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Nested cladistic analysis, phylogeography and speciation in the *Timarcha goettingensis* complex (Coleoptera, Chrysomelidae)

J. GÓMEZ-ZURITA, E. PETITPIERRE and C. JUAN
Laboratori de Genètica, Departament de Biologia, Universitat de les Illes Balears, E-07071 Palma de Mallorca, Balearic Islands, Spain

Abstract

The *Timarcha goettingensis* complex is a monophyletic assemblage of closely related leaf beetles (Chrysomelidae), distributed from the north half of the Iberian Peninsula to Central Europe. Oligophagy, mountainous habitat and apterism are factors which are assumed to promote speciation in these beetles. We have used cytochrome oxidase subunit II mitochondrial DNA genealogies obtained from 31 sampling localities and a nested geographical distance analysis to assess the population structure and demographic factors explaining the geographical distributions of the mtDNA haplotypes in the *T. goettingensis* complex. The results show that there is a significant association between genetic structuring and geography. Inferences about the historical population processes in the species complex are discussed, being in general in accordance with contiguous range expansions and past fragmentations. The use of the cohesion species concept approach suggests the existence of several systematic ranks among the different *T. goettingensis* populations, which is in part supported by ecological traits such as trophic selection and altitudinal distribution.

Keywords: cohesion species concept, mtDNA phylogeny, nested cladistic analysis, phylogeography, population structure and history, *Timarcha* leaf-beetles

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Introduction

Coalescent theory is increasingly being applied to studies relating haplotype frequency, genealogy and geographical distribution of populations, i.e. phylogeography (Avise 1989; Hudson *et al*. 1992; Templeton 1998a, references therein). The information contained in gene genealogies allows us not only to describe genetic structuring in space but to use the evolutionary history to develop hierarchical analysis of the spatial distribution of genetic variation (see Templeton 1998a for a review). In addition, this analysis can give some clues about the population history (range expansion, colonization or past fragmentation) and population structure (Templeton *et al*. 1995). The method relies on a previous estimation of the validity and limits of parsimony, building a network connecting the obtained haplotypes (Templeton *et al*. 1992). This network is then hierarchically subdivided in a nested design (Templeton *et al*. 1987; Templeton & Sing 1993), and the outcome is then used to test for associations of haplotypes to different variables using permutation chi-squared contingency tests (Roff & Bentzen 1989; Hudson *et al*. 1992; Templeton *et al*. 1995). The assessment of geographical subdivision in a set of populations and of the historical processes that led to such subdivision can be studied in more detail using the recently developed 'nested geographical distance analyses' as described in Templeton *et al*. (1995). Predictions from the coalescent theory and analyses on several case studies show that the criteria of the method are valid and robust (Templeton 1998a, references therein). This approach to the analysis of spatial genetic variation is complementary to the use of the traditional *F* and related statistics (Wright 1951; Nei 1982; Slatkin & Barton 1989; Lynch & Crease 1990), basically because classical methods ignore the historical factors affecting the geographical distribution of DNA haplotypes. For instance, *F* statistics can lead to a conclusion of gene flow among genetically uniform populations even in their absence, just because of a recent colonization of the area under study (Larson 1984) or because of shared ancestry

Correspondence: J. Gómez-Zurita. Fax: +34-971-173184; E-mail: dbajgf4@clust.uib.es

of the populations isolated by a past fragmentation event (Templeton *et al*. 1995).

Leaf beetles of the genus *Timarcha* constitute an interesting system to address questions concerning the patterns and factors acting in speciation. Apterism, mountainous habitats, and distributions dependent on the presence of their host plants are factors promoting isolation and eventually leading to speciation in these insects. The *Timarcha goettingensis* complex is a monophyletic assemblage of closely related chrysomelids (Petitpierre 1970a; Gómez-Zurita *et al*. 2000). This species complex is distributed from the north half of the Iberian Peninsula, where they show their highest diversification, to central Europe (Bechyné 1948; Jeanne 1965). The Iberian populations of the complex have allopatric distributions and their morphological variation can be related to altitudinal and latitudinal clines (Petitpierre 1970a). The species complex was originally described based on a common highly conserved karyotype of $2n = 20$, with many synapomorphies in the shape and size of the chromosomes (Petitpierre 1970a, 1970b, 1976). Most species of the complex feed on one or a few closely related species of one plant family (usually Rubiaceae or Plantaginaceae), and therefore a population is adapted to the local host plant species but retains the potential to feed on others of the same genus as some laboratory tests suggest (Jolivet & Petitpierre 1973). However, some *Timarcha* species including those of the complex show plant affiliation to single species belonging to different families (usually Rubiaceae and Plantaginaceae, Dipsacaeae or Asteraceae). Thus, oligophagy is defined for this genus, and particularly for the *T. goettingensis* complex, at the level of plant family and is probably related to the temporal and spatial availability of chemically similar plant resources (Jolivet & Petitpierre 1973; Becerra 1997).

We have recently shown, using a general phylogeny of the genus based on mitochondrial DNA (mtDNA), that there are several evolutionary lineages in the *T. goettingensis* complex concordant with their distribution areas. At least two monophyletic species groups can be clearly distinguished: one of the groups is distributed from Central Europe to northeast of the Iberian Peninsula, and the other is distributed over the remaining north half of the Iberian Peninsula (Gómez-Zurita *et al*. 2000).

In the present study, we use partial cytochrome oxidase subunit II (COII) mitochondrial sequences from beetles in 31 sampling localities and the methods outlined above to assess the contribution of population structure and/or population history as factors explaining the geographical distribution of mtDNA haplotypes in the *T. goettingensis* complex. We are also interested in linking the phylogenetic information and the proposed morphology-based systematics for the taxa of the complex under the assumptions of the cohesion species concept (Templeton 1989).

Materials and methods

Sampling

We have obtained samples from 133 specimens of different taxa in the *Timarcha goettingensis* complex. Figure 1 shows the sampling localities, species names and number of specimens studied from each sampled population.

Data set obtention

DNA extractions of each individual, PCR amplifications of COII and sequencing protocols are the same as given in Gómez-Zurita *et al*. (2000). PCRs for the amplification of the COII gene were performed using the LR-N-13398 and a modified LR-J-12887 (with an extra CT in the 5′ end and 3 nucleotides shorter in the 3′ end) set of primers (Simon *et al*. 1994). PCR products were used for direct sequencing using a modified LR-N-13398, 8 nucleotides shorter in its 5′ end and labelled with digoxigenin. The COII fragment selected for this analysis comprises 354 bp of the 5′ end of the gene, which was unambiguously aligned without gap insertions using Clustal W, version 1.7 (Thompson *et al*. 1994). The nucleotide diversity (π), defined as the average number of nucleotide differences per site between two sequences and its standard error (Nei 1987), was calculated using DnaSP version 3.14 (Rozas & Rozas 1999). The sequence haplotypes have been deposited in EMBL data library under accession numbers AJ389386–AJ389423.

Haplotype network estimation

The phylogenetic reconstruction algorithm was described in Templeton *et al*. (1992). This method was designed to estimate phylogenies at low levels of divergence (intraspecific data) but it also proved to be reliable at higher divergences, outperforming parsimony and parsimony with bootstrapping (Crandall 1994). In summary, this method starts calculating the overall limits of parsimony for the complete data set using a statistic from neutral coalescent theory (Hudson 1989). DnaSP version 3.14 (Rozas & Rozas 1999) was used to estimate Watterson's θ (Watterson 1975) from our sample. Once the use of parsimony is justified, the method estimates a single or several networks with connections having probabilities above the 0.95 limit, although nonparsimonious connections can be considered as well (Templeton *et al*. 1992). These probabilities were estimated using the software ParsProb version 1.0 by David Posada which implement the algorithm in Templeton *et al*. (1992). The software by David Posada used in the present study is available at the web site http://bioag.byu.edu/zoology/crandall_lab/programs.htm. PAUP* vs. 4.0b65 by D. L. Swofford was used to help in the network construction.

