

## ORIGINAL ARTICLE

## Phylogeography of a ground beetle species in La Gomera (Canary Islands): the effects of landscape topology and population history

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*Paraeutrichopus pecoudi* (Coleoptera, Carabidae) is a species endemic to the mountain laurel forests of La Gomera in the Canary archipelago. La Gomera is an island of volcanic origin (9.4 mya), where a well-preserved laurel forest is partly fragmented by valleys and ridges. Historically restricted gene flow with isolation by distance are deduced at different clade levels for *P. pecoudi* populations, an expected scenario arising from landscape discontinuity, which has caused local isolation of these flightless insects. Methodological considerations were investigated for nested clade phylogeographic analysis (NCPA), comparing the results obtained using either (a) pairwise distances between collecting sites measured as surface (route) distances or (b) standard geographical distances calculated from latitude–longitude coordinates. Some differences were found in the NCPA statistics and associated inferences at the higher clade levels in relation to

the assumed distance criteria. Coalescent simulations and posterior automated NCPAs assuming different geographical distances were performed to test the robustness of the method when considering the distances used. These analyses showed no significant differences in NCPA conclusions, and the scenario of restricted gene flow was recovered using both distance calculators at the same rate, although the parameter statistics were slightly different. We suggest that in landscapes with extreme topography, geographical distances separating populations are certainly underestimated by standard techniques. More complex and explicit descriptions of the potential dispersal of terrestrial organisms should be explored for implementation in statistical phylogeography in such cases.

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## Introduction

The Canary archipelago off the northern Atlantic African coast has a rich and unique biodiversity, which is related to the volcanic origin of the islands and to the fact that they support remarkable habitat heterogeneity (Juan *et al.*, 2000). These islands constitute ideal systems in which to study the modes and patterns of speciation that can generate endemic taxa in speciose genera (Emerson and Kolm, 2005; Emerson and Oromí, 2005). In particular, the laurel forest (laurisilva), which is also present on other Macaronesian Islands (Azores and Madeira archipelagos), has received much attention due to its supposed ancient origin and high endemicity. This forest is considered a relict of the South Europe and North Africa Tertiary flora, which became extinct due to the effects of glaciation and desertification in those areas (Cronk, 1992; Médail and Quézel, 1999; Nakamura *et al.*, 2000).

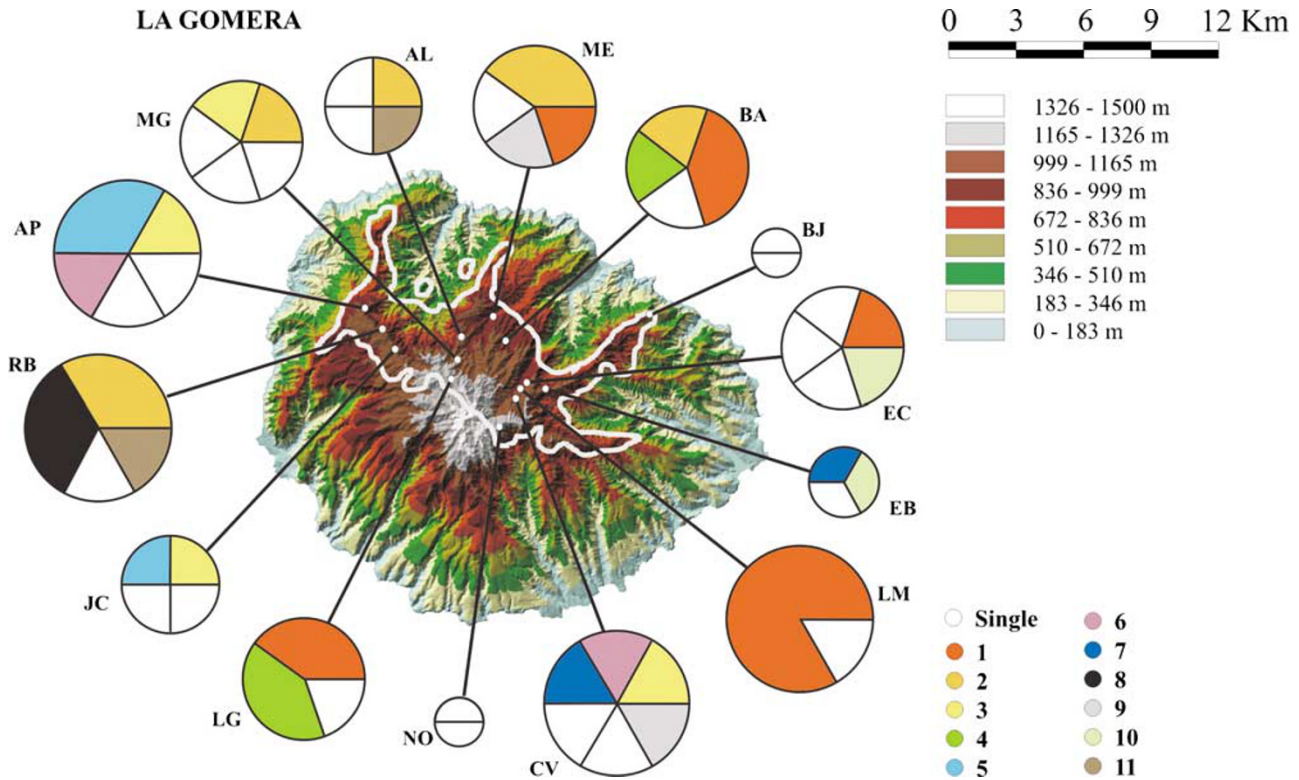
The endemic ground beetle genus *Paraeutrichopus* (Coleoptera, Carabidae, Pterostichinae) is composed of

only two species: *Paraeutrichopus pecoudi* from La Gomera and *P. harpaloides* from El Hierro. *P. pecoudi* occurs exclusively in the humid forests of La Gomera. This island emerged 9.4 mya (Paris *et al.*, 2005), and after a long stage of active shield-building, entered a quiescent period or 'gap stage' (the last eruption occurred 2.8 mya), during which time erosion has reduced the total size to its present 370 km<sup>2</sup> and has lowered its maximum height to the present 1487 m a.s.l. The central area of La Gomera (Garajonay National Park) is covered by humid forest, which includes mixed heath forest (also known as *fayal-brezal*) on the mountaintops and ridges, and laurel forest on the northern slopes and in the valleys. The preferred habitat of *P. pecoudi* is the pristine laurel forest, which is very well preserved here compared with that on the mountain massifs of the neighbouring islands Tenerife and Gran Canaria, although it is partly fragmented by topology, drainage systems and scattered ravines radiating down the volcano (see Figure 1). An estimation of the genetic diversity of *P. pecoudi* using mitochondrial data should provide us with information on the diversification of a species very much influenced by extreme topography and about the persistence of potential barriers to gene flow.

