 1000 masl zone (Φ_{ST}: 0.667, $P = 0.00001$). GENELAND found two geographically defined groups in *Ancylus* C4. Group 1 contains four sampling sites located in the northern and eastern parts of the study area, mainly dominated by haplotype C4_1 (> 1000 masl zone: 2 sites, < 500 masl zone: 2 sites). Group 2 contains 13 sampling sites in the mid and western part of the Montseny, mainly dominated by haplotype C4_3 (< 500 masl zone: 10 sites, 500–1000 masl zone: 3 sites) (Fig. 2c). A maximum of two substitutions were found between haplotypes of *Ancylus* C4 (Fig. 2d).

Discussion

In this study, we investigated the number and distribution of *A. fluviatilis sensu lato* in the Montseny mountain range on the Iberian Peninsula. Our first expectation was that cryptic species of *A. fluviatilis sensu lato* are present in the study area. This expectation was met by the discovery of two molecular clades occurring in the Montseny. Both *A. fluviatilis* clades were initially delimited by Pfenninger *et al.* (2003). While *Ancylus* C1 corresponds to *A. fluviatilis sensu stricto*, *Ancylus* C4 is a yet undescribed species with a Mediterranean distribution, ranging from Portugal through the Southern Iberian Peninsula to Italy (Pfenninger *et al.*, 2003; Albrecht *et al.*, 2006). Although the distribution and ecology of cryptic species within *A. fluviatilis sensu lato* are roughly known on a European scale, our study is the first that allows assessment of the differences regarding small-scale distribution, population structure and bioclimatic preferences of two of them in the same region, allowing for a better understanding of species ecology. In the future, this might help with identification of species and improving stream quality assessments by making it possible to assign correct ecological traits to species.

Our second expectation was that different clades within *A. fluviatilis sensu lato* rarely occur in syntopy, due to possibly different ecological demands or competition. Our results show that this might be true, as *Ancylus* C1 and *Ancylus* C4 occurred in syntopy in only 11.43% of the sites and showed altitudinal partitioning: *Ancylus* C1 was found more often at higher altitudes (median 665 masl), while *Ancylus* C4 was found more often at lower elevations (median 440 masl). Elevation is a good indicator for bioclimatic and environmental conditions such as temperature (− 0.65 °C per 100 m increase in altitude on average; Dodson and Marks, 1997) and flow velocity (due to steeper mountain slopes at higher altitudes). Thus, the observed pattern might hint at different bioclimatic preferences of the two species. However, due to the limited number of samples and the fact that both clades share 71% of the altitudinal range, it cannot be excluded that the pattern observed is mainly due to the limited sampling. The MaxEnt modelling approach results support the observed pattern, which suggests that *Ancylus* C1 occurs

