Molecular analysis of the diversity of terrestrial planarians (Platyhelminthes, Tricladida, Continenticola) in the Iberian Peninsula

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This work is a prospective study to estimate the potential species diversity of terrestrial planarians in the Iberian Peninsula. Live specimens were collected from several Iberian localities and assigned to different morphotypes on the basis of their external morphological characteristics. From the same specimens, sequences from the mitochondrial Cytochrome Oxidasa subunit I (COI) and from the nuclear ribosomal gene 18S rRNA were obtained. Sequences from GenBank of the families Dendrocoelidae, Planariidae, Bipaliidae, Geoplanidae and Rhynchodemidae have been used as outgroups in the phylogenetic analysis. The results showed that terrestrial planarians have a wide distribution in the Peninsula, with all individuals found belonging to the Rhynchodemidae family. Morphological observations indicated the presence of 10 morphospecies confirmed by the molecular analyses. At the same time, COI sequences were successfully used as a molecular marker for species identification in the barcoding mode, which is of great use in groups like this with few external morphological characteristics. The combined data strongly suggest the presence of at least 15 species in the Iberian Peninsula, a number that nearly doubles previous estimates, indicating that terrestrial planarians are more diverse than expected in the region and, as proposed, may be a good biodiversity indicator and model for biogeographical studies.

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Introduction

The general knowledge on terrestrial planarians in Europe is rather poor. This is mainly due to their small size, cryptic mode of life and nocturnal activity, which make their localization difficult. In addition, the taxonomy of the few known species is rather confused, in many cases with poor descriptions lacking the essential information to identify new individuals. Moreover, terrestrial planarians have been proposed as good biodiversity indicators (Sluys 1999) and have appropriate characteristics for the study of factors causing biodiversity distribution patterns (Sunnucks *et al*. 2006). In view of their biological importance, thorough knowledge of their diversity and distribution is desirable.

In Europe, 24 autochthonous species, all belonging to the family Rhynchodemidae Graff 1896, and 17 introduced species, belonging to the families Bipaliidae Stimpson 1857, Geoplanidae Stimpson 1857 and Rhynchodemidae Graff 1896, have been described (Minelli 1977; Ball & Reynoldson 1981; Jones 1988, 1998; Mateos *et al*. 1998; Faubel 2004; Jones *et al*. 2008; Vila-Farré *et al*. 2008).

In the Iberian Peninsula and Balearic Islands, at present, eight autochthonous species belonging to the Rhynchodemidae have been found, five of them being recently described species: *Microplana terrestris* (Muller 1774) (Menorca) (Minelli 1977), *Microplana nana* Mateos, Giribet & Carranza, 1998 (Les Alberes, Girona) (Mateos *et al*. 1998), three new species from the

Sierra de Grazalema (Cádiz) — *Microplana aixandrei* Vila-Farré, Mateos, Sluys & Romero, 2008, *Microplana grazalemica* Vila-Farré, Mateos, Sluys & Romero, 2008 and *Microplana gadesensis* Vila-Farré, Mateos, Sluys & Romero, 2008 (Vila-Farré *et al.* 2008), one new species from the Serra de Collserola (Barcelona) — *Microplana groga* Jones, Webster, Littlewood & McDonald, 2008 (Jones *et al.* 2008; previously missidentified as *Microplana terrestris* by Mateos *et al.* 1998), *Rhynchodemus sylvaticus* (Leidy, 1851) (found in various Spanish localities) (Vila-Farré *et al.* 2008; Boix & Sala 2001); finally, *Microplana scharffi* (Von Graff, 1896) has been cited by Faubel (2004) in mainland Spain and Portugal; although this citation is not well documented it will be considered valid in the present work. In addition, an introduced species, *Bipalium kewense* Moseley, 1878 (family Bipaliidae) has been reported in Barcelona (Filella-Subirá 1983).