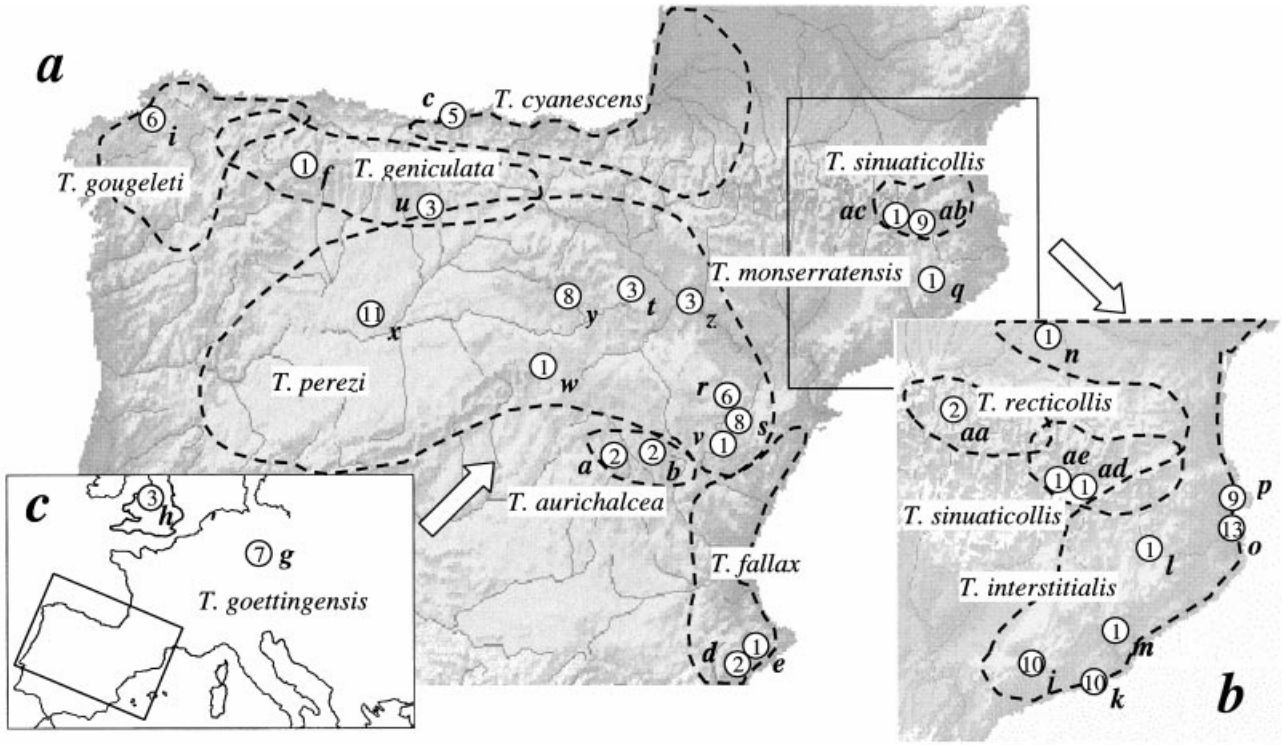

Fig. 1 Map showing sampling localities for the analysed taxa of the *Timarcha goettingensis* complex. Each locality is identified with the same letter code given in Table 1. Sample sizes are indicated in the circles and the geographical distribution of the taxa is delimited by dashed lines.

Nested design and geographical permutational contingency analysis

The nesting algorithm described in Templeton *et al*. (1987) and Templeton & Sing (1993) allows us to identify clades grouped by mutational changes step by step until the final level of nesting comprises the entire tree. As the nesting level is related to divergence, it is supposed to be directly correlated with evolutionary time. The detection of significant geographical associations of haplotypes is performed with exact permutation contingency analysis, where clades with genetic and/or geographical variation within a nested category are tested against their geographical locality (Roff & Bentzen 1989; Hudson *et al*. 1992; Templeton *et al*. 1995). The exact permutation contingency analysis was performed using the software Chiperm version 1.0 by David Posada, randomly permuting 10 000 times the lower-level clade categories in a nesting clade vs. the geographical localities.

Nested geographical distance analysis

In addition to restricted gene flow, some historical population events can produce nonrandom geographical associations, including range expansions, colonization or population fragmentation. In order to distinguish among these situations, Templeton *et al*. (1995) developed a statistical method relating the nested haplotype networks and the geographical distances between sampling localities. Briefly, this method works by estimating two different geographical measures in the context of the hierarchical design: clade distance of a clade X, Dc(X), and nested clade distance of clade X, Dn(X). Dc(X) informs of the geographical extend of clade X, whereas Dn(X) measures how far individuals of clade X are from those in the closest evolutionary sister clades (i.e. clades in the same nesting category as X). The distribution of these distance measures under the null hypothesis of no geographical associations in a given nesting clade is obtained, recalculating both of them after 10 000 permutations of the low-level nesting clades vs. the sampling localities. This procedure allows us to discriminate for nonindependent geographical distributions of haplotypes at the 5% level of significance, and once the null hypothesis of no geographical associations has been rejected it is possible to recognize patterns of short- or long-distance dispersal of populations by a joint analysis of Dc and Dn (Templeton *et al*. 1995).

The same random permutation procedure can be used to test different predictions of several models of population structure and historical events, but in this case a temporal polarity in the network of haplotypes is needed (Templeton *et al*. 1995). According to the neutral coalescent theory, this polarity is provided by the haplotype frequencies

and the topology of the network, after distinguishing between tip and interior clades (Castelloe & Templeton 1994). The limitations in the assessment of relative clade ages of coalescent theory can be ignored in the nested analysis, because the statistical estimations are restricted to evolutionary close clades represented in a nested category, so that interior clades tend to be the older clades and tip clades the younger ones. The expected patterns under each model of restricted gene flow, range expansion and population fragmentation are described elsewhere (Templeton 1993; Templeton *et al*. 1995; Templeton & Georgiadis 1996; 1998a). The geographical distance analysis using permutation testing which was mentioned was performed using version 2.0 of the program GeoDis written by D. Posada, K. Crandall and A. Templeton.

Testing cohesion species

The cohesion species concept synthesizes other approaches based on evolutionary, ecological, isolation and recognition definitions (Templeton 1989; de Queiroz 1998). Testing the null hypotheses of no differentiation across lineages with respect to traits contributing to genetic exchangeability and/or ecological interchangeability has been proposed as an explicit method to identify cohesion species (Templeton 1998b). Two kinds of traits are usually considered: (i) isolating or fertilization mechanisms; and (ii) adaptive and niche characteristics (selective regimes; Baum & Larson 1991; Templeton 1994). The available data concerning isolation mechanisms (either prezygotic or postzygotic isolation) is scarce for *Timarcha*, but oligophagy and alpine habitats have been proposed as mechanisms promoting speciation in many phytophagous insects with narrow distributions (e.g. Dobler *et al*. 1996). Accordingly, we have applied permutational contingency analysis to test the null hypothesis of no association of haplotypes in different nesting categories with trophic selection at the plant family level, and altitude of habitats distinguishing between localities above and below 1000 m, as qualitative variables. Table 1 shows the altitudes of the collection sites and the four trophism categories considered for the taxa included in the analysis. With regard to trophic selection, we have used the data from the literature on the host-plant affiliation for the studied taxa (e.g. Jolivet & Petitpierre 1973). Most of the taxa of the *T. goettingensis* complex feed on several species of Rubiaceae (*Rubia* spp., *Galium* spp.), which is the ancestral food choice for the genus in its Palearctic distribution (Jolivet & Petitpierre 1973; Gómez-Zurita *et al*., 2000). This trophism has been described for *T. gougeleti*, *T. interstitialis*, *T. monserratensis*, *T. recticollis* and *T. sinuaticollis*. The taxa *T. perezi* and *T. geniculata* have a mixed regime on Rubiaceae and Plantaginaceae depending on the seasonal availability of these plants. Other species, such as *T. fallax* and *T. goettingensis* basically feed on Rubiaceae (*Galium*), but also on *Scabiosa maritima* (Dipsacaceae). Finally, *T. cyanescens* feeds both on Rubiaceae and Plantaginaceae, but has also been described feeding on *Centaurea* sp. (Asteraceae) (Tiberghien 1972).