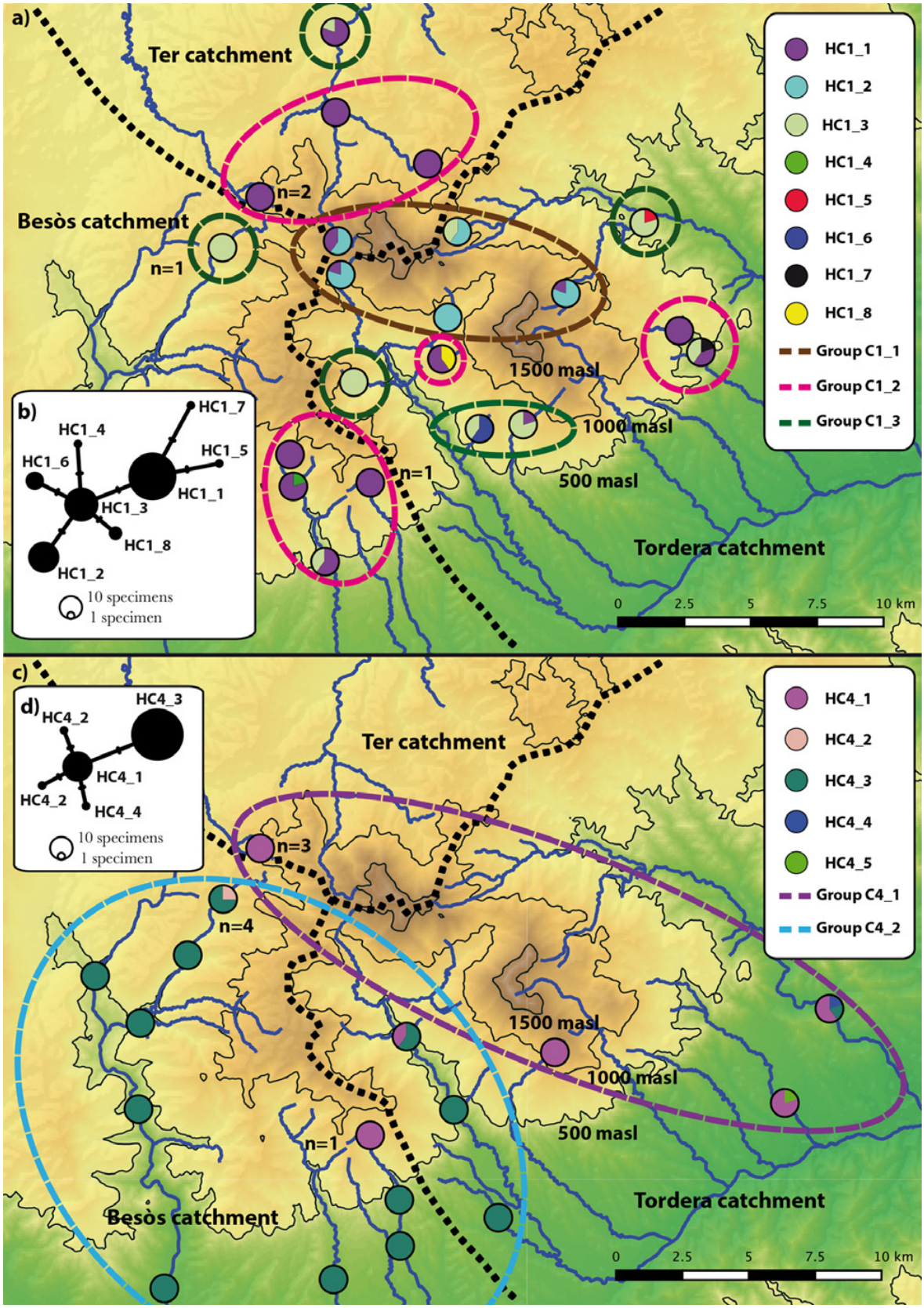

Fig. 2. (a) + (c) Map of the study area indicating the number of studied *Ancylus fluviatilis* specimens per sampling site and haplotypes found. Number of specimens per sampling site is 5, unless stated otherwise. GENELAND groups are shown as coloured dashed lines and catchment boundaries as black dashed lines. (b) + (d) Statistical Parsimony Network of *A. fluviatilis* Clade 1 and *A. fluviatilis* Clade 4 haplotypes, respectively. Dots represent sampled haplotypes, bars represent number of substitutions between haplotypes.