Phylogeographic analysis based on nested clade phylogeographic analysis (NCPA, Templeton *et al.*, 1995) uses pairwise linear distances from geographical

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**Figure 1** Map of La Gomera with the altitude contour lines and sampling sites indicated. The population codes correspond to those presented in Supplementary Table 1 (Supplementary material). Pie chart segments are proportional to the number of individuals collected at each location and are used to illustrate the haplotype distribution with colour codes. White haplotypes are unique to a single population. The present range of humid forest areas (potential distributional area for *Paraeutrichopus pecoudi*) is delimited with a white line.

coordinates between sampling sites (that is, great circle distances) to evaluate the geographical distributions of genotypes and their historical and contemporary causes. However, the habitat peculiarities and dispersal strategies of many organisms (for example, riparian or coastal species) make these distances an inadequate representation of the actual geographical separation between populations (Fetzner and Crandall, 2003). Given that the degree of subdivision due to extreme topography is expected to be greater in mountain habitats, we tested the performance of two different geographical distance estimators. We performed our NCPA using either geographical coordinates (from latitude and longitude) to calculate the distances between sampling sites, or the shorter-route distances separating them, calculated by geographic information system (GIS) procedures. Computer simulations of evolutionary processes can be a valuable synergistic tool for population genetic studies (Ibrahim, 2004; Mardulyn and Milinkovitch, 2005). For instance, simulations have been used to understand the patterns of genetic variation generated by expanding populations (Ibrahim *et al.*, 1996; Ray *et al.*, 2003). Empirical and simulated data have also been used to criticize NCPA for neither providing statistical support for the inferences made nor information regarding the relative likelihood of alternative inferences (Knowles and Maddison, 2002). Here, we undertook a study using simulated data, assuming a model of restricted gene flow with isolation by distance, to assess whether the surface distance calculator performs better in inferring the correct history by NCPA and to compare these

results with our empirical data obtained from *P. pecoudi* populations.

## Materials and methods

### Sampling

Beetles were collected in La Gomera and stored in absolute ethanol until DNA extraction. The sampling locations are shown in Figure 1. Details of the number of sampled individuals, collection localities, their corresponding codes, and haplotype denominations and distributions are presented in Supplementary Table S1 (available in Supplementary material). The sampling regime was designed to cover the humid forest areas of the island (Figure 1), which includes most of the distribution range of the species. Each site was visited on at least three occasions, and similar times and effort were invested in direct collecting, with the aim of sampling at least five specimens per locality. This was possible in only about two-thirds of the visited localities, because the drier southern slopes of the forest fragments were non-productive during the 3 years of fieldwork. Sixty-four individuals of *P. pecoudi* were obtained from 14 localities (Supplementary Table S1 and Figure 1).

### DNA extraction, PCR amplification and sequencing

DNA was purified from a leg of each specimen with the DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA, USA). An aliquot (1  $\mu$ l) of the extract was used for the PCR amplification of a fragment, using 1 U of *Taq* DNA

polymerase (Biolone, London, UK). The primers 5'-ATACCTCGACGTTATTCAGA-3', modified from C1-J-2797 (Simon *et al.*, 1994) and 5'-GCTCCACAAATTTCT-GAGCA-3' (C2-N-3668; formerly designated C2B-605, Simon *et al.*, 1994) were used to amplify a fragment of about 750 bp that included a partial sequence of the mitochondrial region encoding cytochrome oxidase subunits I and II (Cox1 and 2) and the leucine tRNA. PCR and sequencing of both strands were performed using previously published methods (Moya *et al.*, 2004). The sequences used in this study have been deposited in the EMBL database under accession numbers AM262994–AM263028.

### Data analysis

All sequences were aligned using the ClustalX program (Thompson *et al.*, 1997), and the 3'- and 5'-ends were pruned to avoid length differences in the alignment. Modeltest v. 3.7 (Posada and Crandall, 1998) was used to select the substitution model(s) that best described the data, and PAUP\* v. 4.0b10 (Swofford, 2002) was used to calculate sequence divergences. Maximum likelihood (ML) trees were constructed using PAUP\*, with TBR as the branch swapping algorithm. The likelihood ratio test was performed to assess whether the trees obtained followed a molecular clock. Sequences of the sister species *P. harpaloides* (O Moya, unpublished data) were used to estimate the corrected divergence between the two lineages and derive a conservative estimate for the separation time of *P. pecoudi* and *P. harpaloides*. This correction ( $d$ ) is given by  $d = d_{XY} - 1/2 (d_X + d_Y)$ , where  $d_{XY}$  is the mean pairwise genetic distance between haplotypes from lineages X and Y, and  $d_X$  and  $d_Y$  are the mean pairwise genetic distances within the respective lineage (Nei, 1987; Edwards, 1997).

Estimates of mean nucleotide and haplotype diversities ( $\pi$ ) were obtained with DnaSP v. 4.0 (Rozas *et al.*, 2003). Analysis of molecular variance (AMOVA), using Arlequin v. 2.000 (Schneider *et al.*, 2000), was performed to partition the molecular variance into different hierarchical levels. In this analysis, we tested for differences between sampling sites (nested within regional groups) and differences between groups. The significance of the variance components was computed using a nonparametric permutation test (Excoffier *et al.*, 1992) with 10 000 permutations. Populations were grouped by geographical proximity and altitudinal criteria. The grouping that maximized the geographical subdivision was assumed to be the most plausible within those supported by significant variation among regions ( $\Phi_{CT}$ ). A Bonferroni correction was applied (with simple interactive statistical analysis (SISA), available at <http://www.home.clara.net/sisa/bonfer.htm>) to adjust the level of significance used for AMOVA. This correction is necessary when multiple tests based on the same observations are considered. Significance at a 0.05 level was adjusted to a critical value of 0.01 for the five different groupings of the same sampling locations.