Results

COII variation

We have obtained 38 haplotypes from 133 studied individuals (Table 1). Most of the samples in which more than five individuals were sequenced are monomorphic or have a predominant haplotype plus a single variant differing by one to three changes. The only two exceptions are the sample of *Timarcha cyanescens* from locality *c*, which has four individuals with an identical haplotype and one individual having a diverged haplotype showing 13 substitutions with respect to the former ($\pi = 0.015 \pm 0.009$). The second case refers to the sample of *T. interstitialis* from locality *p*, which exhibited five different haplotypes in the nine analysed individuals ($\pi = 0.004 \pm 0.001$). On the other hand, each sampling locality shows unique haplotypes with the exception of Sant Pere de Roda (locality *p*), which shares haplotypes with localities *o* and *k* (all three samples attributed to *T. interstitialis*). The sampling localities *r*, *s*, *t* and *u* share the most common haplotype, although the latter comes from a different morphological species (*T. geniculata*).

The 38 haplotypes of the 354 bp COII fragment showed 45 synonymous and 12 nonsynonymous substitutions (see Fig. 2), this corresponds to an average uncorrected divergence among haplotypes of 3.75 (range 0.28–6.22).

Haplotype network and nested design

Evaluation of the limits of parsimony [equation 10 in Hudson (1989)] yielded a value of $H = 0.087$ ($\theta = 0.028$; $n = 38$), higher than the suggested 0.05 limit value for parsimony, and therefore we proceeded with the cladogram estimation (Templeton *et al*. 1992). This procedure starts calculating the maximum number of mutational steps between haplotypes allowing parsimonious connections with a probability equal or higher than 0.95. The value obtained for our data is eight steps with a probability of $P_8 = 0.950$. The parsimony unrooted cladogram construction using the subsets of haplotypes within this limit yields three disjoint networks (Fig. 3). Network I includes 23 haplotypes obtained from Iberian endemics of the *T. goettingensis* complex collected in 20 different sampling localities (Fig. 1a). Network II (Fig. 3) comprises all the remaining haplotypes sampled from the northeast region in the Iberian Peninsula plus one locality in Southern France (Fig. 1b). These haplotypes belong to European or

Table 1 Taxa, sample localities, geographical coordinates, elevation of sample localities, and number of individuals for *Timarcha goettingensis* complex COII haplotypes. Host plant affiliation for the species included in the analysis are also given

Haplotype number	Taxa	Location	Geographical coordinates*	Location altitude†	Trophic selection‡	No. of individuals
1	*T. aurichalcea*	Tragacete (a)	40.35, −1.85	~1300	R/P	1
2	*T. aurichalcea*	Tragacete (a)	40.35, −1.85	~1300	R/P	1
3	*T. aurichalcea*	Guadalaviar (b)	40.40, −1.72	1790	R/P	1
4	*T. aurichalcea*	Guadalaviar (b)	40.40, −1.72	1790	R/P	1
5	*T. cyanescens*	Cueto (c)	43.48, −3.80	Sea level	R/P/A	4
6	*T. cyanescens*	Cueto (c)	43.48, −3.80	Sea level	R/P/A	1
7	*T. fallax*	Port de la Carrasqueta (d)	38.61, −0.48	1020	R/D	2
8	*T. fallax*	Serra d'Aitana (e)	38.65, −0.32	1200	R/D	1
9	*T. geniculata*	Puerto de Ventana (f)	43.05, −6.00	1587	R/P	1
10	*T. goettingensis*	Jena (g)	50.85, 11.52	< 500	R/D	6
11	*T. goettingensis*	Jena (g)	50.85, 11.52	< 500	R/D	1
12	*T. goettingensis*	Stinchcombe (h)	51.68, 2.38	< 500	R/D	3
13	*T. gougeleti*	Bergondo (i)	43.33, −8.23	Sea level	R	5
14	*T. gougeleti*	Bergondo (i)	43.33, −8.23	Sea level	R	1
15	*T. interstitialis*	Alió (j)	41.28, 1.30	266	R/D	1
16	*T. interstitialis*	Alió (j)	41.28, 1.30	266	R/D	9
	T. interstitialis	Garraf (k)	41.27, 1.97	Sea level	R	9
	T. interstitialis	L'Esquirol (l)	42.03, 2.37	693	R	1
	T. interstitialis	Sant Just Desvern (m)	41.37, 2.08	~165	R	1
17	*T. interstitialis*	Caramany (*n*)	43.53, 1.75	~240	R	1
18	*T. interstitialis*	L'Escala (o)	42.13, 3.13	Sea level	R	13
	T. interstitialis	Sant Pere de Roda (p)	42.32, 3.17	540	R	1
19	*T. interstitialis*	Sant Pere de Roda (p)	42.32, 3.17	540	R	1
20	*T. interstitialis*	Sant Pere de Roda (p)	42.32, 3.17	540	R	5
	T. interstitialis	Garraf (k)	41.27, 1.97	Sea level	R	1
21	*T. interstitialis*	Sant Pere de Roda (p)	42.32, 3.17	540	R	1
22	*T. interstitialis*	Sant Pere de Roda (p)	42.32, 3.17	540	R	1
23	*T. monserratensis*	Collformic (q)	41.80, 2.33	1145	R	1
24	*T. perezi*	Ejulve (r)	40.78, −0.53	~1200	R/P	5
	T. perezi	Puerto de Cuarto Pelado (s)	40.55, −0.55	1600	R/P	8
	T. perezi	Moncayo (t)	41.80, −1.85	> 2000	R/P	3
	T. geniculata	Peña Amaya (u)	42.67, −4.17	1200	R/P	3
25	*T. perezi*	Ejulve (r)	40.78, −0.53	~1200	R/P	1
26	*T. perezi*	Puerto de Linares (v)	40.33, −0.53	1720	R/P	1
27	*T. perezi*	Layna (w)	41.10, −2.30	1100	R/P	1
28	*T. perezi*	Villanubla (x)	41.72, −4.83	710	R/P	11
	T. perezi	Puerto de Oncala (y)	41.95, −2.32	1454	R/P	7
29	*T. perezi*	Puerto de Oncala (y)	41.95, −2.32	1454	R/P	1
30	*T. perezi*	Zaragoza (z)	41.62, −0.92	197	R/P	1
31	*T. perezi*	Zaragoza (z)	41.62, −0.92	197	R/P	2
32	*T. recticollis*	Pla de l'Artiga (aa)	42.75, 0.75	1550	R	1
33	*T. recticollis*	Pla de l'Artiga (aa)	42.75, 0.75	1550	R	1
34	*T. sinuaticollis*	Coll de la Creueta (ab)	42.32, 1.98	2025	R	8
35	*T. sinuaticollis*	Coll de la Creueta (ab)	42.32, 1.98	2025	R	1
36	*T. sinuaticollis*	Vall de Núria (ac)	42.40, 2.13	2200	R	1
37	*T. sinuaticollis*	Queixans (ad)	42.40, 2.07	1125	R	1
38	*T. sinuaticollis*	Viliella (ae)	42.42, 1.72	1565	R	1

*Geographic coordinates are given in decimal degrees.