In this paper we present the results of the molecular analysis of the individuals captured in various campaigns that were carried out between 2004 and 2006 to detect the presence of terrestrial planarians. The aims of the study were to estimate the diversity of terrestrial planarians in the Iberian Peninsula, to establish the relationships among the groups found and to develop a molecular method that would allow the identification of the different species (DNA barcoding). The barcode method also facilitates the analysis of the genetic variability of the group in a geographical area (Stoeckle *et al.* 2005). Two molecules were selected for this purpose, one mitochondrial (Cytochrome Oxidase I, COI) and one nuclear (18S rRNA) gene, allowing us to test whether there was any incongruence between the two genomes.

Materials and methods

Specimens

Live specimens of terrestrial planarians were collected from 16 Iberian localities; 35 were photographed and preserved immediately in 96% EtOH (Table 1 and Fig. 1). From each locality some individuals were fixed in Steinnman fluid and are kept in a reference collection at the Animal Biology Department (Universitat de Barcelona). The animals were initially classified into 10 different morphotypes on the basis of external morphological data for each specimen. The external characters used, in relaxed (fully extended) animals, were as follows:

1 Body length: longitudinal distance from tip to tip.
2 Body shape: flattened, cylindrical
3 Anterior end: pointed, blunt.
4 Pigmentation: different patterns.
5 Shape of eyes: aureolated (central black circular eye bordered by a hyaline circular area), dense (black circular eye).
6 Dorso-lateral position of eyes: dorsal (distance between eyes less than four eye diameters), lateral (distance between eyes more than eight eye diameters).

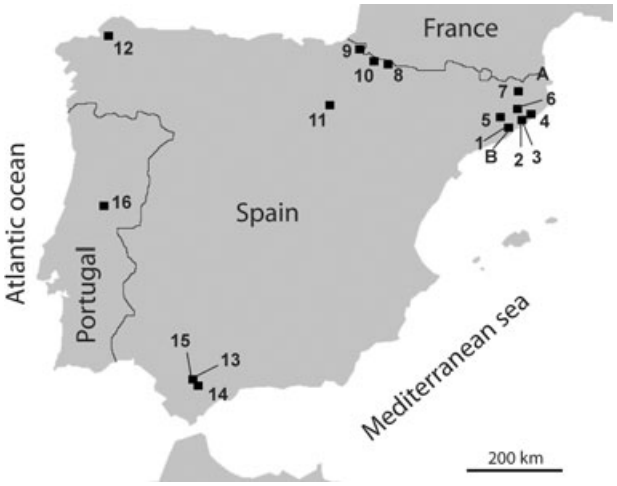

Fig. 1 Localities of terrestrial planarians in the Iberian Peninsula. —A. Type locality of *Microplana nana* (*sensu* Mateos *et al.* 1998). —B. *Microplana terrestris** locality. Locality codes follow Table 1.

7 Antero-posterior position of eyes: distant from the anterior tip (distance from the anterior tip between eight and ten eye diameters), anterior position (distance from the anterior tip between six and four eye diameters), apical position (distance from the anterior tip less than three eye diameters).
8 Creeping sole: reaching the anterior end, not reaching the anterior end.
9 Ventral groove: present in the anterior third of the animal (see Fig. R1a), absent.

In some terrestrial planarian species the colouration of the body is affected by their feeding activity; for example, *Microplana scharffi* may be cream, yellow, pink, orange, brown, violet or grey depending on when and what it was fed (Mcdonald & Jones 2007). Despite this, we considered the colour pattern to be an important character in the definition of morphotypes.

We used sequences from GenBank of limnic species of the families Dendrocoelidae Hallez, 1892, and Planariidae Stimpson, 1857, and of the terrestrial species of the families Bipaliidae Stimpson, 1857, Geoplanidae Stimpson, 1857 and Rhynchodemidae Graff, 1896 (Table 2) as outgroups and to establish the correct situation of the species found within the terricola group. Although the limnic family Dugesiidae have previously been found to be the sister group to the terrestrial planarians, we did not use species of this family in this analysis for two main reasons. In a recent paper, Álvarez-Presas *et al.* (2008) found it difficult to recover the monophyly of the two groups with high support using four genes. Here our aim was to obtain a picture of the situation of Iberian terrestrial planarians within the terricolan framework and then analyse diversification within them. The gene fragments used for this

Table 1 Localities from the Iberian Peninsula in which terrestrial planarians were collected.