in areas with lower mean annual temperature and strong precipitation during the cooler season of the year, while *Ancylus* C4 occurs in areas with strong precipitation seasonality and higher mean annual temperature. These findings correspond to those of Pfenninger *et al.* (2003) who, on a European scale, found *Ancylus* C1 to mainly inhabit cooler, precipitation-rich areas and *Ancylus* C4 to mainly inhabit hotter, seasonally drier areas. Similar patterns have been observed in other aquatic invertebrate species (*e.g.* Monaghan *et al.*, 2005; Múrria *et al.*, 2014). Closely related species co-occurring in the same area often inhabit different habitats due to different bioclimatic preferences or the competition exclusion principle (*e.g.* Fišer *et al.*, 2015). In southern Europe, cooler and precipitation-rich conditions are mainly found at higher elevations, suggesting that *Ancylus* C1 might be close to the southern border of its distribution range in the Montseny. For aquatic species, higher precipitation means that more water is available during at least parts of the year, which is especially important in generally dry areas or areas with high precipitation seasonality. It appears thus possible that *Ancylus* C1 relies more on constant flow in the streams it inhabits, while *Ancylus* C4 might be able to cope with intermittent conditions in streams and generally higher water temperatures in lowland streams. This is especially important in the light of future climate change and ongoing human activities such as water abstraction, which might greatly alter temperature and precipitation patterns, ultimately changing flow regimes and thus possibly driving some species into local extinction while giving other species the possibility to colonize new habitats. Knowing the number and ecology of species in an area allows tracking the impact of such changes and preventing the loss of species. Tests of genetic differentiation, albeit based on a limited number of specimens and on the mitochondrial, maternally inherited COI gene only, hint at both *A. fluviatilis* clades found in the Montseny showing a division into lower altitude and higher altitude populations that differ genetically. *Ancylus* C1 populations from above 1000 masl significantly differed from populations below 1000 masl, which was also confirmed by the GENELAND analyses. *Ancylus* C4 populations from below 500 masl differed significantly from populations located above that altitude, likely due to the fact that the common haplotype C4_3 was found mainly below 500 masl. GENELAND, however, did not confirm the existence of a haplotype group corresponding with altitude in *Ancylus* C4, probably due to the low number of specimens from above 500 masl sampling sites. Although the limited amount of data per catchment and altitudinal zone does not allow us to draw definite conclusions, a pattern of genetic differentiation between higher-altitude and lower-altitude populations could hint at two phenomena, possibly in combination: one possibility is that specimens of *A. fluviatilis sensu lato* are weak dispersers that rarely migrate over longer distances within and between streams; thus over time, populations diverge genetically due to limited gene flow. Weak dispersal capabilities have been found in other freshwater gastropods (Kappes and Haase, 2012). However, at least *A. fluviatilis* sensu stricto has been shown to disperse over longer distances (Cordellier and Pfenninger, 2008), *e.g. via* passive transport (Rees, 1965). The second explanation could be that populations from higher and lower altitudes differ genetically due to selective processes, having adapted to the different bioclimatic conditions. This corresponds to findings of other studies that found high-altitude populations of species to be genetically differentiated and potentially adapted to harsher bioclimatic conditions (*e.g.* McCulloch *et al.*, 2009; Dussex *et al.*, 2016). Fast adaptation to bioclimatic conditions has been found in *Ancylus* C1 (*A. fluviatilis sensu stricto*) (Cordellier and Pfenninger, 2008), possibly making this explanation for the pattern observed in the Montseny more likely. Further studies based on nuclear markers and addressing the ecologies of both species need to be conducted, ideally using a combination of laboratory and field experiments. Overall, it remains possible that the patterns observed are mainly due to generally low genetic variation within the species and the relatively low number of sequenced specimens. The data are based on a limited number of specimens only and need to be interpreted with care. Studies involving nuclear markers and possibly a greater number of specimens per site are needed to verify the observed patterns of genetic variation on a geographically small scale. However, the patterns of population differentiation between altitudinal zones and catchments demonstrate the need to take population structure into account when planning to protect species and ecosystems. Environmental changes in the lower or higher altitude zones or in catchments might lead to the extinction of genotypes adapted to the local conditions. The resulting lower levels of genetic diversity possibly limit the adaptive potential of species and their ability to respond to environmental changes, ultimately leading to loss of genetic diversity in the species as a whole and increasing the risk of extinction (Bálint *et al.*, 2011). Our study highlights the importance and potential of using molecular techniques to study species diversity and genetic diversity of species. Molecular studies can greatly help to understand the impact of climate change and other human stressors on biodiversity (Bálint *et al.*, 2011; Hampe and Jump, 2011; Pauls *et al.*, 2013; Macher *et al.*, 2016). Here, we confirmed the occurrence of the two previously discovered potential cryptic species within a common freshwater taxon and genetic divergence within species on a small geographic scale. Our results show that patterns of genetic diversity, connectivity and bioclimatic preferences can be different even between closely related species, a fact that should be considered in biomonitoring and conservation plans. Knowledge of freshwater species diversity and ecological preferences is also important due to the fact that many bioassessment and monitoring programs worldwide rely on species occurrence data as a metric to measure ecosystem quality (*e.g.* Carter and Resh, 2001; Stark 2001; Haase *et al.*, 2004) and the indication value of species is mostly derived from their ecological demands. Generally, not considering