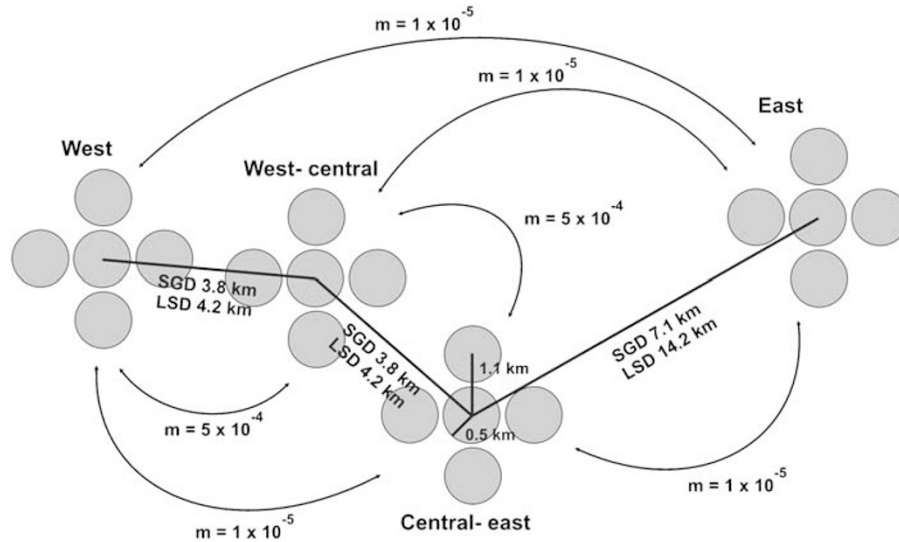
Distances between sampling sites were obtained with GIS software (ArcGis v. 9.1 and 3D Analyst tool). Two different criteria were applied to estimate geographical distances standard geographical distances (SGDs) and landscape surface (or route) distances (LSDs). They were obtained from La Gomera map files with a coordinate

system and contour lines with explicit altitude data in computer-assisted design (CAD) format. SGDs represent the shortest geographical distances between two locations on a flat surface, and were calculated with the *distance* tool. LSDs, corresponding to the direct-walking distance between locations, were obtained from the map files modelling a three-dimensional triangular irregular network of the surface, tracing routes between locations and using the *surface length* tool implemented in ArcGis.

The program TCS v. 1.13 (Clement *et al.*, 2000) was used to infer the parsimony network of haplotypes, linked by the smallest number of mutational steps with a 95% confidence criterion (Templeton *et al.*, 1992). Loops of ambiguity were resolved by criteria based on geography and coalescent theory (Posada and Crandall, 2001). The haplotypes represented in the network were grouped at different nesting levels for NCPA (Templeton *et al.*, 1995) to infer the population history of *P. pecoudi*. Nesting rules (Templeton, 1998) are based on predictions from coalescent theory and are described by Posada and Crandall (2001). Two permutational tests were performed with GeoDis v. 2.4 (Posada *et al.*, 2000) for all clades at each nesting level, with 10 000 random permutations. First, an exact contingency test was performed, treating sample locations as categorical variables and calculating a  $\chi^2$  statistic from the contingency matrix of genetic clades with respect to the geographical locations at which their haplotypes were found. The second test was a geographical distance analysis, in which distance parameters ( $D_c$ , clade distance;  $D_{nr}$ , nested distance; and I-TD<sub>c</sub> and I-TD<sub>nr</sub>, interior-tip contrasts) are specified and checked to determine whether they are significantly different from the values expected for a panmictic population. The last version of Templeton's inference key (released in November 2005, [http://www.darwin.uvigo.es/download/geodisKey\\_11Nov05.pdf](http://www.darwin.uvigo.es/download/geodisKey_11Nov05.pdf)) was used for guidance in the inference process. To evaluate the effects of landscape topology on NCPA inferences, we contrasted the null hypothesis, in which plain linear distances were used (SGDs), with analyses taking into account the increases due to altitudinal differences (estimated as LSDs with GIS procedures).

### Simulations

Computer simulations can potentially assess most precisely the effect of SGD and LSD on NCPA parameter statistics and the corresponding inferences obtained. Firstly, we defined the simulated demographic scenario. Four sampling areas equivalent to our geographical regions in the empirical *P. pecoudi* study were modelled, each of which was divided into five demes (sampling sites) of 0.5 km radius (see Figure 2). We simulated the evolution of populations using Simcoal v. 1.0 (Excoffier *et al.*, 2000). This program simulates the gene history (coalescence) of samples of genes drawn from different demes in a population. This depends on the history and demography of the population, assuming a neutral model, and is independent of the mutational process (Excoffier *et al.*, 2000). We simulated 100 sampled data sets taken from a population with a history of restricted gene flow, with isolation by distance between regions. The following parameters were used in all simulations: effective population size of each deme = 1000; 400



**Figure 2** Geographical and evolutionary scenarios modelled for the coalescent simulations. Grey circles represent demes of 0.5 km radius. Four geographical regions are considered, each composed of five demes separated by 1.1 km. Distances between geographical regions are indicated in kilometres for the two alternative geographical scenarios (see text for details), and migration rates between those in terms of migrants per generation per population are also indicated. Migration between demes of the same region assumes an island model, with  $m = 1 \times 10^{-2}$  (data not shown in figure); other migration rates are indicated.

sequences sampled (20 from each deme); length of the DNA sequence = 1000 bp; mutation rate per sequence per generation =  $1.15 \times 10^{-5}$ ; a transition/transversion bias of 0.67; and  $\gamma$ -distribution with  $\alpha = 0.4$ , using 10 rate classes. The choice of the effective population size is arbitrary because no census size estimate for *P. pecoudi* is available; hence, we used the same effective size value that was used in another coalescent simulation of a beetle study (Mardulyn and Milinkovitch, 2005). The sampled sequences and the length of the DNA sequence were chosen to be similar and to optimize the empirical sampling. The mutation rate per base per generation assumed a standard of 2.3%/million years (Myr) (Brower, 1994) for arthropod mitochondrial DNA and a generation time of 1 year. We assumed an island model in which migration is equal among demes within each geographical region (with a proportion of migrants of 1% per population and per generation ( $m = 1 \times 10^{-2}$ ), thus assuming substantial gene flow) coupled to a linear stepping-stone model among regions ( $m = 5 \times 10^{-4}$ , but see below), but allowing for a low migration rate between non-neighbouring regions ( $m = 1 \times 10^{-5}$ ). An equivalent low migration rate was considered between the east and central-east regions to accommodate their separation by a deep valley.