Morpho	Code	Locality	Province (Country)	Coordinates	Altitude
M4	L1	Serra de Collserola, Can Catà	Barcelona (Spain)	41.474183 N 2.147384 E	85 m
M4,R1	L2	Serra del Corredor, Canyamars creek shore	Barcelona (Spain)	41.598317 N 2.443020 E	260 m
M6	L3	Serra del Corredor, can Rimbles creek shore	Barcelona (Spain)	41.594097 N 2.464440 E	250 m
M1,M9	L4	Serra del Montnegre, sot de la Massaneda	Barcelona (Spain)	41.668975 N 2.602379 E	310 m
M5	L5	Sant Llorenç del Munt, Sot de les Teixoneres	Barcelona (Spain)	41.661680 N 1.997060 E	830 m
M4	L6	Montseny mountains, Montseny town	Barcelona (Spain)	41.758725 N 2.399094 E	550 m
M1,M4	L7	Fageda d'en Jordà	Girona (Spain)	42.149366 N 2.519729 E	570 m
M2	L8	Isaba, Belabarte creek shore	Navarra (Spain)	42.877835 N 0.862635 W	1070 m
M8	L9	Road NA174, Artesiaga river shore	Navarra (Spain)	43.113056 N 1.538889 W	270 m
M2	L10	Irati forest	Navarra (Spain)	43.007606 N 1.201696 W	925 m
M2	L11	Sierra Cebollera, Puente Ra	Logroño (Spain)	42.046260 N 2.685450 W	1240 m
M2,M3	L12	Fragas do Eume	A Coruña (Spain)	43.417185 N 8.063563 W	60 m
M7	L13	Sierra de Grazalema, Llano del Berral	Cádiz (Spain)	36.754280 N 5.453990 W	657 m
M7	L14	Sierra de Grazalema, las Covezuelas	Cádiz (Spain)	36.711273 N 5.368400 W	808 m
M7	L15	Sierra de Grazalema, Majaceite river shore	Cádiz (Spain)	36.773681 N 5.486587 W	278 m
M2	L16	Mata da Margaraça, Serra do Açor	Coimbra (Portugal)	40.216810 N 7.909610 W	600 m
M.na	A	L'Albera, Coll de Banyuls	Girona (Spain)	42.464234 N 3.057014 E	380 m
M.te*	B	Serra de Collserola, Font Groga	Barcelona (Spain)	41.434834 N 2.122644 E	330 m
M.sp	C†	Catalonia	(N-E Spain)	— —	—

M.na: *Microplana nana*; M.te*: *Microplana terrestris**; M.sp.: *Microplana* sp.; †C: Unspecific locality in Catalonia (North Eastern Spain).

purpose are quite short and unable to give good resolution of basal relationships among Dugesiidae and terrestrial planarians. Consequently, we found it more convenient to use a slightly more basal outgroup to analyse the relationships between the populations. This was either the planarioidea (sister group to the Dugesiidae + terricolan clade) or, in the case of 18S, the type I 18S sequences of the same terricolan group (Carranza *et al*. 1999; Álvarez-Presas *et al*. 2008).

