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The Evolutionary History of the Genus *Timarcha* (Coleoptera, Chrysomelidae) Inferred from Mitochondrial COII Gene and Partial 16S rDNA Sequences

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The apterous genus *Timarcha* consists of three subgenera and more than 100 species in its Palearctic distribution, with specialized feeding on few plant families. Fifty-four sequences sampled from 31 taxa of the genus plus three outgroup leaf beetles were studied for their complete cytochrome oxidase II (COII) and a fragment of 16S rDNA mitochondrial genes, representing a total of about 1200 bp. Phylogenetic analyses using maximum-parsimony and distance methods for each gene separately and for the combined data set gave compatible topologies. The subgenus *Metallotimarcha* consistently appears in a basal position and is well differentiated from the remaining *Timarcha,* but no clear monophyletic grouping of *Timarchostoma* and *Timarcha s. str.* subgenera can be deduced from our analysis. Calibration of the molecular clock has been done using the opening of the Gibraltar Strait after the Messinian salinity crisis (about 5.5 MYA) as the biogeographic event causing disjunction of two particular taxa. Accordingly, the COII evolutionary rate has been estimated to be of 0.76×10^{-8} substitution/site/year in *Timarcha.* Relation between phylogeny and host-plant use indicates widening of trophic regime as a derived character in *Timarcha.*

INTRODUCTION

The leaf beetles of the genus *Timarcha* (Coleoptera, Chrysomelidae) are popularly known as "bloody nose beetles" in Europe and "strawberry leaf beetles" in North America. They comprise more than 100 species distributed in Europe, Turkey, and Transcaucasian countries in West Asia, and North Africa from Morocco to Libya, and there are two representatives in the west coast of North America. The latter species, distributed from Vancouver to northern California are possibly relictual taxa. This peculiar disjunct distribution could be the product of vicariance after the formation of the North Atlantic fracture during the Tertiary (Jolivet, 1994). The *Timarcha* species are flightless phytophagous insects feeding mostly on herbaceous Rubiaceae and/or Plantaginaceae, depending on the availability of host plants. However, some taxa feed on Rosaceae (the only host family for the North American taxa), Brassicaceae, Scrophulariaceae, Dipsacaceae, or Asteraceae. Herbaceous Rubiaceae are hypothesized to be the ancestral host plant for the Old World *Timarcha* and switches to *Plantago* and other plants have probably been forced by ecological changes in their habitats (Jolivet and Petitpierre, 1973; Crowson, 1981).

These beetles occur from the high-altitude alpine habitats to humid forests in Central Europe, littoral Mediterranean areas, and even flatland regions or Saharian oases. More than a third of the described species occur as endemics in the Iberian Peninsula, in which they have experienced extensive local diversification. Bechyné (1948) divided the tribe Timarchini into 28 morphological-biogeographical groups and into four subgenera, one in the Nearctic (*Americanotimarcha*) and three in the Palearctic regions (*Timarcha s. str.*, *Timarchostoma*, and *Metallotimarcha*).

The subgenus *Timarchostoma* includes a group of morphological types similar to the species *T. goettingensis*, an alliance presenting many synapomorphies at the morphological and karyological levels. This group has been defined as the *Timarcha goettingensis* species complex (Petitpierre, 1970a), with the implicit proposition that an incipient speciation is occurring among the different local populations within the group. Patched distributions caused by low vagility and host-plant specialization are factors that can restrict gene flow in these beetles, hence promoting geographical isolation and speciation. However, to study this attractive model group of organisms under an evolutionary perspective, firm hypotheses are needed for the phylogenetic relationships within this clade of closely related taxa or for the major groups of *Timarcha*. Adult and larval morphological (Bechyné, 1948; Steinhausen, 1994) and karyological (Petitpierre, 1970b, 1976) data have provided many informative characters for such analysis but, in many cases, defining the polarity of some of the charac-

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ter states is problematic. Data on the extent of genetic variation present in different populations of these taxa in relation to geographical distribution and morphological diversification is also necessary. For these reasons, we have undertaken a study of mitochondrial and nuclear DNA sequences from *Timarcha* in addition to life history.

This paper presents the first insight into the molecular phylogeny of *Timarcha* using the complete mitochondrial cytochrome oxidase subunit II gene (COII) and a fragment of the large ribosomal subunit (16S) gene sequences, representing a total of 1.2 Kb. We have sequenced 54 individuals from 31 taxa representing all three Palearctic subgenera and most (about 75%) of the morphological-biogeographical groupings (Bechyné, 1948).

The COII gene has been extensively used to address phylogenetic questions in insects at different taxonomic levels: among orders (Liu and Beckenbach, 1992), within an order (Frati *et al.*, 1997), and more successfully within genus or species groups, including beetles (Beckenbach *et al.*, 1993; Emerson and Wallis, 1995; Mardulyn *et al.*, 1997, among others). There is a good knowledge of the structure and function of the protein encoded by COII (Capaldi, 1990) that enables one to predict which gene regions can suffer substitutional constraints during their evolution.

Part of the mitochondrial rDNA 16S sequences (16S) used in this paper have been reported elsewhere (Gómez-Zurita *et al.*, 1999). We have expanded considerably this preliminary data in order to perform separate and combined phylogenetic analyses of the two data sets to robustly ascertain the evolutionary relationships within *Timarcha*.

We address the following questions: (1) are the Palearctic subgenera monophyletic groupings?; (2) is the subgenus *Metallotimarcha* basal to the remaining *Timarcha*, as has been proposed?; (3) what is the level of genetic divergence and substitution rate for the major clades and, more specifically, within the *Timarcha goettingensis* species complex?; and finally, (4) can we infer the history of host-plant affiliation for this genus?

MATERIAL AND METHODS

Sampling

The complete list of taxa analyzed, their sources, and the classical subgeneric and morphological grouping are given in Table 1. For outgroup comparisons we have used sequences obtained from the chrysomelids *Coptocephala scopolina* and *Macrolenes dentipes* (Clytrinae) and a seed beetle, *Bruchus* sp. (Bruchidae), which some authors consider as belonging to chrysomelids. These outgroup taxa are part of two lineages that are believed to be basal divergences in the family Chrysomelidae (Reid, 1995; Farrell, 1998). Voucher specimens of all studied taxa have been deposited in the UIB Laboratory of Genetics beetle collection.

DNA Extraction, PCR, and Sequencing

DNA extraction and purification from single individuals were performed from abdomen (after removing the digestive tract) or head–thorax homogenates following standard techniques (Juan *et al.*, 1993). Primers used were a modified TL2-J-3037 (5′ TAATATGGCAGATTAGTGCATTGGA 3′) and a modified and slightly longer TK-N-3785 (5′ GAGACCATTACTTGCTTTCAGTCATCT 3′), following the primer nomenclature of Simon *et al.* (1994) for the amplification of a fragment of 777 bp containing part of the tRNA-Leu and the whole COII gene. LR-N-13398 (5′ CGCCTGTTTATCAAAAACAT 3′) and a modified LR-J-12887 (5′ CTCCGGTTTGAACTCAGATCA 3′) were used for the amplification of a fragment of about 550 bp of 16S rDNA. PCR conditions were as follows: 4 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a final single extra extension step at 72°C for 10 min. COII PCR products were sequenced in the two directions with digoxigenin-labeled primers and performing cycle sequencing reaction with the DIG *Taq* DNA Sequencing Kit for Standard Cycle Sequencing (Boehringer) following manufacturer's instructions. Sequences were run in a GATC 1500-System with Direct Blotting Electrophoresis. For 16S rDNA, PCR products were cloned using the pMOS*Blue* blunt-ended cloning kit (Amersham) and sequenced as above.