molecular data and cryptic species ecologies in monitoring programs can lead to strongly biased assessment results and ultimately to unsuitable management plans. The latter is especially problematic as management programmes often need to focus on protecting the maximum amount of biodiversity with the least amount of monetary effort. Using molecular methods can help to identify and effectively protect species and intraspecific diversity.

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Data accessibility

CO1 DNA sequences:

GenBank accession numbers for *Ancylus* Clade 1: KY012061-KY012162. Will be available upon publication and numbers will be added accordingly.

GenBank accession numbers for *Ancylus* Clade 4: KY012163-KY012240.

Conflict of Interest

None to declare.

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Appendix

Table A1. Clade assignments, specimen IDs, sampling site IDs and sampling site coordinates (Lat/Long, WGS84) for *Ancylus fluviatilis* specimens from the Montseny.

Ancylus fluviatilis clade	Specimen ID *Ancylus fluviatilis*	Sampling site ID	Latitude	Longitude
1	A_Di04_1	Di4	2.44	41.75
1	A_Di04_2	Di4	2.44	41.75
1	A_Di04_3	Di4	2.44	41.75
1	A_Di04_4	Di4	2.44	41.75
1	A_Di04_5	Di4	2.44	41.75
1	A_Di05.1_1	Di5.1	2.42	41.75
1	A_Di05.1_2	Di5.1	2.42	41.75
1	A_Di05.1_3	Di5.1	2.42	41.75
1	A_Di05.1_4	Di5.1	2.42	41.75
1	A_Di05.1_5	Di5.1	2.42	41.75
1	A_Di08_1	Di8	2.51	41.78
1	A_Di08_2	Di8	2.51	41.78
1	A_Di08_3	Di8	2.51	41.78
1	A_Di08_4	Di8	2.51	41.78
1	A_Di08_5	Di8	2.51	41.78
1	A_Di10_1	Di10	2.52	41.77
1	A_Di10_2	Di10	2.52	41.77
1	A_Di10_3	Di10	2.52	41.77
1	A_Di10_4	Di10	2.52	41.77
1	A_Di10_5	Di10	2.52	41.77
1	A_Do01_5	Do1	2.3	41.81
1	A_DoH_1	DoH	2.4	41.77
1	A_DoH_2	DoH	2.4	41.77
1	A_DoH_3	DoH	2.4	41.77
1	A_DoH_4	DoH	2.4	41.77
1	A_DoH_5	DoH	2.4	41.77
1	A_Fr01_1	Fr01	2.36	41.81
1	A_Fr01_2	Fr01	2.36	41.81
1	A_Fr01_3	Fr01	2.36	41.81
1	A_Fr01_4	Fr01	2.36	41.81
1	A_Fr01_5	Fr01	2.36	41.81
1	A_Fr02.1_3	Fr2.1	2.32	41.83
1	A_Fr02.1_5	Fr2.1	2.32	41.83
1	A_Fr04_1	Fr4	2.36	41.85
1	A_Fr04_2	Fr4	2.36	41.85
1	A_Fr04_3	Fr4	2.36	41.85
1	A_Fr04_4	Fr4	2.36	41.85
1	A_Fr04_5	Fr4	2.36	41.85