DNA extraction, gene amplification and sequencing

High molecular weight DNA was purified from 35 ethanol-fixed (Table 2) specimens using a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. Initially, universal primers were used to amplify a 381-bp fragment of the COI gene, but these primers did not yield any product and new primers specific for terricolans were designed: COIPra2S (forward): 5′-AGC TGC AGT TTT GGT TTT TTG GAC ATC CTG-3′ and COIPrb2S (reverse) 5′-CTT GGC AAA TGC TTT CGC-3′. PCR conditions were as follows: a hot start was used (94 °C for 10 min and 80 °C for 5′ to introduce GoTaq), followed by five cycles of 1 min at 94 °C, 1 min at 40 °C and 1 min at 72 °C; 35 cycles of 1 min at 94 °C, 1 min at 44 °C and 1 min at 72 °C, and finally a 10 min final extension at 72 °C. For 18S the primer pair 3F18S (forward 5′-TAT CAG TAA GCG GAG GAA AAG-3′) and 5R18S (reverse, 5′-CCT TGG GTC CGT GTT TCA

Table 2 List of species and morphotypes and associated GenBank accession numbers.

			GenBank accession number		
Species/morphospecies (collection date, collectors)	code	Locality	COI	18S-TI	18S-TII
Dendrocoelidae					
Dendrocoelum lacteum Oerste, 1844			AF178312		
Planariidae					
Polycelis tenuis Ijima, 1884			AF178321		
Bipaliidae					
Bipalium sp.			AF178307		
Bipalium adventitium Hyman, 1943			AF178306		DQ666000
Bipalium kewense Moseley, 1878				AF033039	
Bipalium nobile Kawakatsu & Makino, 1982					DQ666001
Novibipalium venosum (Kaburaki, 1922)					DQ666019
Geoplanidae					
Caenoplaninae					
Arthurdendyus testaceus (Hutton, 1880)			AF178305		DQ666010
Arthurdendyus triangulatus (Dendy, 1895)			DQ666027	AF033038	
Artioposthia sp.			AF178325		
Australoplana sanguinea (Moseley, 1877)			DQ666028	AF033041	AF050434
Caenoplana caerulea Moseley, 1877			DQ666030	AF033040	
Caenoplana sp. 1			DQ666031		AF048765
Caenoplana sp. 4			DQ666032		
Newzealandia sp.					AF050431
Geoplaninae					
Geoplana burmeisteri Schultze & Müller, 1857			DQ666039		DQ666004
Geoplana ladislavii von Graff, 1899			AF178315		DQ666005
Geoplana quagga Marcus, 1951			DQ666040		
Geoplana sp.			DQ666043		DQ666016
Rhynchodemidae					
Rhynchodeminae					
Dolichoplana sp.			DQ666037		DQ666003
Platydemus manowkari Beauchamp, 1962			AF178320		AF048766
R1 (12 May 2004, EM)	PT161	L2	FJ969946*		FJ969980
Microplaninae					
Microplana kwiskea K2 Jones *et al.* 2008			EU334574*		
Microplana kwiskea K8			EU334575*		
Microplana kwiskea K9			EU334576*		
Microplana nana Mateos, Giribet & Carranza, 1998		A	AF178317	AF033042	GQ179648
*Microplana terrestris** (Muller, 1774)		B	AF178318		
Microplana terrestris T2			EU334580*		
Microplana terrestris T4			EU334581*		
Microplana terrestris T7			EU334582*		
Microplana terrestris G1			EU334583*		
Microplana terrestris G4			EU334584*		
Microplana sp.		C	DQ666045		DQ666017
Microplana scharffi (von Graff, 1896)			DQ666044		AF050435
Microplana scharffi S1			EU334577*		
Microplana scharffi S2			EU334578*		
Microplana scharffi S5			EU334579*		
M1 (19 November 2004, EM)	PT108	L7	FJ969947*		FJ969981*
M1 (18 November 2005, EM	PT163	L4	FJ969948*		
M2 (01 July 2004, EM)	PT71	L12	FJ969949*		FJ969982*
M2 (19 August 2004, EM & MV)	PT75	L10	FJ969950*		
M2 (21 August 2004, EM & MV)	PT86	L8	FJ969951*		
M2 (15 July 2005, EM)	PT151	L12	FJ969952*		FJ969983*
M2 (13 July 2005, EM)	PT155	L11	FJ969953*		FJ969984*
M2 (13 July 2005, EM)	PT157	L11	FJ969954*		
M2 (13 July 2005, EM)	PT158	L11	FJ969955*		
M2 (05 November 2005, EM)	PT162	L16	FJ969956*		FJ969985*