Sequence and Phylogenetic Analyses

COII sequence alignment was done by hand. At the 3′ end of the gene, the *Timarcha* sequences were difficult to align with those of the outgroups and alignment was performed by means of the inferred amino acid sequences. The codon position assignation was by comparison with those reported in Liu and Beckenbach (1992) and the inference of the amino acid sequences was obtained by using the *Drosophila* mitochondrial genetic code (De Bruijin, 1983).

The alignment of the 16S sequences was done with CLUSTAL W, version 1.7 (Thompson *et al.*, 1994). We found a highly variable region in ingroup/outgroup comparisons between position 241 and position 298. Different alignment conditions were tried for this region using MALIGN version 1.99 (Wheeler and Gladstein, 1992–1994).

Sequence analyses (nucleotide composition, substitution rates, etc.) were performed with test version 4.0d64 of PAUP* written by David L. Swofford, Li93 program (Li, 1993), and MacClade v. 3.0.3 (Maddison and Maddison, 1992). Estimates of nucleotide diversity and testing of natural selection with Tajima (1989) and Fu and Li (1993) tests were done with the program DnaSP v. 2.0 (Rozas and Rozas, 1997).

Maximum-parsimony (MP) and distance-based methods for phylogenetic inferences were applied using 4.0d64 test version of PAUP*. For MP analyses we used characters equally weighted, the heuristic algorithm, random stepwise addition with 25 replicates, ACCTRAN, and TBR swapping options. Distance analyses were performed using neighbor-joining and the Kimura two-parameter correction. Support for particular nodes was assessed by 500 bootstrap resamplings. To use COII and 16S data sets in a combined phylogenetic analysis, we assessed congruence between both partitions using the ILD test (Farris *et al.*, 1994; partition homogeneity test in PAUP*). This test is based on the incongruence index defined by Mickevich and Farris (1981), and the distribution of the ILD statistic is computed after randomization of the original data set partitions, constructing random data sets of the same size as the original and calculating their corresponding incongruence index. The incongruence threshold for this test is $P < 0.05$, although it is considered too conservative (Cunningham, 1997). The ILD test was performed using PAUP* with a heuristic search of 5000 replicates, TBR in effect, MULPARS not selected, and removing all invariant characters (Cunningham, 1997).

MacClade v. 3.0.3 (Maddison and Maddison, 1992) was used to map chromosome numbers and host plant affiliation on the cladograms and to calculate the minimum shifts expected under accelerated (ACCTRAN) or delayed (DELTRAN) transformations. Permutation tests (T-PTP option in PAUP*) were used to ascertain whether there are associations with the mitochondrial phylogeny and the observed distribution of character states for both chromosome numbers and trophic selection. This was accomplished by performing 10000 random permutations of the associated character state of the taxa using the topology of the mitochondrial phylogeny as a constraint tree. Finally, a distribution of tree lengths was obtained that allows testing whether the original tree length is significantly shorter than expected under a random model (Maddison and Slatkin, 1991).

Rate Constancy Test

In order to determine whether there is an evolutionary rate constancy at different divergence levels of COII and 16S *Timarcha* sequences, we applied the "relative-rate test" by Sarich and Wilson (1973) and the "two-cluster test" by Takezaki *et al.* (1995). The latter was computed using the software available at the ftp site of the authors. The two-cluster test is based on the comparisons of average substitution rates for two clusters (each with one or more sequences) branching off from an internal node in a given tree. Under the assumption of a molecular clock, the difference between the accumulation of substitutions per site in each of the two clusters is not expected to be significantly different from zero. The deviation of this difference from zero and its significance can be tested by a two-tailed normal test (Takezaki *et al.*, 1995).

RESULTS

COII Gene

All *Timarcha* species show 685 nucleotides (nts) for this gene encoding to 228 amino acids. As reported by Liu and Beckenbach (1992) for several insects, only the first position of the stop codon, a single T, is present in all studied *Timarcha* plus the outgroup *Bruchus* sp. In the two Clytrinae representatives (*Coptocephala scopolina* and *Macrolenes dentipes*) the stop codon seems to be complete (TAA). There is variability for the initiation codon as well; most *Timarcha* and *Bruchus* sp. show ATT, while the remaining *Timarcha* species show ATC (both being isoleucine codons). Finally, the Clytrinae sequences present ATG (methionine) as initiation codon. Similar variation has been described previously for COII sequences of several insects (Liu and Beckenbach, 1992). A comparison of the inferred amino acid sequences of *Timarcha* shows 52 variable positions of which 26 are conservative (with similar physicochemical properties) changes (French and Robson, 1983). Only 21 of these changes are informative under parsimony, and 3 (positions 95, 119, and 156) define unequivocally a group formed by *T. espanoli*, *T. rugosa*, and *T. punctella*.

Table 2 summarizes the nt composition and substitution patterns in both the COII and the studied fragment of 16S (see below). The sequences are on average 74.4% A + T biased, a well-established fact in insect mitochondrial sequences (Clary and Wolstenholme, 1985; DeSalle *et al.*, 1987). This is in part caused by a preference for codons ending in A or T (A + T percentage rises to 90.7% at third codon positions; see Table 2).

Two to seven individuals from different localities were sequenced for nine of the *Timarcha* species. The estimated nucleotide diversity π (Table 3) was maximum for four sequences of *T. intermedia* (3.11%) and minimum for five sequences of *T. interstitialis* (0.91%). On average, nucleotide diversity is relatively high for these taxa, i.e., 16 positions of every 1000 differ between two random sequences of a *Timarcha* species. Neither Tajima's *D* (Tajima, 1989) nor Fu and Li's (1993) tests show evidence of natural selection for these sequences.

Divergences corrected by the Kimura two-parameter method show a range of 0.30–12.28 (average: 7.36) in the comparisons within *Timarcha*, within *Timarchostoma*, and between both subgenera. Divergences among pairwise comparisons of species of the two subgenera are in a similar range. Comparisons among the two representatives of the subgenus *Metallotimarcha* and the remaining *Timarcha* taxa give values of 11.36–16.09 (average: 12.97). About one-third of the total

TABLE 1

List of Studied Taxa, with Their Taxonomic and Morphological Group Placement, Sampling Localities, and Sequence Accession Nos.