†Altitudes are given in meters. Several instances in which the altitude of collection site is approximate are indicated with appropriate symbols.

‡Trophic categories are specified for each species at the plant family level: R (Rubiaceae), R/P (Rubiaceae/Plantaginaceae), R/D (Rubiaceae/Dipsacaceae) and R/P/A (Rubiaceae/Plantaginaceae/Asteraceae).

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                    11111111111111111122222222222222233333333333
  12334556666779011233445566668899111333447788890011223334
  48136214036927454595714032356692803614907693895394514035 4
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  *              **  *  *     * *        * *  * **  *     **
1 TATTGCAAATTTTAATTGTTTTCTCTCATACTAATATCCCTTAATAAAAACATTTAC
2 .............................................G............
3 ............................G.........T................
4 ............................G.........T.C..............
5 ...C......CC...C.A.C..T..CT.CGT..T...T...........T......
6 ...CATG....C..GC...C..T..C....T..T.................G.C...
7 ...C...........G.........C...GT...........G..G..CA......
8 ...C..................T..C...GT..............G........C.
9 ...C....T.C...GC...C..TC.C..CGT..T................T......
10 .T.C..........CC.A....T....TC.T..T.GA..T....CG....T......
11 .T.C........C.CC.A....T....TC.T..T.GA..TC...CG....T..C...
12 .T.C..........CC.A....T....TC.T..T.GA..T.C..CG....T......
13 ...CAT........GC...C..T..C....T..TC...........G...T..C...
14 ...CAT........GC...C..T..C....T..TC..............T..C...
15 .T.C.....C..?CGC..C...T....T..T..T..CT....T.CG....T.C....
16 .T.C.....C....GC..C...T....T..T..T..CT....T.CG....T.C....
17 .T.C.....C....GC..C...T....T..T.GT...T....T.CG....T.C....
18 .T.C.....C....GC.AC...T....T..T..T..CT....T.CG....T.C....
19 .T.C.....C....GC......T....T..T..T..CT....T.CG....T......
20 .T.C.....C....GC..C...T....T..T..T..CT....T.CG....T......
21 .T.C.....C....GC.AC...T....T..T..T..CT....T.CG....T......
22 .T.......C....GC.AC...T....T..T..T..CT....T.CG....T.C....
23 ...C..........GCA.CA.CT...T...T..T...T.A..........T......
24 ...C......C...GC...C..TC.C..CGT..T................T......
25 ...C......C...GC...C..TC.C..CGT..T..............G.T......
26 ...C..........GC...C..TC....C.T..T................T...C.T
27 ..CC......C...GC......T..C..CGT..T................T......
28 ...C......C....C...C..T..CT..GT..T...T...........T......
29 ...C......C....C...C..T..CT..GT..T................T......
30 ...CATG....C..GC...C..T..C....T..T.......C...........C...
31 ...CATG....C..GC...C..T.TC....T..T..................C...
32 AT.....G.C.....C......T....T..T..T...T....T.......T......
33 AT.....G.C.....C......T....T..T..T...TT...T.......T......
34 ...C..........GCA..A.CT...T...T..T...T.A..........T......
35 ...C..........GCA..A.CT...T.C.T..T...T.A.......G..T......
36 ..............GCA..AA.T...T.C.T..T.....A..........T......
37 .T.C.....C....GC..C...T....T..TC.T..CT....T.CG....T.C....
38 .T.C.....C....GC..C...T..??...T..T..CT....T.CG....T.C....
   X       X    XX  X  X                    X XX  X     XX
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Fig. 2 Sequence alignment of the variable positions found within 38 mtDNA COII haplotypes. Only variable positions are shown and dots indicate identity with the COII haplotype 1. First and second codon positions are identified with one or two asterisks, respectively. An 'X' symbol below a particular position indicates that the corresponding polymorphism determines an aminoacid change.

Pyrenean endemics which are supposed to have colonized the Iberian Peninsula through the Oriental Pyrenees (Bechyné 1948; Jeanne 1965). Network II shows a loop of ambiguity due to homoplasy in the mtDNA sequences resulting in four most parsimonious trees. Finally, the three haplotypes of network III were obtained from populations of the *T. goettingensis s. str.* from central Germany and England (Fig. 1c). These three clades are concordant with the general *Timarcha* species mitochondrial phylogeny published elsewhere (Gómez-Zurita *et al*. 2000), in which the COII and 16S mtDNA sequences gave similar and compatible topologies (although the one based on the latter is less resolved) for the *T. goettingensis* species complex.

Alternative topologies to the most parsimonious networks can be assessed with the formula (formula 9) given in Templeton *et al*. (1992), which calculates the cumulative probability for both parsimonious and non-parsimonious connections. If this probability is above the 0.95 confidence limit, near-parsimony connections can also be considered in cladogram estimation. The exact limit of parsimony for our data is 21 substitutional steps. Therefore, the three networks can be merged in a single one with connections involving 9 (between networks II and III) and 11 (between networks I and II) mutational steps (Fig. 4). The link between networks II and III determine one loop of ambiguity intersecting with the one found in network II.

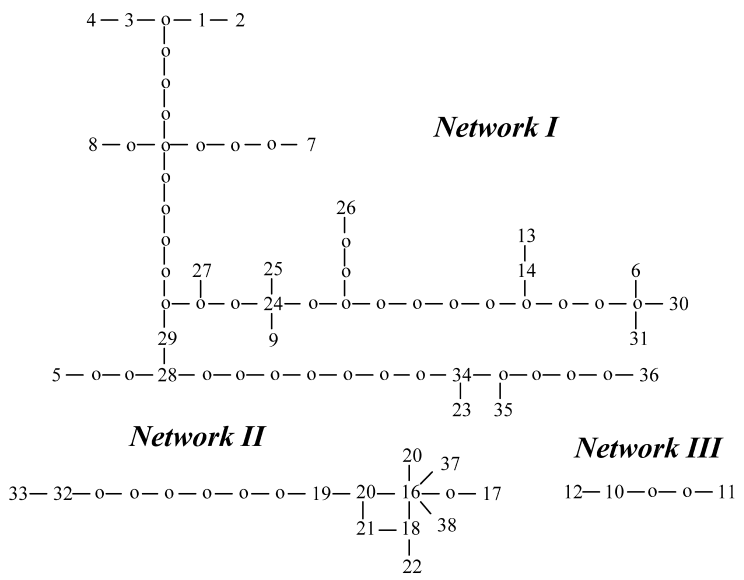

Fig. 3 The 8-step haplotype networks for the COII haplotypes from the analysed samples of the *Timarcha goettingensis* complex. These networks represent the most parsimonious connections for the set of haplotypes within the statistical limits of parsimony for the data set using the algorithm in Templeton *et al*. (1992). Each bar represents a single mutational step and zeros indicate inferred intermediate haplotypes not observed in the sample.