For the NCPA, sequences obtained in simulations were analysed in terms of the alternative geographical settings described above with the automated NCPA software AneCA v. 0.9 (Panchal, 2007) (available at <http://www.rubic.rdg.ac.uk/~mahesh/software.html>), complemented by Perl scripts, which run the software on multiple replicates obtained with Simcoal (developed by M Panchal). AneCA first uses TCS v. 1.18 (Clement *et al.*, 2000) to automatically build a statistical parsimony haplotype network. Then, both observed and inferred haplotypes are automatically nested following the rules modified by Posada and Crandall (2001). NCPA distance statistics (based on geographical coordinates or pairwise distances) and their significances were then calculated

using GeoDis v. 2.2 (Posada *et al.*, 2000), and finally, the version of the inference key implemented in AneCA corresponding to the one dated 14th July 2004 (Templeton, 2004) was used to interpret the summary statistics. This program allows simulation testing because it automates the NCPA procedure.

To compare the effects of the two distance regimes, we used the same 100 simulated data sets and analysed them in NCPA with different pairwise distances. The results of coalescent simulations analysed with AneCA were compared, testing for the stability of the significance of NCPA distance parameters ( $D_c$ ,  $D_n$ , I-TD<sub>c</sub> and I-TD<sub>n</sub>) in the simulated clades of the two scenarios (number of clades with significant results in the null hypothesis that change in significance in the landscape scenario and *vice versa*). The number of contradictory inferences (fragmentation, long-distance colonization or range expansion instead of the expected restricted gene flow inference) obtained was also recorded to quantify whether the landscape distance calculator performs better in inferring the actual simulated history.

## Results and discussion

### Sequence data

The DNA of 64 *Paraeutrichopus* individuals was sequenced for 744 mitochondrial nucleotide positions and yielded 35 different haplotypes. The sequences were AT-biased (75.5%), showing 39 variable positions, 16 of which were informative under the parsimony criterion. No substitutions were observed in the tRNA-leucine gene. For the 31 variable positions of Cox2 (513 bp in total), 29.0% were in first, 9.7% in second, and 61.3% in third codon positions. Cox1 (165 bp) varied at only eight positions, 25% of which were in first and 75% in third codon positions.

The Modeltest hierarchical likelihood ratio test suggested HKY+I+G (Hasegawa *et al.*, 1985) as the

substitutional model that best explained our data set (in-group plus out-group species). In contrast, the Akaike Information Criterion favoured the GTR+I+G model (Rodríguez *et al.*, 1990), but included HKY+I+G as a plausible alternative (cumulative Akaike weight = 0.9953). The low divergence observed in our sequences makes it advisable to select the latter because it is the less parameterized model favoured by both criteria. Under HKY+I+G (with  $\alpha = 0.8412$  for the  $\gamma$ -distribution and a proportion of invariable sites of 0.8148), the maximum haplotype pairwise divergence within the *P. pecoudi* sequences was 1.86%. Using the ML tree obtained (data not shown), the likelihood ratio test could not reject the null hypothesis of clock-like evolution along the tree branches ( $\Delta L = 34.23$  at d.f. = 33;  $P = 0.05$ ). Diverse beetle groups (for example, Emerson *et al.*, 2000a; Barraclough and Vogler, 2002) show mtDNA rates close to the widely accepted 2.3%/Myr pairwise divergence for insect mtDNA (Brower, 1994), although ranging for particular beetle genera and mitochondrial genes from 0.5 to 3% (for example, Prüsser and Mossakowski, 1998; Farrell, 2001). If we tentatively apply the (Brower, 1994) 2.3% rate to *P. pecoudi* mtDNA sequences, it derives that the maximum divergence of the La Gomera *P. pecoudi* haplotypes (0.0186) gives an estimated age of about 800 000 years for the most recent common ancestor of this species. This estimate does not account, however, for the bias associated with ancestral coalescence (Arbogast *et al.*, 2002), so we used sequences obtained from the sister species *P. harpaloides* (O Moya, unpublished data) to calculate a corrected estimate of genetic divergence (Nei, 1987; Edwards, 1997) between two species. This divergence is  $0.0129 \pm 0.0008$ , suggesting a separation time (mean  $\pm$  s.d.) for the two lineages of  $565\,000 \pm 36\,000$  years ago, assuming of 2.3%/Myr rate (430 000–2 600 000 years using the extreme rates of 3 and 0.5%/Myr, respectively). This shows, despite the considerable uncertainty and associated errors, that the sequences reflect a relatively ancient divergence for an endemic island insect species with a very restricted distribution range. However, this is still recent in relation to the ancient tertiary origin of the Macaronesian laurisilva where this species lives (Emerson *et al.*, 1999, 2000b; Emerson and Oromí, 2005).

#### Population analyses and phylogeography

The haplotype diversity value for the total island population was  $\pi = 0.00618 \pm 0.0004$ . AMOVA showed high and statistically significant ( $P < 0.01$ )  $\Phi_{CT}$  values (fixation index among groups) in most comparisons among *P. pecoudi* populations when locations were grouped geographically, in particular when the eastern and western regions were compared, but not when they were grouped according to altitudinal differences (see Table 1).

It has been shown that network approaches may be more effective than classical phylogenetic ones in representing intraspecific evolution (Posada and Crandall, 2001). TCS analysis included all *P. pecoudi* haplotypes in a single network. The maximum number of substitutions, before homoplasy was likely under the 95% probability criterion, was estimated to be 11, whereas the maximum number of empty haplotypes (either extinct or not sampled) between two sampled