Subgenus Taxon	Code	Morphological group[a]	Locality[b]	Accession Nos.[c]
Metallotimarcha Motsch.				
Timarcha metallica (Laich.)	MET1	1	Schlagebachtal, Thuringia, GER	AJ236315/AJ236367
T. metallica (Laich.)	MET2	1	Fôret d'Anlier, Anlier, BEL	Y18821/Y18826
Timarchostoma Motsch .				
T. interstitialis Fairm.	INS1	3	Alió, Tarragona, SP	AJ236316/AJ236368
T. interstitialis Fairm.	INS2	3	L'Esquirol, Barcelona, SP	AJ236317/AJ231148
T. interstitialis Fairm.	INS3	3	Garraf, Barcelona, SP	AJ236318/AJ236369
T. interstitialis Fairm.	INS4	3	Caramany, FR	AJ236319/AJ236370
T. interstitialis Fairm.	INS5	3	Sant Pere de Roda, Girona, SP	AJ236320/AJ236371
T. goettingensis (L.)	GOE	3	Jena, Thuringia, GER	AJ236321/AJ231142
T. sinuaticollis Fairm.	SIN1	3	Vall de Núria, Girona, SP	AJ236322/AJ231156
T. sinuaticollis Fairm.	SIN2	3	Queixans, Girona, SP	AJ236323/AJ236372
T. sinuaticollis Fairm.	SIN3	3	La Creueta, Girona, SP	AJ236324/AJ235373
T. montserratensis Bech.	MON	3	Collformic, Barcelona, SP	AJ236325/AJ236374
T. recticollis Fairm.	REC	4	Pla de l'Artiga, Lleida, SP	AJ236326/AJ231154
T. cyanescens Fairm.	CYA	5	Cueto, Cantabria, SP	AJ236328/AJ231139
T. perezi Fairm.	PER1	6	Villanubla, Valladolid, SP	AJ236329/AJ8078
T. perezi Fairm.	PER2	6	Zaragoza, SP	AJ236330/AJ231152
T. perezi Fairm.	PER3	6	Ejulve, Teruel, SP	AJ236331/AJ236375
T. perezi Fairm.	PER4	6	Puerto de Linares, Teruel, SP	AJ236332/AJ236376
T. perezi Fairm.	PER5	6	Pto. de Cuarto Pelado, Teruel, SP	AJ236333/AJ236377
T. perezi Fairm.	PER6	6	El Moncayo, Zaragoza, SP	AJ236334/AJ236378
T. perezi Fairm.	PER7	6	Layna, Soria, SP	AJ236335/AJ236379
T. geniculata (Germ.)	GEN1	7	Puerto de Ventana, León, SP	AJ236336/AJ231141
T. geniculata (Germ.)	GEN2	7	Peña Amaya, Burgos, SP	AJ236337/AJ236380
Timarcha sp.	TSP	6–7	Puerto de S. Isidro, León, SP	AJ236338/AJ231160
T. gougeleti Fairm.	GOU	8	Fiobre-Bergondo, A Coruña, SP	AJ236339/AJ236381
T. strangulata Fairm.	STR	9	Pla de l'Artiga, Lleida, SP	AJ236340/AJ231157
T. hispanica H.-S.	HIS1	12	Sao Vicente Cape, Algarve, POR	AJ23641/AJ231144
T. hispanica H.-S.	HIS2	12	Sao Torpes beach, Alentejo, POR	AJ236342/AJ236382
T. hispanica H.-S.	HIS3	12	Pto. de Caracuel, Ciudad Real, SP	AJ236343/AJ236383
T. calceata Pérez	CAL	14	Vegacervera, León, SP	Y18823/Y18828
T. fallax Pérez	FAL	15	Pto. de la Carrasqueta, Alicante, SP	Y18822/Y18827
T. aurichalcea Bech.	AUR	15	Tragacete, Cuenca, SP	AJ236327/AJ231136
T. granadensis Bech.	GRA	15	Sierra de Guillimona, Granada, SP	AJ236344/AJ231143
T. intermedia H.-S.	INM1	16	Arenales del Sol, Alicante, SP	AJ236345/AJ236384
T. intermedia H.-S.	INM2	16	Isla Nueva Tabarca, Alicante, SP	AJ236346/AJ231147
T. intermedia H.-S.	INM3	16	Rodalquilar, Almería, SP	AJ236347/AJ231146
T. intermedia H.-S.	INM4	16	Nacimiento, Almería, SP	AJ236348/AJ236385
T. balearica Gory	BAL1	17	Esporles, Mallorca, SP	AJ236349/AJ231137
T. balearica Gory	BAL2	17	Alcoufar, Menorca, SP	AJ236350/AJ236386
T. balearica Gory	BAL3	17	Puig de Randa, Mallorca, SP	AJ236351/AJ236387
T. insparsa Rosenh.	INP	18	Pico Veleta, Granada, SP	AJ236352/AJ231145
T. marginicollis Rosenh.	MAG	18	Pico Veleta, Granada, SP	AJ236353/AJ231150
T. coarcticollis Fairm.	COA	19	Los Barrios, Cádiz, SP	AJ236354/AJ231138
T. lugens Rosenh.	LUG	21	Pico Veleta, Granada, SP	AJ236355/AJ231149
Timarcha s. str. Dej.				
T. maroccana Weise	MAC	22	Tanout-ou-Fillali, Moyen Atlas, MO	AJ236356/AJ231151
T. espanoli Bech.	ESP	24	L'Altet, Alicante, SP	AJ236357/AJ231140
T. rugosa L.	RUG	24	Lengua de Tierra, Nador, MO	AJ236358/AJ231155
T. pimelioides H.-S.	PIM	26	C. da Cugno Vasco, Sicily, IT	Y18819/Y18824
T. tenebricosa F.	TEN1	27	Planoles, Girona, SP	AJ236359/AJ231159
T. tenebricosa F.	TEN2	27	Port de la Bonaigua, Lleida, SP	AJ236360/AJ231158
T. nicaeensis Villa	NIC1	27	Mt. Zatta, Genova, IT	AJ236361/AJ236388
T. nicaeensis Villa	NIC2	27	S. Cataldo, Puglia, IT	Y18820/Y18825
T. punctella Mars.	PUN1	28	Lengua de Tierra, Nador, MO	AJ236362/AJ231153
T. punctella teleuetica Bech.	PUN2	28	Kelaa des Sahrna, Moyen Atlas, MO	AJ236363/AJ236389
Outgroups				
Bruchus sp.		—	Campus UIB, Mallorca, SP	AJ236364/AJ231161
Coptocephala scopolina (L.)		—	Campus UIB, Mallorca, SP	AJ236365/AJ231162
Macrolenes dentipes (O1.)		—	Campus UIB, Mallorca, SP	AJ236366/AJ231165

[a] The systematic arrangement of the species analyzed and the morphological group assignation follow the proposal by Bechyné (1948).

[b] Abbreviations correspond to the name of the country of the sampling site: BEL, Belgium; FR, France; GER, Germany; IT, Italy; MOR, Morocco; POR, Portugal; SP, Spain.

[c] The first EMBL accession no. refers to COII sequences and the second to the 16S fragment sequences.

TABLE 2

Summary of Nucleotide Composition for COII and 16S Data of *Timarcha*

Marker	A%	C%	G%	T%	Total sites	Variable sites	Informative sites	Ts	Tv	Tv AT	Ts + Tv
COII	36.37	14.36	11.22	38.05	685	259	182	144	52	41	63
1st Pos.	34.97	15.57	19.66	29.81	229	49	35	30	12	7	7
2nd Pos.	26.87	19.68	12.47	40.98	228	29	4	20	8	4	1
3rd Pos.	47.28	7.83	1.48	43.41	228	181	143	94	32	30	55
16S[a]	39.40	15.52	9.12	35.97	496	97	66	43	31	27	23
Stem	38.14	16.99	10.79	34.07	302	54	40	18	20	17	16
Loop	41.35	13.22	6.52	38.92	194	43	26	25	11	10	7

Note. Ts, transitions and Tv, transversions calculated as minimum observed from the data matrix.
[a] Excluding the G3 loop (positions 255–281) ambiguously aligned.

positions of COII are variable, nearly 70% of which occur at third codon positions (see Table 2). Transitional changes are more abundant than transversions, which in turn are mostly composed of A–T changes. Figure 1 shows a plot relating the proportion of transitional changes as a function of divergence in *Timarcha* COII. As has been repeatedly shown elsewhere (Simon *et al.*, 1994, and references therein), there is a drop in the transitional changes as divergence increases. This is, in fact, caused by a monotonic increment of transversions, which begin to show a saturation effect at about 8–10% divergence. Figure 2 shows a plot of synonymous (Ks) substitutions as a function of the total number of changes in pairwise comparisons.