1	A_Fr06_1	Fr6	2.36	41.88
1	A_Fr06_2	Fr6	2.36	41.88
1	A_Fr06_3	Fr6	2.36	41.88
1	A_Fr06_4	Fr6	2.36	41.88
1	A_Fr06_5	Fr6	2.36	41.88
1	A_Fr07_1	Fr7	2.38	41.9
1	A_Fr07_2	Fr7	2.38	41.9
1	A_Fr07_3_	Fr7	2.38	41.9
1	A_Fr07_4	Fr7	2.38	41.9
1	A_Fr07_5	Fr7	2.38	41.9
1	A_Fr08_1	Fr8	2.4	41.84
1	A_Fr08_2	Fr8	2.4	41.84
1	A_Fr08_3	Fr8	2.4	41.84
1	A_Fr08_4	Fr8	2.4	41.84
1	A_Fr08_5	Fr8	2.4	41.84
1	A_Fr09.1_1	Fr9.1	2.46	41.79
1	A_Fr09.1_2	Fr9.1	2.46	41.79
1	A_Fr09.1_3	Fr9.1	2.46	41.79
1	A_Fr09.1_4	Fr9.1	2.46	41.79
1	A_Fr09.1_5	Fr9.1	2.46	41.79
1	A_Fr09_1	Fr09	2.41	41.81
1	A_Fr09_2	Fr09	2.41	41.81
1	A_Fr09_3	Fr09	2.41	41.81
1	A_Fr09_4	Fr09	2.41	41.81
1	A_Fr09_5	Fr09	2.41	41.81
1	A_Mi05_1	Mi5	2.37	41.73
1	A_Mi05_2	Mi5	2.37	41.73
1	A_Mi05_3_	Mi5	2.37	41.73
1	A_Mi05_4	Mi5	2.37	41.73
1	A_Mi07_1	Mi7	2.34	41.73
1	A_Mi07_2_	Mi7	2.34	41.73
1	A_Mi07_3_	Mi7	2.34	41.73
1	A_Mi07_4_	Mi7	2.34	41.73
1	A_Mi07_5	Mi7	2.34	41.73
1	A_Mi08_1	Mi8	2.34	41.74
1	A_Mi08_2	Mi8	2.34	41.74
1	A_Mi08_3_	Mi8	2.34	41.74
1	A_Mi08_4_	Mi8	2.34	41.74
1	A_Mi08_5	Mi8	2.34	41.74
1	A_Mi09_1	Mi9	2.35	41.7
1	A_Mi09_2	Mi9	2.35	41.7
1	A_Mi09_3	Mi9	2.35	41.7
1	A_Mi09_4	Mi9	2.35	41.7
1	A_Mi09_5	Mi9	2.35	41.7
1	A_Mo03_1	Mo3	2.36	41.76
1	A_Mo03_2	Mo3	2.36	41.76
1	A_Mo03_3	Mo3	2.36	41.76
1	A_Mo03_4	Mo3	2.36	41.76
1	A_Mo03_5	Mo3	2.36	41.76
1	A_Mo06_1	Mo6	2.36	41.8
1	A_Mo06_2	Mo6	2.36	41.8
1	A_Mo06_3	Mo6	2.36	41.8
1	A_Mo06_4	Mo6	2.36	41.8
1	A_Mo06_5	Mo6	2.36	41.8
1	A_Mo07_1	Mo7	2.41	41.78
1	A_Mo07_2	Mo7	2.41	41.78
1	A_Mo07_3_	Mo7	2.41	41.78
1	A_Mo07_4	Mo7	2.41	41.78
1	A_Mo07_5	Mo7	2.41	41.78
1	A_Sa04.1_1	Sa4.1	2.5	41.82
1	A_Sa04.1_2	Sa4.1	2.5	41.82
1	A_Sa04.1_3	Sa4.1	2.5	41.82
1	A_Sa04.1_4	Sa4.1	2.5	41.82
1	A_Sa04.1_5	Sa4.1	2.5	41.82
4	A_Di01_1	Di1	2.46	41.76
4	A_Di01_2	Di1	2.46	41.76
4	A_Di01_3	Di1	2.46	41.76
4	A_Di01_4	Di1	2.46	41.76
4	A_Di01_5	Di1	2.46	41.76
4	A_Di11_1	Di11	2.56	41.74
4	A_Di11_2	Di11	2.56	41.74
4	A_Di11_3	Di11	2.56	41.74
4	A_Di11_4	Di11	2.56	41.74
4	A_Di11_5	Di11	2.56	41.74
4	A_Do01_1	Do1	2.3	41.81
4	A_Do01_2	Do1	2.3	41.81
4	A_Do01_3	Do1	2.3	41.81
4	A_Do01_4	Do1	2.3	41.81
4	A_Do02_1	Do2	2.29	41.79
4	A_Do02_2	Do2	2.29	41.79