**Table 1** Regional structure of *Paraeutrichopus pecoudi* tested by AMOVA

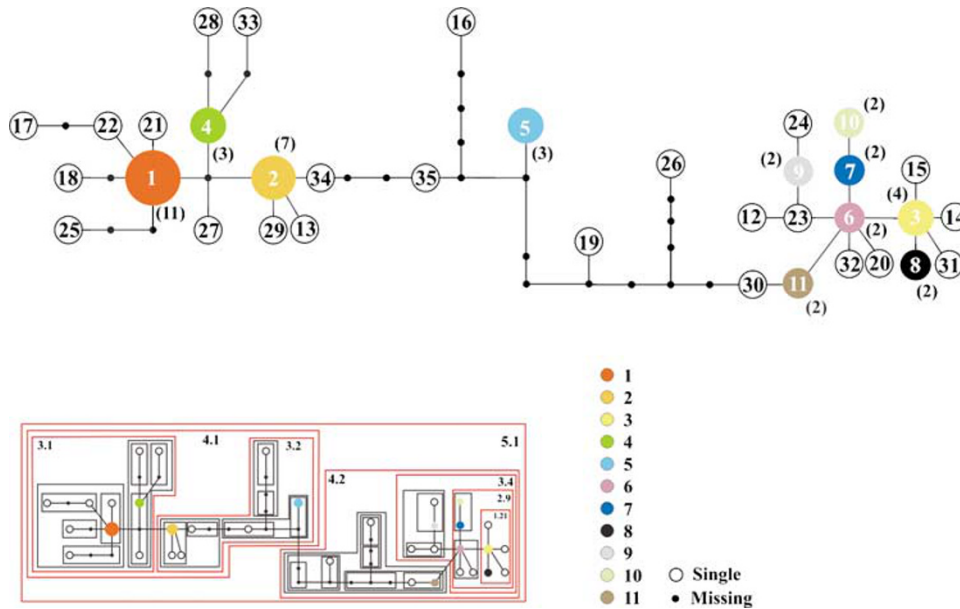
	$\Phi$ -Statistics	% Total	P-value
<i>West: AP, RB, JC / Central: AL, MG, LG, ME, BA / East: NO, CV, LM, EC, EB, BJ</i>			
Among groups	0.136	13.6	<0.01
Among populations within groups	0.041	3.6	NS
Within populations	0.171	82.9	<0.01
<i>West: AP, RB, JC / West-Central: AL, MG, LG / Central-East: ME, BA / East: NO, CV, LM, EC, EB, BJ</i>			
Among groups	0.114	11.4	<0.01
Among populations within groups	0.051	4.5	NS
Within populations	0.159	84.1	<0.01
<i>West and Central: AP, RB, JC, AL, MG, LG, ME, BA / East: NO, CV, LM, EC, EB, BJ</i>			
Among groups	0.037	3.7	NS
Among populations within groups	0.121	11.7	<0.01
Within populations	0.153	84.7	<0.01
<i>&gt; 1200 m: NO, LG / 1000–1200 m: AP, RB, JC, MG, AL, CV, LM / &lt; 1000 m: ME, BA, EC, EB, BJ</i>			
Among groups	0.007	0.7	NS
Among populations within groups	0.134	13.3	<0.01
Within populations	0.140	86.0	<0.01
<i>&gt; 1000 m: NO, LG, RB, JC, AP, MG, AL, CV, LM / &lt; 1000 m: ME, BA, EC, EB, BJ</i>			
Among groups	-0.001	-0.1	NS
Among populations within groups	0.138	13.8	<0.01
Within populations	0.137	86.3	<0.01

Abbreviations: AMOVA, analysis of molecular variance; NS, non-significant.

Localities are grouped on the basis of geographical proximity or altitude (expressed in metres above sea level). Fixation indices  $\Phi_{ST}$  (within populations),  $\Phi_{SC}$  (among populations within groups),  $\Phi_{CT}$  (among groups of populations in the species) and their significances are shown. Probabilities of  $P > 0.01$  are considered NS. The percentage of total variance attributed to each level of population subdivision is also indicated.

haplotypes consisted of eight mutational steps. The haplotype network and nesting design is represented in Figure 3. Haplotype 1 is the most frequent, and is present in 11 of the 64 sampled individuals. Nesting resulted in 24 one-step clades that were further nested into higher-order groups until the total cladogram was obtained.

The historical restriction of gene flow with isolation by distance was inferred for the five (using LSDs) or seven (using SGD) clades with significant non-random geographical patterns in the NCPA. For the total species cladogram, two alternative inferences are possible, depending on the ancestry of the four-level clades. Assuming the ancestry of clade 4.1 (based on the frequency and interior nature of haplotype 1) makes contiguous range expansion the most likely explanation at the level of the entire cladogram. However, note that this inference is sensitive to the chosen distance measure (Table 2). Fu's test of neutrality (Fu, 1997) gave a statistically significant negative value for this clade ( $F_s = -23.54$ ;  $P = 0.000$ ) indicating population growth, thus supporting the SGD inference. Although AMOVA is a much more limited method than NCPA in terms of the possible scenarios considered, the two analyses agree in pointing to a pattern of population structuring related to persistent barriers to gene flow.



**Figure 3** Parsimony networks and nesting design for *Paraeutrichopus* mtDNA haplotypes. Black unlabelled circles represent haplotypes not sampled (either extinct or not found). Numbers in parentheses are the number of copies found for a particular haplotype. Only clades with significant results are labelled and delimited by red boxes. Haplotype colour codification is the same as in Figure 1.

**Table 2** Permutational contingency test  $\chi^2$  values, NCPA probabilities and inferences obtained with landscape surface distance (LSD) and standard geographical distance (SGD)

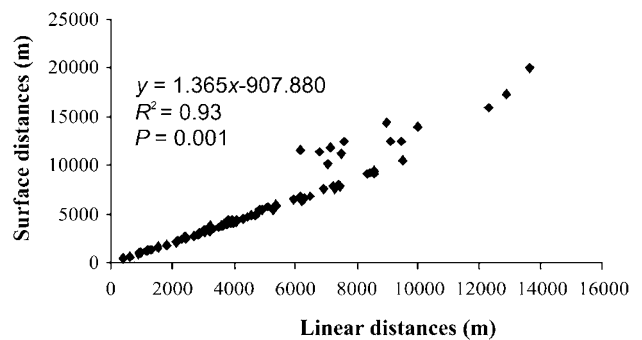
Clade	$\chi^2$	P	SGD	LSD
			Inference	Inference
1.21	16.88	0.5823	Restricted gene flow with isolation by distance	Restricted gene flow with isolation by distance
2.9	20.93	0.0648	Restricted gene flow/dispersal but with some long distance dispersal	Restricted gene flow/dispersal but with some long distance dispersal
3.1	13.60	0.1639	Restricted gene flow with isolation by distance	Restricted gene flow with isolation by distance
3.2	26.50	0.0230	Restricted gene flow with isolation by distance	Restricted gene flow with isolation by distance
3.4*	13.93	0.1170	Restricted gene flow with isolation by distance	Restricted gene flow with isolation by distance
4.1*	21.58	0.0167	Restricted gene flow with isolation by distance	Null hypothesis of random distribution cannot be rejected
5.1*	21.91	0.0407	Contiguous range expansion (4.1 interior)	Null hypothesis of random distribution cannot be rejected

Abbreviations: LSD, landscape surface distance; NCPA, nested clade phylogeographic analysis; SGD, standard geographical distance. Only clades with significant distance patterns are listed. Clades for which geographical distance analysis statistics vary depending on the metrics applied are marked with an asterisk. Note that the inference obtained depends on the distance metrics for the higher-level clades 4.1 and 5.1.