16S rDNA Sequences

The same species and individuals used for obtaining COII data were sampled for a fragment of 16S rDNA. Sequence multialignment was unambiguous except for a highly variable segment between position 241 and position 298, in particular in the comparisons involving the outgroups. The exclusion of this region in the phylogenetic analysis resulted in loss of information and subsequent lack of resolution in the 16S trees (*sensu* Gatesy *et al.*, 1993). Prediction of the secondary structure based on that proposed for *Drosophila yakuba* 16S rRNA (Gutell and Fox, 1988) allowed determination of the nt homologies of the stems in the ingroup subset. This is particularly important in the case of the variable segment, reducing the ambiguity to 27 nts of the G3 loop, following the nomenclature by De Rijk *et al.* (1996). To assess the effect of different alignment alternatives involving ingroup/outgroup comparisons and bearing in mind the secondary structures for this variable region, we used MALIGN, assigning different gap-to-nt change costs from 1:1 to 20:1. All the alignments obtained gave essentially the same phylogenetic estimate with respect to resolution and support of the nodes.

The A + T content is similar to that of COII (75.4%) but is lower in stems than in loops (see Table 2). This could be related to selective constraints to maintain a minimum of the more stable G–C pairs in stems compared to the loop regions. Intraspecific variation measured by π is on average three times lower in the 16S fragment than in COII (Table 3).

Interspecific divergence (corrected by the Kimura two-parameter method) ranges from 0 to 6.9% (average 3.5%) in the ingroup comparisons, excluding *Metallotimarcha*, and 4.6–7.3% (average 5.7%) among *Timarcha–Metallotimarcha* comparisons. Thus, divergence in 16S is between two and three times lower than that observed for COII. For all the ingroup pairwise comparisons, 19.6% of the positions are variable, of which almost two-thirds are phylogenetically informative (Table 2). Nevertheless, variation on the 16S segment is heterogeneous along the sampled sequence and levels of variation in different regions can be related to their corresponding A + T content (Han and McPheron, 1997). No clear-cut relationship has been found between transition/transversion ratio and nucleotide divergence for 16S in our data (not shown). In addition, analysis of the substitutions present among *Timarcha* shows that, in the G3 stem, pairs A–T in some taxa are substituted by pairs T–A in others, which can be interpreted as the effect of compensatory mutations.

TABLE 3

Nucleotide Diversity (π) ± Standard Errors (SDE) within *Timarcha* Species for the Two Mitochondrial Markers Studied

Species	Sample size	COII			16S		
		n	π	SDE (π)	*n*	π	SDE (π)
T. balearica	3	654	0.0214	0.0083	491	0.0068	0.0032
T. hispanica	3	685	0.0039	0.0018	489	0	0
T. intermedia	4	655	0.0099	0.0024	491	0.0065	0.0015
T. interstitialis	5	679	0.0091	0.0022	488	0.0025	0.0010
T. perezi	7	675	0.0227	0.0046	488	0.0076	0.0021
T. sinuaticollis	3	675	0.0311	0.0105	490	0.0109	0.0045

Note. n, Number of nucleotide positions analyzed.

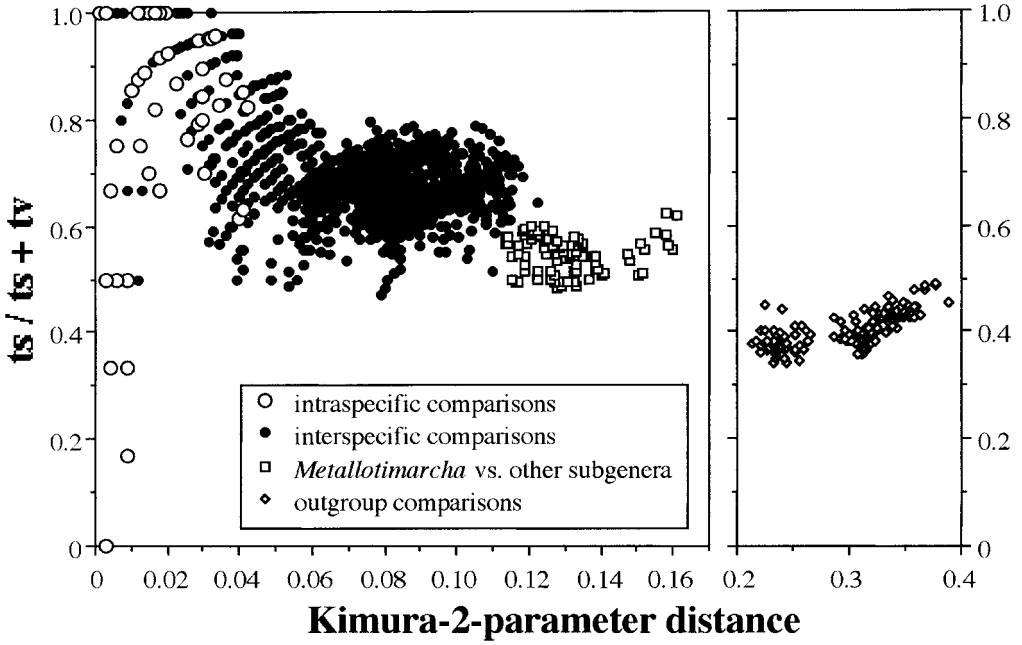

FIG. 1. Proportion of transitions among all changes in pairwise comparisons of *Timarcha* COII as a function of total nucleotide divergence. Comparisons at different taxonomic levels are indicated with symbols. Comparisons with outgroups are shown in a different scale for clarity.

Some stems show noncanonical G–T pairs which also help in maintaining stem stability (Gutell *et al.*, 1994).

Phylogenetic Analysis

COII and 16S data were used separately to reconstruct the gene phylogenies. Figure 3a shows a strict consensus obtained under parsimony analysis using *Coptocephala*, *Macrolenes*, and *Bruchus* sequences as outgroups. In this analysis 558 equally parsimonious trees were recovered (one single island of 1073 steps; C.I. = 0.468; R.I. = 0.650). Neighbor-joining (NJ) COII trees obtained using divergence estimates corrected assuming several substitutional models rendered essentially the same topology (not shown). Using the same assumptions as above, the 16S sequences gave the MP tree shown in Fig. 3b (one single island of 290 MP trees, 426 steps; C.I. = 0.629; R.I. = 0.758), which also is compatible with the NJ analysis. As mentioned above, alternative alignments of the variable region obtained using a range of gap-to-nt change costs gave the same topology. As evidenced by the COII topology, many of the basal nodes obtained in the 16S tree were weakly supported by bootstrap resampling. A comparison of the COII- and 16S-based topologies shows that there is little conflict between the two phylogenetic hypotheses. Namely, *T. intermedia* and *T. lugens* appear monophyletic for COII but not for 16S, and conflicts for *T. tenebricosa–T. nicaeensis* and *T. punctella–T. rugosa* interrelationships are also present in the two gene-based phylogenies. All other major clades are the same in the two topologies and the bootstrap supports for them are similar. Furthermore, the ILD test has been computed, rendering a *P* value of 0.195, which is nonsignificant; so we concluded that both data sets are combinable. Figure 4 shows a parsimony tree based on the combined data set and computed as a strict consensus of the 96 MP trees obtained (981 steps; C.I. = 0.460; R.I. = 0.705), in which bootstrap values higher than 50% are shown for both parsimony and NJ analyses. Long-branch attraction has been described elsewhere