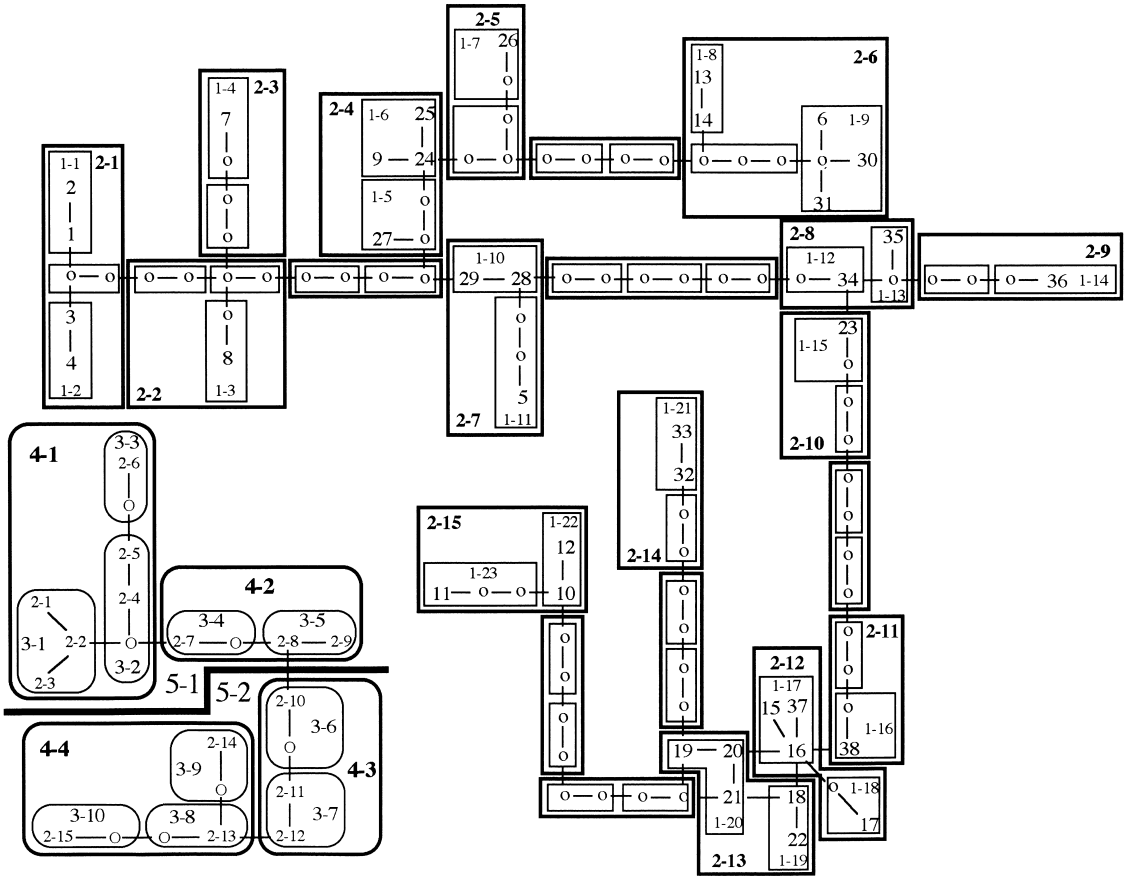

Fig. 4 Unrooted COII gene tree for the *Timarcha goettingensis* populations and associated nested statistical design. The haplotype network is the estimated 95% plausible set of cladograms obtained for COII haplotypes with the algorithm in Templeton *et al*. (1992). Bars represent parsimonious connections between haplotypes with a probability higher than 95% and each one corresponds to one mutational step. Intermediate haplotypes missing in the sample are represented by '0'. Thin-lined boxes indicate 1-step clades or nestings of haplotypes and are designated with '1-*n*', where *n* corresponds to the specific number of clade. Thick-lined boxes are 2-step clades grouping 1-step clades. For clarity, higher nesting levels are shown in the bottom-left inset. 3-step clades are indicated with thin-lined boxes with rounded corners, 4-step clades with thick-lined boxes with rounded corners and the two 5-step clades in which the entire network is subdivided are separated by a thick line. Two intersecting loops of ambiguity in the cladogram do not affect the nesting categories.

Table 2 Nested contingency analysis of geographical associations

Clade	Permutational chi-square statistic	Probability
1–6	23.579	0.059
1–9	4.000	0.500
1–10	1.451	0.427
1–17	23.210	0.261
1–19	6.964	0.136
1–20	0.381	1.000
1–22	9.000	0.013*
2–1	4.000	0.339
2–4	22.000	0.046*
2–6	10.000	0.004*
2–7	23.000	< 0.001*
2–12	23.000	0.129
2–13	16.143	< 0.001*
2–15	0.476	1.000
3–1	14.000	0.028*
3–2	23.000	0.088
3–5	10.000	0.099
3–7	24.000	0.209
4–1	80.000	0.000*
4–2	33.000	0.000*
4–3	25.000	0.208
4–4	70.000	0.000*
5–1	69.770	0.000*
5–2	56.297	0.000*
Entire cladogram	133.000	0.000*

*Significant at the 0.05 level.

Figure 4 also shows the results of the nested design using the rules given in Templeton *et al*. (1987) and Templeton & Sing (1993). The above-mentioned loops of ambiguity represent 15 alternative most parsimonious trees that do not affect the resolution of the nested design.

Nested contingency analysis for geographical subdivision

Table 2 shows the results of the nested contingency analysis for the COII haplotypes in the 31 localities included in this study. This test was performed for nested categories with genetic and geographical variation. Probabilities were calculated permuting the one-step lower categories within the analysed clade vs. the sampling localities included in that clade. Only one of the 1-step clades (1–22) shows clear geographical association of its haplotypes, while four 2-step clades (2–4, 2–6, 2–7 and 2–13) have a significant level of geographical subdivision. The null hypothesis of no geographical association can be rejected with high significant values in all but four higher-level categories (see Table 2).

Figure 5 shows the results of the nested geographical distance analysis (see the Materials and methods for details), whereas the inferences about the population structure and demography deduced in the *T. goettingensis* complex following Templeton *et al*. (1995) are outlined in Table 3.

Nested contingency analysis and cohesion species concept

As stated above, there are multiple evolutionary lineages in the set of studied populations, and therefore potentially each evolutionary lineage can be a species under the cohesion definition (Templeton 1989, 1994; 1998b). The null hypothesis of a single lineage in our sample is rejected at different hierarchical levels, and this is particularly clear for the two higher-nesting categories in the haplotype network, which are the best candidates to delimit distinct species. Nevertheless, as indicated by Templeton (1994, 1998b), the identification of different lineages is a necessary but not sufficient condition to infer the existence of more than one cohesion species.

The left half of Fig. 6 shows the evolutionary structured lineages identified in our samples using the permutation contingency test procedure (Table 2). As can be seen, we have found lineages concordant with a single taxon or morphospecies (e.g. *T. aurichalcea*, *T. fallax*, *T. gougeleti*, *T. goettingensis*, and *T. recticollis*). On the contrary, two types of conflicts with current taxonomy can be distinguished in the remaining populations: first, two or more closely related taxa constitute a single lineage (e.g. clade 3–2 containing *T. perezi* and *T. geniculata*) and, second, a single morphospecies shows haplotypes falling into different lineages (as it is deduced for *T. perezi*, *T. cyanescens*, *T. sinuaticollis* and *T. interstitialis*). These conflicts could be either a result of erroneous taxonomic assignment or the result of mitochondrial introgression. Permutational contingency tests randomly permutating the clades within the analysed category vs. the current taxonomic status of the specimens included in it have been used to statistically test the above-mentioned associations. Here, the null hypothesis is of no association between the currently recognized morphospecies and the obtained haplotype genealogy. The general pattern obtained using this approach is an agreement between these variables, but there are also some conflicts between parapatric closely related taxa, as for *T. perezi*, *T. geniculata* and *T. cyanescens* in clade 5–1, or *T. interstitialis* and *T. sinuaticollis* in clade 5–2.