### SGDs vs LSDs

A remarkable increase (average of 12%) in the pairwise geographical distances was observed in our study when we corrected for surface landscape (Figure 4). This increase is not randomly distributed but occurs predominantly in measures including sampling site BJ, a locality separated from the others by a deep valley (Figure 1). If we do not consider this locality, the average increase is only 6.2%. There is a weak correlation between surface separation and distance increase with respect to geographical standard measures when localities in La Gomera are compared (data not shown).

NCPA results for empirical sampling are clearly dependent on the geographical distance used (Table 2). A comparison of the  $D_c$ ,  $D_{nv}$  and I-T distances obtained applying either SGD or LSD, and their associated NCPA statistics is available in the Supplementary material (Supplementary Table S2). The distributions of haplotypes within clades 4.1 and 5.1 were not significantly different from a random distribution when LSDs were



**Figure 4** Plot of standard geographical distances (SGDs) vs landscape surface distances (LSDs) for sampling sites in La Gomera.

considered, rendering non-significant patterns of geographical distribution, which is in contrast to the results obtained using SGDs (Table 2). Other clades, such as 3.4, showed differences in the significance of  $D_n$  and I-TD<sub>n</sub>

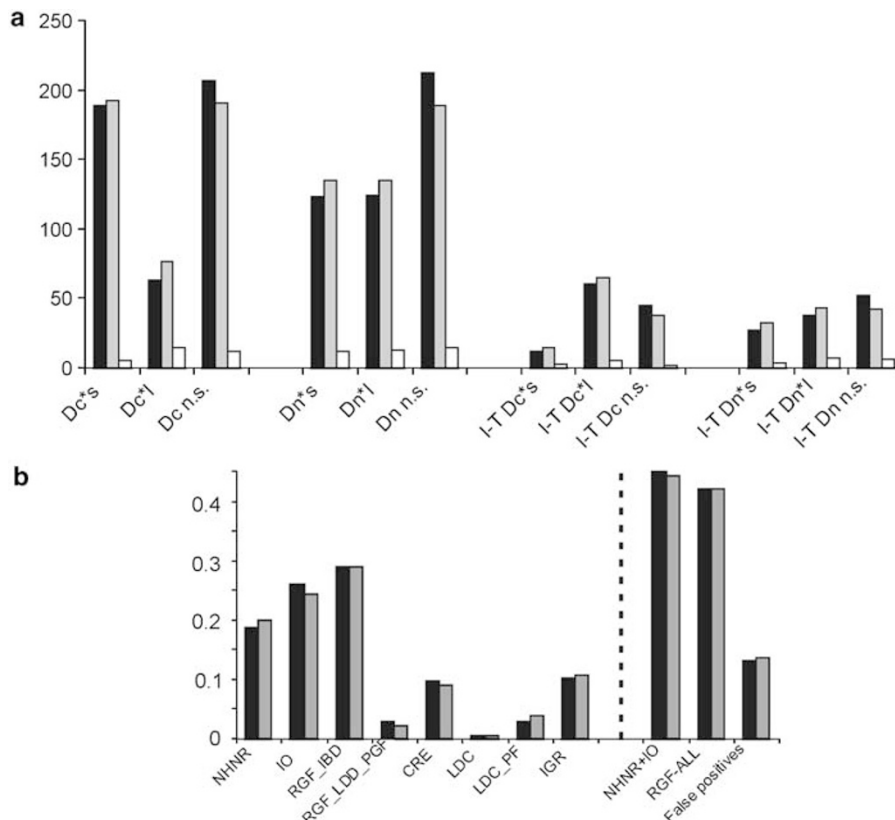
tests depending on the distances used in the analysis, but without changing the associated inference of restricted gene flow. The analyses showed remarkable discrepancies in clades that included haplotypes from locality BJ, a population that is greatly influenced by the geographical metrics chosen, as mentioned above. These differences involved inferring significantly non-random haplotype (or clade) distributions when using SGDs, in contrast to obtaining marginally significant or non-significant probabilities if surface distances were applied (Supplementary Table S2).

### Simulations

NCPA has been criticized for discrepancies obtained in empirical studies (for example, Paulo *et al.*, 2002; Alexandrino *et al.*, 2002; Masta *et al.*, 2003) and inconsistencies found in computer simulations (Knowles and Maddison, 2002; M Panchal, personal communication). Knowles and Maddison (2002) used simulated data to

test the performance of NCPA in simple scenarios of fragmentation and colonization with isolation by distance, and concluded that the performance of the method is very poor and can even lead to mistaken conclusions. Templeton (2004) argued that the modelled fragmentation events in that study were too shallow and that the retention of ancestral polymorphisms across isolates made it difficult for any technique to recover the correct population history. There has also been criticism of the inadequate sampling scheme used for these simulations. Automation of NCPA now provides an easier way to replicate analyses and assess their results.

Our coalescent simulations aimed to test the performance of NCPA using alternative geographical distance calculators in a context of restricted gene flow between geographical regions (but not within them). To do that, we simulated 100 replicates and used them twice in NCPA with the alternative distances. The nesting of haplotypes generated in the simulation replicates resulted in 173 clades with genetic and geographical variations that were