Table A1. *Continued*

Ancylus fluviatilis clade	Specimen ID *Ancylus fluviatilis*	Sampling site ID	Latitude	Longitude
4	A_Do02_3	Do2	2.29	41.79
4	A_Do02_4	Do2	2.29	41.79
4	A_Do02_5	Do2	2.29	41.79
4	A_Do03_1	Do3	2.27	41.77
4	A_Do03_2	Do3	2.27	41.77
4	A_Do03_3	Do3	2.27	41.77
4	A_Do03_4	Do3	2.27	41.77
4	A_Do03_5	Do3	2.27	41.77
4	A_Do04.1_1	Do4.1	2.27	41.74
4	A_Do04.1_2	Do4.1	2.27	41.74
4	A_Do04.1_3	Do4.1	2.27	41.74
4	A_Do04.1_4	Do4.1	2.27	41.74
4	A_Do04.1_5	Do4.1	2.27	41.74
4	A_Do10_1	Do10	2.28	41.68
4	A_Do10_2	Do10	2.28	41.68
4	A_Do10_3	Do10	2.28	41.68
4	A_Do10_4	Do10	2.28	41.68
4	A_Do10_5	Do10	2.28	41.68
4	A_Do11_1	Do11	2.25	41.78
4	A_Do11_2	Do11	2.25	41.78
4	A_Do11_3	Do11	2.25	41.78
4	A_Do11_4	Do11	2.25	41.78
4	A_Do11_5	Do11	2.25	41.78
4	A_Fr02.1_1	Fr2.1	2.32	41.83
4	A_Fr02.1_2	Fr2.1	2.32	41.83
4	A_Fr02.1_4	Fr2.1	2.32	41.83
4	A_Mi03_1	Mi3	2.38	41.71
4	A_Mi03_2	Mi3	2.38	41.71
4	A_Mi03_3	Mi3	2.38	41.71
4	A_Mi03_4	Mi3	2.38	41.71
4	A_Mi03_5	Mi3	2.38	41.71
4	A_Mi05_5	Mi5	2.37	41.73
4	A_Mi10_1	Mi10	2.39	41.69
4	A_Mi10_2	Mi10	2.39	41.69
4	A_Mi10_3	Mi10	2.39	41.69
4	A_Mi10_4	Mi10	2.39	41.69
4	A_Mi10_5	Mi10	2.39	41.69
4	A_Mi11_1	Mi11	2.36	41.68
4	A_Mi11_2	Mi11	2.36	41.68
4	A_Mi11_3	Mi11	2.36	41.68
4	A_Mi11_4	Mi11	2.36	41.68
4	A_Mi11_5	Mi11	2.36	41.68
4	A_Mo01_1	Mo1	2.41	41.74
4	A_Mo01_2	Mo1	2.41	41.74
4	A_Mo01_3	Mo1	2.41	41.74
4	A_Mo01_4	Mo1	2.41	41.74
4	A_Mo01_5	Mo1	2.41	41.74
4	A_Mo02_1	Mo2	2.39	41.76
4	A_Mo02_2	Mo2	2.39	41.76
4	A_Mo02_3	Mo2	2.39	41.76
4	A_Mo02_4	Mo2	2.39	41.76
4	A_Mo02_5	Mo2	2.39	41.76
4	A_Mo09_1_	Mo09	2.43	41.7
4	A_Mo09_2	Mo09	2.43	41.7
4	A_Mo09_3	Mo09	2.43	41.7
4	A_Mo09_4_	Mo09	2.43	41.7
4	A_Mo09_5	Mo09	2.43	41.7
4	A_Sa01_1	Sa1	2.58	41.77
4	A_Sa01_2	Sa1	2.58	41.77
4	A_Sa01_3	Sa1	2.58	41.77
4	A_Sa01_4	Sa1	2.58	41.77
4	A_Sa01_5	Sa1	2.58	41.77