To assess if these lineages are candidates to be treated as different cohesion species, we tentatively applied the ecological cohesion criteria of trophic selection and altitudinal distribution (see the Materials and methods section and legend to Fig. 6 for details of the qualitative variables considered for each species). The results of the permutation contingency tests using these variables and the corresponding nested categories are summarized in the right half of Fig. 6, where the statistically significant cases of differential cohesion patterns are identified. The analysis

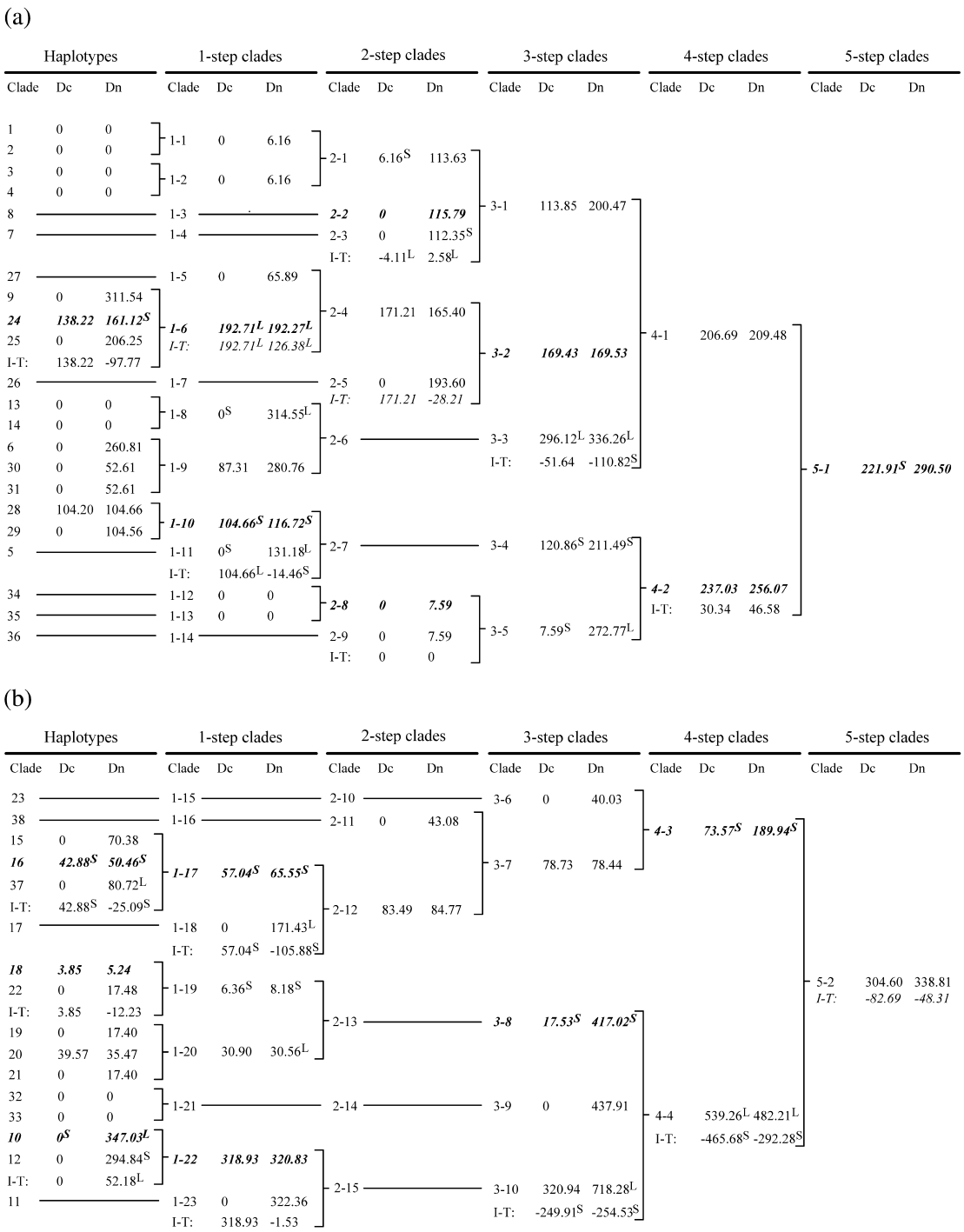

Fig. 5 Results of the nested geographical distance analysis for the *Timarcha goettingensis* complex COII haplotypes in 5–1 (a) and 5–2 (b) clades. The hierarchical structure represented in this figure is the same as the one given in Fig. 4. From left to right, the different columns indicate increasing nesting levels, from haplotypes to 5-step clades. Brackets reflect the nesting structure, grouping in a given clade the immediate low-level nesting categories. For each clade both clade (Dc) and nested clade (Dn) distances are given (for details see Templeton *et al*. 1995). When the nested geographical distance analysis detects a significant difference between the observed and the expected distances under a situation of random geographical distribution of haplotypes, it is indicated with a superscripted 'S' (for distances significantly smaller than expected) or 'L' (for distances significantly larger than expected). In clades where tip/interior status can be determined, the result of the interior vs. tip clades test is indicated with 'I-T:' in the corresponding clade. In these cases, the interior clade is given in italics and bold type. For the entire cladogram and clades 2–4 and 3–2, the tip/interior status is inferred using the outgroup method for the former and predictions from coalescent theory for the latter two (Castelloe & Templeton 1994), and the result of the interior vs. tip clades test is given in italics.

Table 3 Demographic inferences using the results of the nested geographical distance analysis and the inference key given in Templeton *et al*. (1995)

Clade	Inference chain	Inferred pattern
1–6	1–2-11–17–4-9-NO	Past fragmentation
1–17	1–2-11–12-NO	Contiguous range expansion
1–22	1–2-11–12–13–14-NO	Sampling design inadequate to discriminate between contiguous range expansion and long-distance colonization
2–4	1–2-3–4-9–10-NO	Sampling design inadequate to discriminate between fragmentation and isolation by distance
2–7	1–2-3–5-15-NO	Past fragmentation
2–12	1–2-11–12-NO	Contiguous range expansion
3–1	1–2-3–4-9–10-NO	Sampling design inadequate to discriminate between fragmentation and isolation by distance
4–1	1–2-11–12-NO	Contiguous range expansion
4–4	1–2-11–12-NO	Contiguous range expansion
5–2	1–2-11–12-NO	Contiguous range expansion
Total	1–2-3–4-9-NO	Past fragmentation