Table 2 *Continued.*

Species/morphospecies (collection date, collectors)	code	Locality	GenBank accession number		
			COI	18S-TI	18S-TII
M2 (12 April 2006, EM)	PT189	L12	FJ969957*		FJ969986*
M2 (12 April 2006, EM)	PT191	L12	FJ969958*		
M2 (12 April 2006, EM)	PT193	L12	FJ969959*		FJ969987*
M2 (12 April 2006, EM)	PT194	L12	FJ969960*		FJ969988*
M3 (12 April 2006, EM)	PT196	L12	FJ969961*		FJ969989*
M3 (12 April 2006, EM)	PT197	L12	FJ969962*		FJ969990*
M4 (28 March 2004, EM)	PT58	L1	FJ969963*		FJ969991*
M4 (04 October 2004, EM)	PT95	L2	FJ969964*		FJ969992*
M4 (11 October 2004, EM)	PT97	L2	FJ969965*		FJ969993*
M4 (19 November 2004, EM)	PT104	L7	FJ969966*		FJ969994*
M4 (05 Marvh 2006, EM)	PT166	L6	FJ969967*		FJ969995*
M5 (11 March 2004 , EM)	PT55	L5	FJ969968*		FJ969996*
M6 (17 March 2006, EM)	PT171	L3	FJ969969*		FJ969997*
M6 (17 March 2006, EM)	PT172	L3	FJ969970*		FJ969998*
M6 (17 March 2006, EM)	PT173	L3	FJ969971*		FJ969999*
M7 (05 December 2004, EM & MV)	PT117	L15	FJ969972*		
M7 (05 December 2004, EM & MV)	PT125	L13	FJ969973*		
M7 (05 December 2004, EM & MV)	PT126	L13	FJ969974*		
M7 (05 December 2004, EM & MV)	PT127	L13	FJ969975*		
M7 (06 December 2004, EM & MV)	PT135	L14	FJ969976*		FJ970000*
M7 (07 December 2004, EM & MV)	PT142	L13	FJ969977*		
M7 (07 December 2004, EM & MV)	PT143	L13	FJ969978*		
M8 (14 May 2006, EM & MV)	PT218	L9	FJ969979*		FJ970001*
M9 (12 January 2004, EM)	PT51	L4			FJ970002*

*indicates new sequences. Locality codes follow Table 1. Collectors: EM = Eduardo Mateos; MV = Miquel Vila-Farré.

AGA-3′) was used to amplify a 544 bp long fragment. The PCR thermal regime consisted of one cycle of 1 min at 94 °C; 25 cycles of 30 min at 94 °C, 45 min at 45 °C and 1 min at 72 °C and a final extension of 5 min at 72 °C. On some occasions a hot start was used, adding a cycle of 10 min at 94 °C, 5 min at 80 °C; and 5 cycles of 1 min at 94 °C, 1 min at 40 °C and 1 min at 72 °C, as a step prior to the initial conditions. Amplification products were cleaned to eliminate nucleotides and primers and were sequenced directly using the same primers used in the PCR reaction. In all cases both chains were sequenced using BigDye (3.1, Applied Biosystems) and an automated sequencer ABI Prism 3730 (Unitat de Genòmica dels Serveis Científico-Tècnics de la UB).

Phylogenetic analyses

Sequencher (v. 4.2.2, *Gene Codes*) was used to revise the chromatograms and obtain the definitive sequences. ClustalX (v. 1.8; Thompson *et al*. 1997) was used to align the sequences, and the alignments were finally revised by hand with the help of a computer editor (BioEdit vs. 7.0.5; Hall 1999). COI sequences were aligned on the basis of the amino acid sequences. For 18S, regions of ambiguous alignment were discarded before the phylogenetic analyses, resulting in a final alignment 500 bp long. The final alignments are available from the authors.