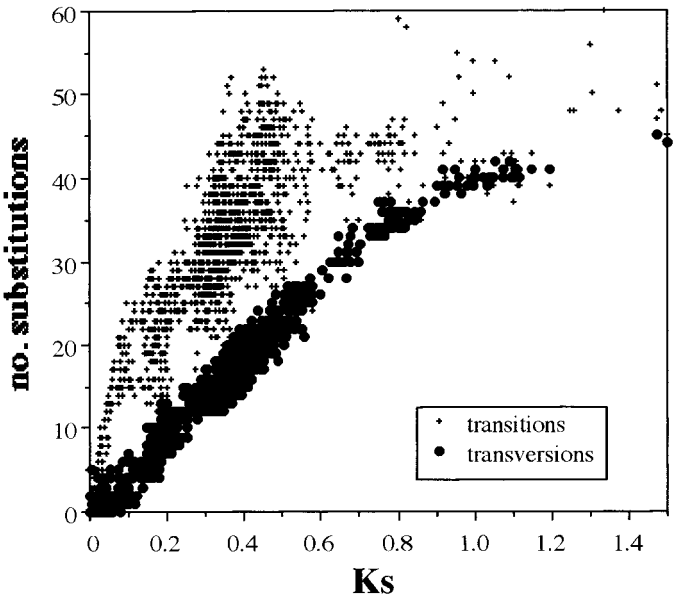

FIG. 2. Relationship between number of substitutions that are transitions or transversions and synonymous rate (Ks) calculated according to Li (1993) in COII of *Timarcha*.

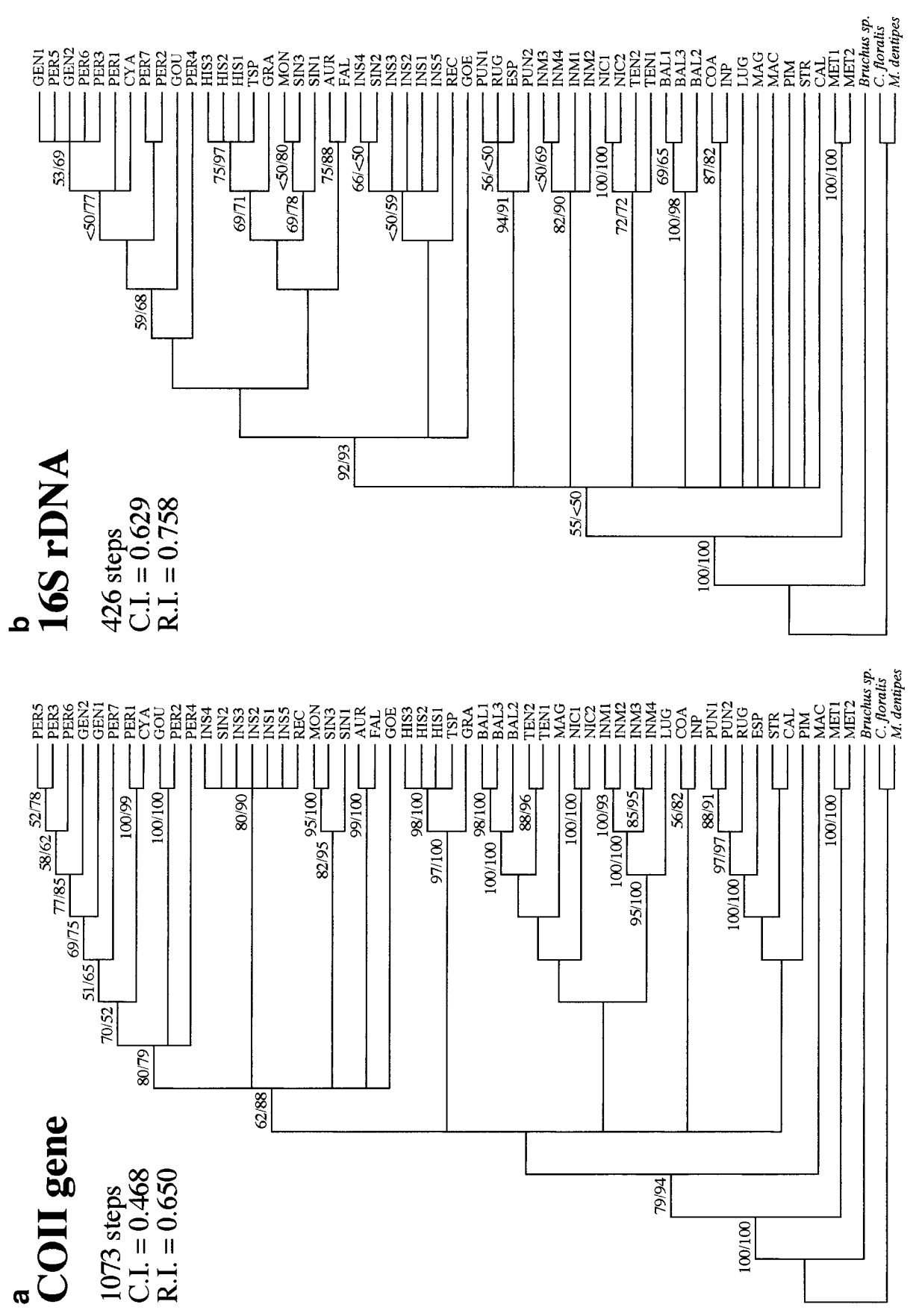

FIG. 3. COII (a) and 16S rDNA (b) MP strict consensus trees constructed using equal weights for all characters, with TBR and ACCTRAN in effect. The trees represent the phylogenetic relationships inferred from each marker using *Coptocephala floralis* and *Macrolenes dentipes* as outgroups considered monophyletic. Confidence levels of tree bifurcations were assessed by 500 bootstrap replicates; values higher than 50% are indicated for each node both for MP (first value) and NJ (second value) analyses.