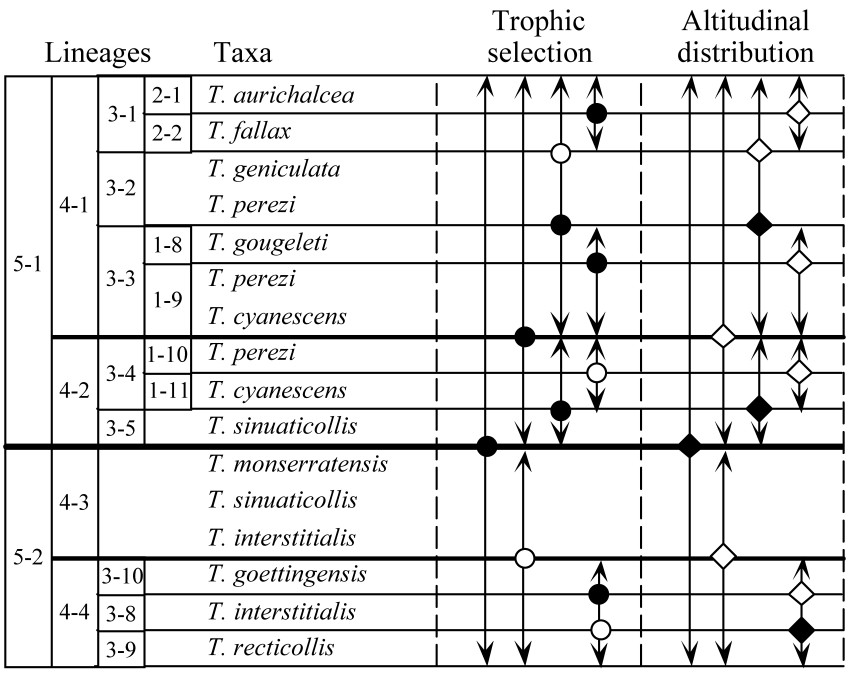

Fig. 6 Summary of the results of permutation contingency tests obtained applying the cohesion species concept in the samples of the *Timarcha goettingensis* complex. Horizontal lines delimitate the inferred genealogical lineages with interrupted gene flow and thus the candidates that are cohesion species (Templeton 1989). For each lineage the associated morphospecies are given. Vertical dashed lines delimit the results of the exact permutational contingency tests for the selected criteria of demographic interchangeability (e.g. trophic selection and distribution in altitude). The tests were applied to clades where low-level nesting categories constitute statistically different lineages; arrows show the categories tested in each case. The tests performed 10 000 random permutations of lower-level clade samples within a given clade vs. the categories for both tested variables (see Table 1). Rejection of the null hypothesis of no association between lineages in the tested clade and ecological traits are indicated with full circles (trophism) and squares (altitude). Empty circles or squares indicate nonsignificant results for the nesting categories tested.

rendered three instances in which both trophic selection and altitudinal distribution reinforce the lineage independence (5–1 and 5–2; 3–3 with respect to 3–1 and 3–2; 3–4 and 3–5). Other lineages show differences based on one of the cohesion mechanisms or suggest that there is not a clear interrelationship between evolutionary lineage and the tested ecological traits (Fig. 6).

Discussion

Population structure and demographic inferences

It has been shown elsewhere that nested analyses of haplotype cladograms with geographical data are more robust than *F*-statistics analyses to detect genetic and geographical associations (Templeton 1998a, references therein). Although the method, based on statistical parsimony, was originally designed for intraspecific data, it should also work consistently in the case of low divergences such as the species complex described here (Crandall 1994). The data obtained for *Timarcha goettingensis* and closely related taxa indicate a high level of geographical structuring of these beetle populations (Table 2). The rejection of the hypothesis of population structuring (i.e. random distribution of the haplotypes) is less common for higher-level nestings as can be expected if they are composed of divergent lineages. However, according to Templeton *et al*. (1995), small or inadequate sampling can

produce the same result by a lack of statistical power of the test, and this is more likely to occur in the lower-level nestings.

Nested geographical distance analysis and its associated predictions for different models of population structure and historical events allows us to infer the more probable causes of the observed geographical associations. Overall, the most recovered pattern after the application of the inference chain suggested by Templeton *et al*. (1995) is a model of contiguous range expansion (Table 3). This model was described by Cann *et al*. (1987), and assumes that the expansion of populations is due to dispersal of individuals in a short range. Examples of range expansion are found at all hierarchical levels of the nested design: clades 1–17, 2–12, 4–1, 4–4 and 5–2. However, in the case of the higher nestings, in which the hypothetical range expansions involve more than one lineage with allopatric distribution (i.e. 4–1), it is reasonable to believe that these demographic events predate the establishment of isolation mechanisms among taxa.

Expansion of populations of the taxon *T. interstitialis* can be inferred among the lower nestings, as exemplified in clade 1–17. These populations were sampled from localities in the coastal mountain chain of Catalonia to interior regions in the southern Pyrenees, and to the southeast of France across the Pyrenees (the result of the inference chain for clade 2–12 which includes 1–17 is a contiguous range expansion as well). The range expansion for these populations has probably occurred from coastal areas in the southern part of their distribution (older interior clades in the network) to northern inland areas in their range (younger tip clades). Another nesting series where contiguous range expansion is the inferred explanation for the observed distribution pattern of haplotypes is that of clades 4–4 and 5–2. This result can be related to a scenario of population expansion following European Pleistocene glaciations. Many different lines of evidence indicate that present distributions of western European taxa can be explained by a northward dispersal from a southern Iberian refugium (see Taberlet *et al*. 1998 for a review). In accordance with this explanation, the northern populations of the *T. goettingensis* complex (represented in our study by two very distant sampling localities) seem to harbour very few and closely related haplotypes ($\pi = 0.003 \pm 0.001$). Clade 1–22 includes two of the three haplotypes obtained in the two sampled locations of *T. goettingensis s. str.* Although the observed geographical structuring in clade 1–22 can be explained by range expansion, the absence of sampling in intermediate locations, where the species is actually present, does not allow us to distinguish between contiguous range expansion and long-distance colonization.

Clade 4–1 includes almost all haplotypes found for the Iberian endemics of the *T. goettingensis* complex. The only exceptions are haplotypes in clade 2–7, assigned to two *T. perezi* populations (localities *x* and *y*) and four out of five individuals of *T. cyanescens*. A model of contiguous range expansions seems to be the inference for this clade when applying the inference key, with an expansion centre origin in the northern Iberian plateau (interior clade 3–2), and two main dispersal routes (Fig. 7): northwards across the mountain barrier constituted by the Cantabrian and Sistema Ibérico chains (tip clade 3–3), and to the southeast following the Sistema Ibérico reaching the southern limit of the *T. goettingensis* complex distribution area (tip clade 3–1). The exact contingency test indicates a restricted gene flow among clades included in nesting level 4–1 ($P < 0.001$; Table 2). All taxa belonging to clade 3–3 were collected in lowland areas, while the samples represented in clade 3–2 are constituted by populations living from moderate to high altitudes (~1000 to > 2000 m). Therefore, altitudinal differences could act as an ecological barrier to gene flow between these two groups of taxa.

Another apparent barrier to gene flow is deduced between clades 3–1 and 3–2. The taxa in these two clades present a continuous geographical distribution following the Sistema Ibérico mountains, occurring in similar ecological habitats but showing marked morphological differences. They are classified as *T. perezi* and *T. aurichalcea* in the interior regions of the mountain chain and *T. fallax* in the littoral mountains of southeast areas (Fig. 1a). Interestingly, the closely related *T. perezi* ($2n = 20$, nine autosomal pairs + Xy_p) and *T. aurichalcea* ($2n = 18$, eight autosomal pairs + neoXY) differ chromosomally due to a presumed X–4th chromosome translocation in the latter species which changes the sex chromosome system (Petitpierre 1970b, 1976; J. Gómez-Zurita *et al*. unpublished results). This chromosomal fixed shift can very probably act as a strong barrier to gene flow between the two populations by hybrid disadvantage.

The population structuring observed within clade 3–1 (two samples of *T. aurichalcea* plus two samples of *T. fallax*) cannot be explained unambiguously, but the possible inferences are past fragmentation or isolation by distance (Table 3). Nevertheless, the outcome of the test indicates that more sampling effort in the intermediate areas occupied by these populations is needed to discriminate between both types of inference in this case. The result for clade 3–1 includes the possibility of population fragmentation, which is the second kind of demographic inference for the populations in the *T. goettingensis* complex. A past fragmentation inference is also obtained for clade 2–7 and this is a likely explanation for clades 1–6 and 2–4 as well, but again in the latter a more adequate geographical sampling scheme is needed to clearly distinguish between fragmentation and isolation by distance.