**Figure 5** (a) Histogram showing the number of NCPA distance parameters either significantly smaller or larger than expected under the null hypothesis at the 5% level ( $Dc^*s$ ,  $Dc^*l$ ,  $Dn^*s$ ,  $Dn^*l$ ,  $I-TDc^*s$ ,  $I-TDc^*l$ ,  $I-TDn^*s$ , and  $I-TDn^*l$ ) or non-significant ( $Dc$  n.s.,  $Dn$  n.s.,  $I-TDc$  n.s., and  $I-TDn$  n.s.) among all replicates of the simulation analyses. Black and grey bars show the frequencies obtained under the SGD and LSD models, respectively, whereas white bars show changes in parameter significance when simulations under LSD are compared with the results obtained under SGD. (b) Frequency histograms of inferences made by NCPA among all replicates, and compared with those from the SGD and LSD scenarios (black and grey bars, respectively) using the inference key dated 14th July 2004 and implemented in AneCA vs 0.9. The clade level at which the inference was made was ignored in the analysis. Each column refers to a particular inference made by the inference key as follows. CRE, contiguous range expansion; false positives, inferences obtained that contradict the simulated scenario of restricted gene flow (see text for details); IGR, insufficient genetic resolution to discriminate between range expansion/colonization and restricted dispersal/gene flow; IO, inconclusive outcome; LDC, long-distance colonization, possibly coupled to subsequent fragmentation or past fragmentation followed by range expansion; LDC\_PF, long-distance colonization and/or past fragmentation (not necessarily mutually exclusive); NHNR, null hypothesis was not rejected; NHNR+IO, null hypothesis was not rejected and inconclusive outcome; RGF\_ALL, inferences in which restricted gene flow is included; RGF\_IBD, restricted gene flow with isolation by distance; RGF\_LDD\_PGF, restricted gene flow with some long-distance dispersal over intermediate areas not occupied by the species, or past gene flow followed by the extinction of intermediate populations.

analysed with Geodis, that is, clades with a minimum of two haplotypes sampled from two or more different locations. Changes in the statistical significance of one or more NCPA distance parameters were observed in 49 of the 144 clades tested (clades with significance in some of the distance parameters) in comparisons between the LSD and SGD scenarios. Among these clades, a total of 97 distance parameters ( $D_c$ ,  $D_{iv}$ ,  $I-TD_c$  and  $I-TD_{iv}$ ) changed the statistical significance at the 5% level, 65% of which changed from non-significant to significant. The distribution of the distance parameters obtained in the two alternative simulations (SGD and LSD) is shown in Figure 5a.  $\chi^2$ -Tests showed non-significant differences in the total number of parameters obtained in the two treatments. However, the comparison shows that the LSD metrics deviate slightly on NCPA compared with the SGD metrics, because the number of significant statistics is always higher and the non-significant results decrease moderately. In relation to the inferences obtained, only nine of the 49 clades with differences in distance parameters produced contradictory inferences to explain the non-random geographical distribution of the simulated haplotypes (14 clades, if we include changes from an inconclusive outcome to a non-rejection of the null hypotheses and *vice versa*; see Supplementary Table S3 provided in Supplementary material). Figure 5b shows the frequency distributions of the actual inferences obtained under either SGD or LSD. Restricted gene flow (or some inference in which this possibility is included) was recovered in the majority of cases, in a similar way and independently of the distance calculator used (Figure 5b). Other inferences (contiguous range expansion, long-distance colonization and/or past fragmentation) can be considered false positives given the *a priori* gene flow scenario used in the model, whereas the remaining (about 45%) are inconclusive or cases in which the null hypothesis cannot be rejected. Therefore, both distance metrics lead to similar NCPA conclusions, and although the parameter statistics differ to some extent, the historical demographic scenarios recovered are essentially the same.

#### Concluding remarks

Species' dispersal strategies and habitat peculiarities are of importance when estimating geographical distances for NCPA. Fetzner and Crandall (2003) showed that river distances are often much longer than geographical distances, and observed remarkably different NCPA results in golden crayfish populations depending on the riparian or geographical distances considered. Topographic considerations were incorporated into a population dynamics study of the mountain beetle *Moneilema appressum* (Cerambycidae) in relation to habitat shifts generated by Pleistocene interglacial climate changes (Irwin and Farrell, 2005). This species occurs in mountain refuges of the so-called 'Sky Islands' (Heald, 1951) in south-west North America. Great circle distances between collection localities and 'topographic barriers to dispersal', defined as the lowest elevation along a straight-line route between two populations, were considered in this study, showing that topography is a key determining isolation factor in this species.

Different conclusions were drawn with NCPA when surface distances were used instead of 'air-line' distances between *P. pecoudi* populations. It is expected that the degree of subdivision due to extreme topography is

greater than simply the extra distance incurred when going up from and down to the valley floor on an island such as La Gomera. It is interesting that this effect can be recognized by adding the extra distance estimated by LSD, even at a very small geographical scale, which also relates to the flightlessness and presumably low dispersal ability of this species. According to our simulation study, although a remarkable number of NCPA distance parameters changed in significance when the surface landscape distance calculator was used, not much was gained by using LSDs in terms of the inferences themselves. These results contrast with the empirical ones, in which the distance metrics had some impact on the inferences obtained. Indeed, other features relating to habitat heterogeneity (that is, unsuitable or patchy habitat) and a more explicit description of species-specific dispersal routes in alpine species could be incorporated into statistical phylogeography to obtain more accurate estimates of gene flow. The incidence of false positives in the simulations illustrates the need of using alternative tools to assess the adequacy of NCPA inferences in empirical studies.

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#### References

- Alexandrino J, Arntzen JW, Ferrand N (2002). Nested clade analysis and the genetic evidence for population expansion in the phylogeography of the golden-striped salamander, *Chioglossa lusitanica* (Amphibia: Urodela). *Heredity* **88**: 66–74.
- Arbogast BS, Edwards SV, Wakeley J, Beerli P, Slowinski JB (2002). Estimating divergence times from molecular data on phylogenetic and population genetic timescales. *Annu Rev Ecol Syst* **33**: 707–740.
- Barracough TG, Vogler AP (2002). Recent diversification rates in north American tiger beetles estimated from a dated mtDNA phylogenetic tree. *Mol Biol Evol* **19**: 1706–1716.
- Brower AVZ (1994). Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc Natl Acad Sci USA* **91**: 6491–6495.
- Clement MD, Posada D, Crandall KA (2000). TCS: a computer program to estimate gene genealogies. *Mol Ecol* **9**: 1657–1659.
- Cronk QCB (1992). Relict floras of Atlantic islands: patterns assessed. *Biol J Linn Soc Lond* **46**: 91–103.
- Edwards SV (1997). Relevance of microevolutionary processes for higher level molecular systematics. In: Mindell DP (ed).