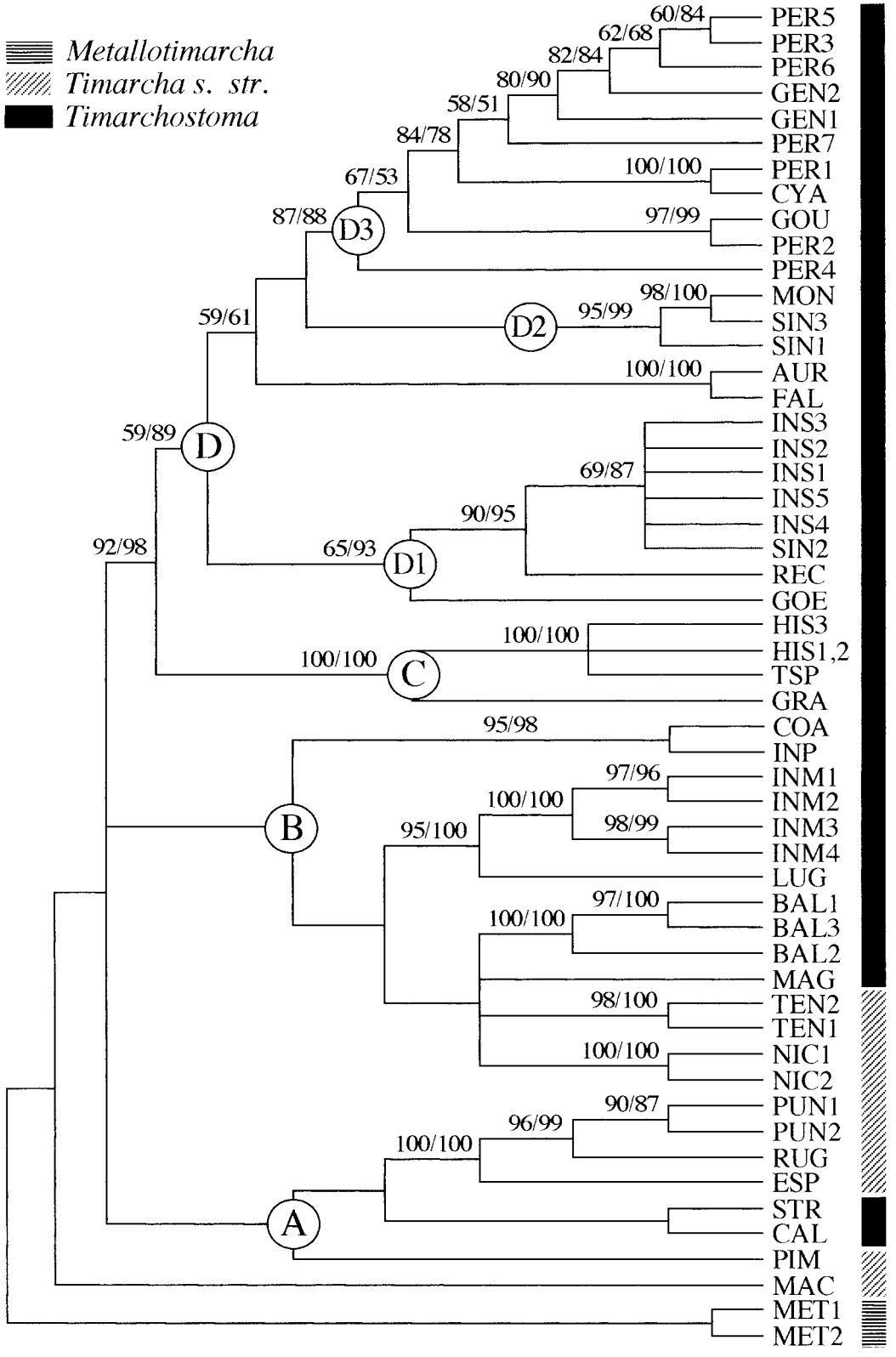

FIG. 4. Phylogenetic hypothesis for the evolutionary relationships in *Timarcha* based on the combination of COII and 16S rDNA data sets. The tree was reconstructed under a maximum parsimony criterion and is the strict consensus of the 96 equally parsimonious trees obtained (981 steps; C.I. = 0.460; R.I. = 0.705). Sequences of *T. metallica* were specified as outgroup (see text for details). Robustness was assessed by bootstrap resampling (500 replicates) and values higher than 50% are given for the MP (first value) and NJ (second value) analyses. Circled letters designate nodes and species groups discussed in the text. Vertical bars with different patterns show the current subgeneric classification for the species studied.

(Hendy and Penny, 1989) as a problem in phylogenetic estimation. In our sequences, long divergences leading to the outgroups could cause distortion. To avoid this effect and as previous single-data-set analyses firmly demonstrate the basal placement of *T. metallica* with respect to the other *Timarcha* sampled taxa, we have rooted this tree accordingly. The tree obtained reinforces most of the supported nodes of single-gene topologies and favors the grouping of *T. lugens* and *T. intermedia* with a high bootstrap support, while it does not confirm monophyly for *T. nicaeensis* and *T. tenebricosa*. All the nodes weakly supported by single-gene analyses remain unsupported in the combined analysis.

Some clades of the mitochondrial phylogeny (indicated in Fig. 4 with circled letters) are of biological interest and will be discussed below: (A) a group of morphologically heterogeneous species characterized by having the highest chromosome numbers in the Palearctic *Timarcha* ($2n = 26–30$) (Petitpierre *et al.*, 1988); (B) European *Timarcha s. str.* and southeastern Iberian and Balearic lineages, well-defined from a morphological point of view; (C) well-supported *T. hispanica* and *T. granadensis* clade, species clearly related on morphological grounds (Bechyné, 1948); and (D) the *T. goettingensis* species complex (Petitpierre, 1970a).

DISCUSSION

Phylogenetic Inferences

The phylogenetic analyses using COII and 16S data show clearly the basal position of *T. metallica* within the Palearctic lineage of the genus. This species and other allied taxa (*T. gibba, T. corinthia,* and *T. hummeli*) belong to a distinct clade classified at the subgeneric rank (*Metallotimarcha*), based on adult and larval morphological characters (Bechyné, 1948; Iablokoff-Khnzorian, 1966; Steinhausen, 1994). Our genetic data, although limited to only one species of this group, are consistent with this interpretation, and the subgeneric-level classification is reinforced by the relatively high divergence found with respect to the remaining *Timarcha* (almost 13% for COII and 5.7% for 16S). Bechyné (1948), in his *Timarcha* monograph, and, later, Stockmann (1966) considered two further Palearctic subgenera: *Timarchostoma* and *Timarcha s. str.*, which have been more controversial (e.g., Stockmann, 1966). This proposal was based on several adult morphological characters, some of which are probably of little phylogenetic value, such as the relative length of male tarsi and adult size, among others. The polarity implicitly applied to character states led to the conclusion that the clade *Timarcha s. str.* is relatively recent, while *Timarchostoma* and *Metallotimarcha* represent more ancestral lineages. This phylogenetic hypothesis, in addition to the geographical distribution of the three subgenera, argued for a northeast–southwest expansion of the genus with an origin in Central Asia and/or secondary diversification and colonization areas in the Iberian Peninsula and North Africa (Jolivet, 1994).

The mitochondrial-based phylogenies obtained do not show any evidence for monophyletic *Timarchostoma* and *Timarcha s. str.* groupings. Neither parsimony nor distance methods support a *Timarcha s. str.* clade, since the phylogenetic relationships of the sampled taxa are unresolved (they fall in the basal polytomy), although they are always positioned close to the root of the tree. The A group (Fig. 4) consists mainly of species of the *Timarcha s. str.* subgenus. However, two of the species included in this group, *T. strangulata* and *T. calceata* (subgenus *Timarchostoma*), have been related to other species groups, based on morphological synapomorphies and their distribution areas (Bechyné, 1948). Interestingly, both of them separate clearly from these species groups in their karyotypes, which are otherwise very homogeneous and conserved. For instance, *T. strangulata,* a very localized high-altitude endemic species of the Central Pyrenees, has been related to the other Pyrenean endemics of the *T. goettingensis* complex (*T. sinuaticollis, T. recticollis,* and others) (Bechyné, 1948), but this species shows $2n = 28$, contrasting with the $2n = 20$ for this species complex (Petitpierre *et al.*, 1988). Similarly, *T. calceata,* from mountain areas in Central Spain, which is morphologically related to *T. hispanica* among the Iberian species, differs from the latter in having a higher chromosome number of $2n = 30$ (Petitpierre, 1970b, 1976).

The mitochondrial relationships among the *Timarchostoma* representatives show three groups of taxa (B, C, and D). The group B falls in the basal polytomy and is characterized by species with shared morphological character states (shape of mesosternum, shape of pronotum, elytral punctuation, etc.) and $2n = 20–22$ chromosomes. Another monophyletic clade (C) (100% bootstrap value) is formed by the *T. hispanica–T. granadensis* haplotypes. Finally, the sister clade of the former includes all the analyzed species of the *T. goettingensis* complex (D), a group highly homogeneous on morphological and karyological grounds (Bechyné, 1948; Petitpierre, 1970b, 1976). Interestingly, the *T. hispanica–T. granadensis* taxa (group C) share morphological character states with group B (same bifidous mesosternum shape) and with group D (e.g., conspicuous elytral punctuation and non-heart-shaped pronotum, among others) (Bechyné, 1948).