The higher nesting level in the *T. goettingensis* complex haplotype network forms two clades (5–1 and 5–2) which

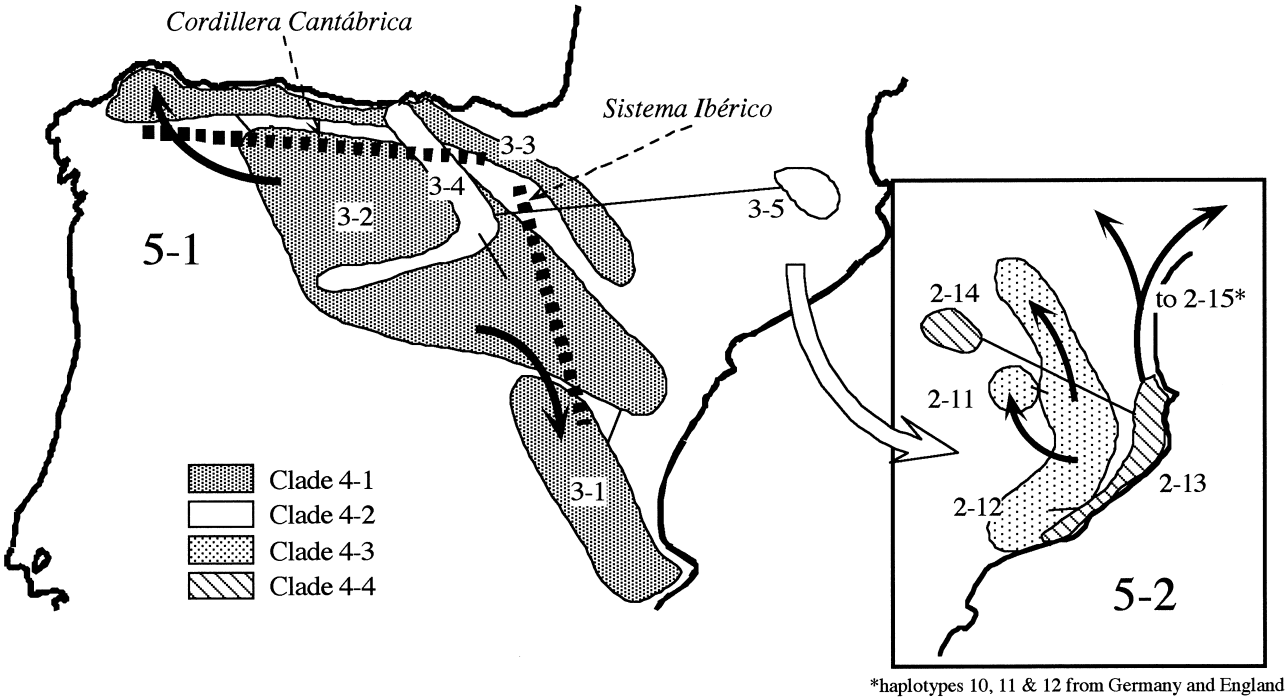

Fig. 7 An approximate geographical overlay of the main categories from the hierarchical design in the *Timarcha goettingensis* complex mtDNA haplotype network. The two highest nesting categories, clades 5–1 and 5–2, are given in different maps, the haplotypes of *T. goettingensis s. str.* (from Germany and England) are not included in the figure. Different shading patterns are used to approximately identify the 4-step clades, and connections among haplotypes from different clades are indicated with a single line. Arrows are used to show the instances in which an inference of contiguous range expansion of populations can be deduced.

have a strong geographical association of haplotypes ($P < 0.001$). The relative ages of these clades, prior to determining older (interior) and younger (tip) clades in the nested geographical distance analysis, are unknown. However, the most probable root for the analysed taxa of the complex would lie in an undetermined position in the 5–1 clade based on the results of the mitochondrial phylogeny for the whole genus (Gómez-Zurita *et al*. 2000). If we tentatively place the root in clade 5–1, the inference obtained for the geographical structure of the complex in the Iberian Peninsula is compatible with a population model of past fragmentation. There is a remarkable divergence between the 5–1 and 5–2 clades, with samples showing nonoverlapping distribution areas except for a group of localized taxa in interior mountain habitats of northeast Iberia and their corresponding haplotypes (haplotypes 23, 34, 35 and 36). In fact, for one of the taxa in this narrow overlapping area, *T. sinuaticollis*, we found haplotypes characteristic of both clades: haplotypes 34, 35, 36 in clade 5–1 and 37, 38 in clade 5–2.

The large genetic breakage between clades 5–1 and 5–2 can be related to vicariance caused by the geological history of the region. It has been suggested that palaeogeological events during the middle Miocene led to the formation of saline interior lakes in the central–north regions of the Iberian Peninsula, which could in turn be responsible for a vicariant speciation in several organisms. This factor has been invoked to explain the phylogenies of freshwater snails, fishes, midwife toads and mammals (Anadón *et al*. 1989; Altaba 1997).

Species inferences

Although food specialization in phytophagous insects has often been assumed to be a key factor in their speciation (Mitter *et al*. 1988; Farrell 1998), statistical tests able to discriminate among alternative hypotheses have rarely been applied. A 'cohesion species' has been defined as a group of organisms constituting a particular evolutionary lineage that represents a reproductive unit in genetic or adaptational/ecological sense (Templeton 1989, 1994). An application of the cohesion species concept (see de Queiroz 1998 for a critical review of species concepts and speciation criteria) to the different lineages found in the *T. goettingensis* complex and their associated ecological traits (see Fig. 6), allows us to assume the existence of different species within the analysed populations. Permutational chi-square contingency tests show that samples included in clades 5–1 and 5–2 tend to differentiate in terms of the plants they eat and in elevation. Interestingly, the taxa in the two clades present clear morphological fixed differences such as the absence or presence of a complete basal

margin in the pronotum, a character which has been used in the *Timarcha* taxonomy (Jeanne 1965). Lineage 5–1 can still be subdivided into two species under the cohesion criterion which are therefore supported by phylogenetic and ecological evidence.

A potential problem in the species inferences presented above lies in the fact that the evolutionary lineages are based upon a single locus gene genealogy (COII mtDNA sequences). Indeed, the majority of intraspecific or low-divergence phylogenies use mtDNA markers because of the advantages of this organelle genome (haploid, no recombination and shorter coalescent times than nuclear DNA). However, mitochondrial introgression (and ancestral lineage sorting) can potentially complicate the establishment of evolutionary lineages and, therefore, the species inferences deduced from them. Interspecific hybridization has been suggested for the pair of morphospecies *T. interstitialis* and *T. sinuaticollis* (Gómez-Zurita *et al*. 2000), which have overlapping distributions (Bechyné 1948; Jeanne 1965). To confirm this fact, both mitocondrial and nuclear data should be obtained from the same samples. Assuming that both kind of data would be available for the same individuals, the comparison of gene genealogies obtained from each marker can still be problematic given the different evolutionary dynamics of mitochondrial and nuclear sequences.

In summary, the results show that the *T. goettingensis* species complex constitutes an interesting model system to address questions such as phylogeographic patterns, the effect of historical and population factors in the present geographical distribution, speciation and the subjacent mechanisms of demographic interchangeability. The nested geographical cladistic analysis provides a valuable statistical tool to test several evolutionary hypotheses concerning these taxa. The results obtained in the present study show the importance of contiguous range expansion and past fragmentation of populations of *Timarcha* caused by palaeogeological events in southwest Europe and host plant specialization as ecological factors able to lead to speciation in these phytophagous insects.

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This work is part of the PhD of Jesús Gómez-Zurita dealing with phylogenetics and population genetics of *Timarcha* using molecular approaches. Carlos Juan is a lecturer in Genetics at the Balearic Islands University and has been carrying out research on Canary Islands beetle phylogenies for the last 6 years. He is interested in molecular evolutionary genetics and phylogeography. Eduard Petitpierre has been Professor of Genetics at the same University since 1981. He leads a research group with interest in evolutionary genetics of beetles both under chromosomal and molecular aspects.