- Avian Molecular Systematics and Evolution*. Academic Press: New York. pp 251–278.
- Emerson BC, Kolm N (2005). Species diversity can drive speciation. *Nature* **434**: 1015–1017.
- Emerson BC, Oromí P (2005). Diversification of the forest beetle genus *Tarphius* on the Canary Islands, and the evolutionary origins of island endemics. *Evolution* **59**: 586–598.
- Emerson BC, Oromí P, Hewitt GM (1999). MtDNA phylogeography and recent intra-island diversification among Canary Island *Calathus* beetles. *Mol Phylogenet Evol* **13**: 149–158.
- Emerson BC, Oromí P, Hewitt GM (2000a). Tracking colonization and diversification of insect lineages on islands: mitochondrial DNA phylogeography of *Tarphius canariensis* (Coleoptera: Colydiidae) on the Canary Islands. *Proc Biol Sci* **267**: 2199–2205.
- Emerson BC, Oromí P, Hewitt GM (2000b). Interpreting colonization of the *Calathus* (Coleoptera: Carabidae) on the Canary Islands and Madeira through the application of the parametric bootstrap. *Evolution* **54**: 2081–2090.
- Excoffier L, Novembre J, Schneider S (2000). Simcoal: a general coalescent program for the simulation of molecular data in interconnected populations with arbitrary demography. *J Hered* **91**: 506–509.
- Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**: 479–491.
- Farrell BD (2001). Evolutionary assembly of the Milkweed fauna: cytochrome oxidase I and the age of *Tetraopes* beetles. *Mol Phylogenet Evol* **18**: 467–478.
- Fetzner JW, Crandall KA (2003). Linear habitats and the nested clade analysis: an empirical evaluation of geographic versus river distances using an Ozark crayfish (Decapoda: Cambaridae). *Evolution* **57**: 2101–2118.
- Fu Y-X (1997). Statistical tests of neutrality mutations against population growth, hitchhiking and background selection. *Genetics* **147**: 915–925.
- Hasegawa M, Kishino K, Yano T (1985). Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**: 160–174.
- Heald WF (1951). Sky islands in Arizona. *Nat Hist* **60**: 56–63.
- Ibrahim KM (2004). Simulations of human colonization history. *Heredity* **93**: 124–125.
- Ibrahim KM, Nichols RA, Hewitt GM (1996). Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity* **77**: 282–291.
- Irwin C, Farrell BD (2005). Phylogeography of the longhorn cactus beetle *Moneilema appressum* LeConte (Coleoptera: Cerambycidae): was the differentiation of the Madrean sky islands driven by Pleistocene climate changes? *Mol Ecol* **14**: 3049–3065.
- Juan C, Emerson BC, Oromí P, Hewitt GM (2000). Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. *Trends Ecol Evol* **15**: 104–109.
- Knowles LL, Maddison WP (2002). Statistical phylogeography. *Mol Ecol* **11**: 2623–2635.
- Mardulyn P, Milinkovitch MC (2005). Inferring contemporary levels of gene flow and demographic history in a local population of the leaf beetle *Gonioctena olivacea* from mitochondrial DNA sequence variation. *Mol Ecol* **14**: 1641–1653.
- Masta SE, Laurent NM, Routman EJ (2003). Population genetics structure of the toad *Bufo woodhousii*: an empirical assessment of the effects of haplotype extinction on nested cladistic analysis. *Mol Ecol* **12**: 1541–1554.
- Médail F, Quézel P (1999). Biodiversity hotspots in the Mediterranean Basin: setting global conservation priorities. *Conserv Biol* **13**: 1510–1513.
- Moya O, Contreras-Díaz HG, Oromí P, Juan C (2004). Genetic structure, phylogeography and demography of two ground-beetle species endemic to the Tenerife laurel forest (Canary Islands). *Mol Ecol* **13**: 3153–3167.
- Nakamura UW, Wildpret de-la-Torre W, DelArco-Aguilar MJ, Reyes-Betancort JA (2000). A phytosociological study on the Mediterranean laurel forest area of Tenerife, Canary Islands, in comparison with Japanese laurel forest landscape area of Izu, Central Japan. *Phytocoenologia* **30**: 613–632.
- Nei M (1987). *Molecular Evolutionary Genetics*. Columbia University Press: New York.
- Panchal M (2007). The automation of nested clade phylogeographic analysis. *Bioinformatics* **23**: 509–510.
- Paris R, Guillou H, Carracedo JC, Pérez FJ (2005). Volcanic and morphological evolution of La Gomera (Canary Islands), based on new K-Ar ages and magnetic stratigraphy: implications for oceanic island evolution. *J Geol Soc London* **162**: 501–512.
- Paulo OS, Jordan WC, Bruford MW, Nichols RA (2002). Using nested clade analysis to assess the history of colonization and the persistence of populations of an Iberian Lizard. *Mol Ecol* **11**: 809–819.
- Posada D, Crandall KA (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
- Posada D, Crandall KA (2001). Intraspecific gene genealogies: trees grafting into networks. *Trends Ecol Evol* **16**: 37–45.
- Posada D, Crandall KA, Templeton AR (2000). GeoDis: a program from the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Mol Ecol* **9**: 487–488.
- Prüsser F, Mossakowski D (1998). Low substitution rates in mitochondrial DNA in Mediterranean carabid beetles. *Insect Mol Biol* **7**: 121–128.
- Ray N, Currat M, Excoffier L (2003). Intra-deme molecular diversity in spatially expanding populations. *Mol Biol Evol* **20**: 76–86.
- Rodríguez F, Oliver JF, Marín A, Medina JR (1990). The general stochastic model of nucleotide substitutions. *J Theor Biol* **142**: 485–501.
- Rozas J, Sánchez-Del Barrio JC, Messegyer X, Rozas R (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**: 2496–2497.
- Schneider S, Roessli D, Excoffier L (2000). *Arlequin, a Software for Population Genetics Data Analysis*. Version 2.000. Genetics and Biometry Laboratory: University of Geneva, Geneva.
- Simon C, Frati F, Beckenbach AT, Crespi B, Liu H, Flook P (1994). Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am* **87**: 651–701.
- Swofford DL (2002). *PAUP\*: Phylogenetic Analysis Using Parsimony (and Other Methods)*. Version 4.0b10. Sinauer: Sunderland.
- Templeton AR (1998). Nested clade analysis of phylogeographic data: testing hypothesis about gene flow and population history. *Mol Ecol* **7**: 381–397.
- Templeton AR (2004). Statistical phylogeography: methods of evaluating and minimizing inference errors. *Mol Ecol* **13**: 789–809.
- Templeton AR, Crandall KA, Sing CF (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. III. Cladogram estimation. *Genetics* **132**: 619–633.
- Templeton AR, Routman E, Phillips CA (1995). Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger Salamander, *Ambystoma tigrinum*. *Genetics* **140**: 767–782.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**: 4876–4882.

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