Chromosomal data gathered from 34 taxa of *Timarcha* (Petitpierre *et al.*, 1988) have been used to hypothesize an ancestral diploid number of $2n = 20$ for the genus, from which increases occurred in several lineages (Petitpierre, 1973). Chromosomal changes have been traced on the 50%-majority rule consensus tree for the combined analysis of COII and 16S in *Timarcha*

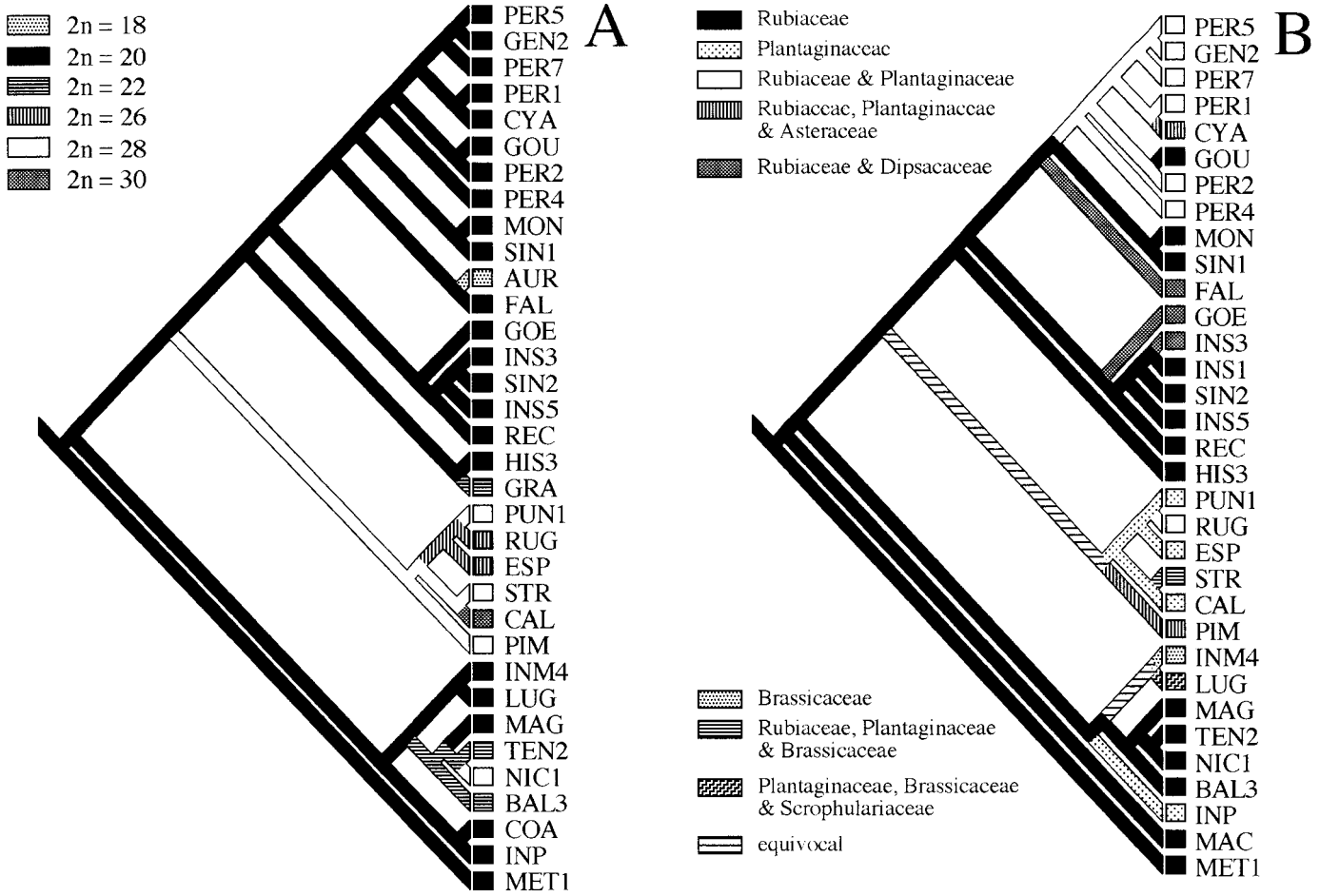

FIG. 5. (A) Character tracing of chromosome numbers in karyologically studied species of *Timarcha* using ACCTRAN character optimization on a phylogenetic hypothesis represented by the majority-rule consensus of the 96 equally parsimonious trees (COII + 16S) obtained in the MP analysis. Each chromosome number is treated as a single category. (B) Tracing of host-plant family use of *Timarcha* beetles using ACCTRAN. The T-PTP test shows that the trophic selection is significantly correlated with the phylogeny of *Timarcha* ($P < 0.001$). Plant affiliation is given according to Jolivet and Hawkeswood (1995). *T. aurichalcea, T. coarcticollis,* and *T. granadensis* are excluded as their trophisms are not well established.

using both ACCTRAN and DELTRAN algorithms. The T-PTP test shows that the chromosomal data is significantly correlated with the phylogeny of *Timarcha* ($P = 0.0294$). The pattern obtained (Fig. 5A) suggests that from an ancestral $2n = 20$ a shift to $2n = 22$ has occurred independently in the lineages of *T. balearica, T. tenebricosa,* and *T. granadensis* (a single shift in the ancestor of *T. tenebricosa* and *T. balearica,* assuming accelerated transformation algorithm). An increase in chromosomal number to $2n = 28$, subsequently followed by decreases to $2n = 26$ (*T. espanoli* and *T. rugosa*) and an increase to $2n = 30$ (*T. calceata*) is deduced in clade A. Finally, the only documented decrease from the basic $2n = 20$ refers to *T. aurichalcea,* in which a translocation involving an autosomal pair and sex chromosomes has reduced the diploid number to $2n = 18$ (Gómez-Zurita *et al.*, unpubl.).

The branching order in our phylogenetic estimate shows conflicts with the systematic propositions based on nonmolecular data. In the phylogeny obtained, the most recent radiation is unequivocally that of the C and D lineages, which are considered ancestral in the classical systematics of the genus. Furthermore, this evolutionary scenario is more compatible with southwest–northeast expansion from North Africa and southeast of Iberia (excluding the more ancient *Metallotimarcha* lineage), which is the geographical region showing the deeper mitochondrial divergence and more diversity of taxa.

The Timarcha goettingensis Species Complex

The *T. goettingensis* species complex (clade D in Fig. 4) has been defined as a cluster of taxa morphologically very plastic but similar to *T. goettingensis,* which are allopatrically distributed and show a process of incipient speciation (Petitpierre, 1970a, 1973). Most of the taxa are mountainous endemics in the Iberian Peninsula, although *T. goettingensis* and a few other taxa are