Table A2. Clade assignments, Spearman's Rank Correlation results, retained WorldClim variables, per cent of contribution of variables, permutation importance of variables and AUC values for all MaxEnt models run on both *Ancylus fluviatilis* Clade 1 and *Ancylus fluviatilis* Clade 4 from the Montseny.

Ancylus fluviatilis clade	Spearman's rank correlation	WorldClim variables	Per cent contribution	Permutation importance	AUC value	*Ancylus fluviatilis* clade	Spearman's rank correlation	WorldClim variables	Per cent contribution	Permutation importance	AUC value
1	0.7	bio15	49.6	78.9	0.93	4	0.7	bio15	58.8	72.9	0.84
		bio18	30.9	14.2				bio18	22.5	9.3	
		bio19	19.4	6.9				bio19	18.7	17.8	
	0.8	bio7	33	19.4	0.96		0.8	bio7	40.4	24.5	0.89
		bio19	30.1	8.7				bio15	36.1	52.9	
		bio9	26.7	6.2				bio19	11.3	12.2	
		bio18	8	9.3				bio18	6.8	0.3	
		bio15	1.5	55.9				bio9	3.1	0.2	
		bio11	0.7	0.5				bio11	2.3	10.1	
	0.9	bio7	31.4	22.2	0.96		0.9	bio7	33	13.9	0.89
		bio9	23.2	8.1				bio15	23.6	26.2	
		bio19	20	0.3				bio8	17.5	0	
		bio16	9.2	40.4				bio16	14.8	40.5	
		bio13	4.7	11.7				bio13	5.7	11.3	
		bio18	4.1	0				bio19	2.6	2.3	
		bio10	3.8	0				bio9	1.5	0	
		bio8	2.5	2				bio10	1.2	5.4	
		bio15	1.2	15.3				bio11	0.2	0.5	
		bio11	0	0				bio18	0	0	

Table A3. Schoener's D bioclimatic niche overlap and range overlap calculated for occurrence likelihood > 50% for *Ancylus fluviatilis* Clade 1 and *Ancylus fluviatilis* Clade 4 in the Montseny, calculated with ENMTools.

Ancylus fluviatilis	Bioclimatic niche overlap	Range overlap (occurrence likelihood > 50%)
Clade 1 + Clade 4	0.634	0.728

Table A4. ΦST values between catchments and ΦST values between altitude zones for populations of *Ancylus fluviatilis* Clade 1 and *Ancylus fluviatilis* Clade 4 in the Montseny. Asterisks indicate $P = < 0.05$

Φ_{ST} values between catchments				Φ^{ST} values between altitude zones			
Ancylus fluviatilis Clade 1				*Ancylus fluviatilis* Clade 1			
	Tordera	Besòs	Ter		< 500 masl	500–1000 masl	> 1000 masl
Tordera	0.00			< 500 masl	0.00		
Besòs	0.32214*	0.00		500–1000 masl	0.00	0.00	
Ter	0.16665*	0.0664	0.00	> 1000 masl	0.34319*	0.27394*	0.00
Ancylus Clade 4				Ancylus Clade 4			
	Tordera	Besòs	Ter		< 500 masl	500–1000 masl	> 1000 masl
Tordera	0.00			< 500 masl	0.00		
Besòs	0.40835*	0.00		500–1–00 masl	0.33475*	0.00	
Ter	0.15213	0.87648*	0.00	> 1000 masl	0.66737*	0.13043	0.00