flatland species with larger distribution areas. Karyologically, all the morphs share a highly conserved chromosome set of $2n = 20$ chromosomes with small differences that can be attributed mainly to pericentric inversions. The latter could have produced reproductive isolation in some of the taxa (Petitpierre, 1973). Herbaceous oligophagy, limited patchy mountainous distribution, and low vagility are factors which should account for small deme sizes and isolation by distance processes leading to speciation in these beetles. The mitochondrial sequences obtained for the taxa included in the complex confirm the monophyly of the group and its sister relationship to the *T. hispanica–T. granadensis* clade. Within this complex, at least three different species subgroups that are geographically structured can be distinguished. *T. goettingensis* + *T. recticollis* + *T. interstitialis* (including one haplotype of *T. sinuaticollis*) form one of these subgroups (D1), distributed from Central Europe to the northeast of the Iberian Peninsula. Divergence within this subgroup is relatively recent, as exemplified by the 3 and 1% for COII and 16S values, respectively, between *T. goettingensis* and *T. interstitialis*. Two haplotypes of *T. sinuaticollis* plus the *T. monserratensis* form another subgroup (D2) of closely related haplotypes (COII: 0.74%; 16S: 0.21%), but a *T. sinuaticollis* haplotype is clearly part of the former clade (is closest to *T. interstitialis* from Caramany). This could either be the product of introgressive gene flow, as the samples come from populations in close proximity, or be due to retention of an ancestral polymorphism (Avise, 1994, and references therein). Data on nuclear sequences and more detailed sampling should distinguish between these two possibilities. Another subgroup (D3) is constituted by quite divergent haplotypes (maximum of 4.24% for COII) belonging to Iberian endemics reaching the southwest of France. The haplotypes of this taxa group appear to be paraphyletic, despite the morphological divergence from the nominal species from which they originated. Some of the closest haplotypes (*T. gougeleti* from A Coruña and *T. perezi* from Zaragoza: 1.63% for COII and 0.62% for 16S; *T. cyanescens* from Cueto and *T. perezi* from Villanubla: 1.20% for COII and 0.41% for 16S) are found in populations that are very distant (more than 600 km) or are geographically split by high mountains; so any present contact between them probably has to be discarded. Therefore, the apparent conflict between taxonomy and mitochondrial divergence can be better explained by past gene flow in relatively recent evolutionary time, although we cannot exclude the possibility of lack of congruence between morphology and genotype due to incomplete lineage sorting. The evolutionary scenario for the *T. goettingensis* species complex is compatible with an ancestral lineage having a continuous geographic distribution that was progressively fragmented by orogenic and glaciation events during the Pleistocene, producing local diversification, speciation, and possibly secondary-contact zones.

A Molecular Clock for Dating the Timarcha Diversification

The use of the relative rate test (Sarich and Wilson, 1973) showed no significant (5% level) deviations from a constant rate (not shown) at different divergence levels of COII and 16S *Timarcha* sequences. Rate constancy was further assessed by the more stringent "two-cluster test" according to Takezaki *et al.* (1995). In this case, using the total number of substitutions corrected by the Kimura two-parameter method, some of the basal lineages showed rates that were significantly different from the average. When using only transversion rates, statistically significant rate discrepancies (5% level) were still obtained for the lineages of *T. marginicollis*, *T. pimelioides*, *T. strangulata*, *T. balearica*, and *T. espanoli*.

As no fossil evidence is known, date estimates for the separation of the *Timarcha* lineages have to be based on biogeographical considerations. The Messinian salinity crisis (about 5.5 MYA) (Hsü *et al.*, 1977; Maldonado, 1985; Jaeger, 1994) has been invoked to explain the origin of the present-day distribution of several organisms in the western Mediterranean region, such as the freshwater adaptation of gobiid fishes or the disjunct *Carabus* (Coleoptera) distributions recently reported by Penzo *et al.* (1998) and Prüser and Mossakowski (1998), respectively. In the latter study, using flightless beetles of the genus *Carabus* and ND1 sequences, molecular clock calibration with the Messinian geological event gave rates below 1×10^{-8} substitution/site/year (Prüser and Mossakowski, 1998). This rate is slower than the estimated mitochondrial average of 2×10^{-8} substitution/site/year for Hawaiian *Drosophila* (ND1) (DeSalle *et al.*, 1987) and several Arthropoda (2.3×10^{-8} substitution/site/year from RFLP, sequence and DNA–DNA hybridization data; Brower, 1994).

Morphological-karyological evidence shows that three species, *T. punctella–T. rugosa* and *T. espanoli*, are clearly derived from the same ancestor and their distribution is likely to be the product of vicariance caused by the opening of the Gibraltar strait after the Mesinian salinity crisis. Using this biogeographical event separating the two clades tentatively fixed at 5.3 Myr, we have obtained rate estimates of 0.76×10^{-8} substitution/site/year for COII and 0.45×10^{-8} substitution/site/year for 16S. These *Timarcha* estimates are 2.6 to 5 times lower than the DeSalle *et al.* (1987) or Brower (1994) estimates, depending on use of COII or 16S sequence divergences, respectively. Alternatively, if we apply the standard 2.3×10^{-8} rate, then we should assume a much more recent gene flow across the Gibraltar strait for both *Timarcha* and *Carabus* populations. More data based on several mitochondrial genes of a range of organisms showing different dispersal capacities are

necessary to assess the significance of these rate discrepancies. Also, independent biogeographical evidence is needed to test whether the Mesinian salinity crisis is the main paleogeographical event explaining the present distribution of south Mediterranean organisms.

Tracing Ancestrality of Host-Plant Affiliation

Herbivorous beetles have been increasingly used as model organisms to address the question of specialization on resource use (Kelley and Farrell, 1998, and references therein). Several studies estimating the phylogenies of chrysomelid genera, such as *Oreina*, *Ophraella*, and *Chrysolina*, show the relationship between phylogeny and food specialization (Funk *et al.*, 1995; Mardulyn *et al.*, 1997; Garin *et al.*, 1999). In a recent study, Kelley and Farrell (1998) asked the question whether specialized taxa tend to be derived, representing an evolutionary "dead end." They showed that a *Dendroctonus* bark-beetle COI phylogeny is compatible with the ancestrality of generalist species, giving rise to specialized taxa in the tips of the tree.

Timarcha beetles can be considered as trophic specialists, as some species feed on one or a few closely related plant species of a particular botanical family, while others use single species belonging to different families, depending on their availability. As examples of the last category, *T. lugens* feeds on *Plantago nivalis* (Plantaginaceae) and *Veronica fruticulosa* (Scrophulariaceae) at altitudes below 2700 m, shifting to *Alyssum spinosum* (Brassicaceae) at higher altitudes (Jolivet, 1954). For other species, such as *T. maritima*, trophism depends on the seasonal availability of their two host plants, *Galium arenarium* (Rubiaceae) as the habitual trophic choice and *Plantago lanceolata* (Plantaginaceae) in winter, when the former is not available (Chevin and Tiberghien, 1968). This strongly suggests that *Timarcha* food specialization is dependent on geographical distribution and hence on availability of host plants, although historical factors, such as feeding on chemically similar hosts, have been proposed for other phytophagous beetles (Becerra, 1997). This implies that a *Timarcha* species feeding on one plant family is not necessarily more restricted than another species with different populations feeding on two or more different families in their distribution area.

The molecular phylogenetic hypothesis obtained for *Timarcha* allows inference of the evolutionary history of host-plant affiliation for this genus. A character tracing of *Timarcha* family host plants on a majority-rule tree obtained using the combined COII + 16S data set is shown in Fig. 5B. In this analysis we have considered only the trophic affiliations that are well-documented in the literature, and character status is of specialization at the family level. Character optimization using either accelerated or delayed transformation showed the clear basal affiliation to Rubiaceae for the Palearctic *Timarcha*. None of the host-plant shifts from this food regime appear as single events in the phylogeny but are products of convergence or reversal. The derived condition in this genus seems therefore to be the widening of the ecological niche, probably as a result of selection pressure imposed by differences in seasonal or altitudinal availability of the appropriate edible plants. We suggest that this could be interpreted as transitional food regimes occurring in marginal populations in the species' distribution area changing toward new specialized food regimes.

In conclusion, these results show that the mitochondrial phylogeny of *Timarcha* allows one to address such topics as the ancestrality of *Metallotimarcha* in the Palearctic lineages, the compatibility with current systematic proposals, and the evolution of host-plant affiliation. The use of other sequences with different substitution rates, including nuclear sequences, and sampling of the New World and Eastern European lineages should provide a more complete picture of the time scale and evolution of the genus.

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