A duplicated ribosomal gene cluster in Dugesiidae and terricolan planarians has previously been reported (Carranza *et al*. 1996, 1998, 1999); the two copies of the cluster having been named type I and type II. To assess the orthology of the 18S sequences (i.e., whether they were type I or type II) a phylogenetic tree (not shown) was inferred including terricola sequences of type I and type II 18S from GenBank. All sequences obtained in this study turned out to be type II.

Genetic distances from COI sequences were calculated using DNAdist (PHYLIP package, Felsenstein 1993), correcting the distances with the two-parameter method of Kimura (1980). ML trees were inferred using RAxML (Stamatakis 2006), and 1000 bootstrap replicates were run. BI trees were inferred using MrBAYES vs. 3.0 (Ronquist & Huelsenbeck 2005) with the model recommended by Modeltest (v. 3.6, Posada & Crandall 1998) under the Akaike information criterion (GRT both for COI and 18S). One million generations for each of two independent runs were performed. To calculate the consensus tree the first 400 000 generation trees in the sample were removed to avoid including trees sampled before likelihood values had reached a plateau.

Results

Morphotypes

A total of 35 specimens of terrestrial flatworms collected from the Iberian Peninsula were included in the analysis. On the basis of external morphology the individuals collected were assigned to 10 well-differentiated morphotypes (Table 2, Fig. 2).

Morphotype R1: body length 7–20 mm; shape: elongated flattened body with pointed anterior end; pigmentation: light brown with two dark longitudinal stripes along the animal's back, medial dorsal black circular spot; eyes: two large dense eyes, in lateral position and distant from the anterior tip; creeping sole: absent in the anterior third of the body, which presents a ventral groove.

Morphotype M1: body length 5–17 mm. Shape: elongated cylindrical body that tapers anteriorly to a blunt anterior end; pigmentation: variable colour, from dark brown to light grey, with small dark spots; eyes: two aureolated in lateral and anterior position; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M2: body length: 10–60 mm. Shape: elongated cylindrical body with blunt anterior tip; pigmentation: colour variable, from dark brown to light brown with small dark spots; eyes: two aureolated in lateral and anterior to apical position, sometimes deeply buried in the parenchyma, making its dorsal observation difficult; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M3: body length: 38–42 mm. Shape: elongated cylindrical body that flattens when resting, with a blunt anterior tip; pigmentation: orange; eyes: two small and dense in lateral and apical position; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M4: body length: 20–25 mm. Shape: elongated slender cylindrical body with blunt anterior tip; pigmentation: cream with small darker spots covering the dorsal surface; eyes: two aureolated in lateral and anterior position; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M5: body length: 8 mm. Shape: elongated cylindrical body with blunt anterior tip; pigmentation: cream with black spots, apical end whitish; no eyes; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M6: body length: 4–9 mm. Shape: elongated cylindrical body with blunt anterior tip; pigmentation: cream with black spots, apical end whitish when the animal stretches; eyes: two small, dense in dorsal and anterior position; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M7: body length: 2–15 mm. Shape: elongated cylindrical body with blunt anterior tip; pigmentation: white, nearly transparent; eyes: two very small in lateral and apical position (only visible under dissecting microscope); creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M8: body length: 25 mm. Shape: elongated cylindrical body with blunt anterior tip; pigmentation: brown with a white apical tip (possible regenerating blastema), a short black line between the eyes; eyes: two, dense in lateral and anterior position; creeping sole: reaching the anterior end; ventral groove absent.

Morphotype M9: body length: 15 mm. Shape: elongated cylindrical body with blunt anterior tip, when resting the body flattens dorso-ventrally; pigmentation: cream, with a dark mid-dorsal line running the length of the body, and a lighter anterior tip; eyes: large, asymmetric number, three on the right side and two on the left; creeping sole reaching the anterior end; ventral groove absent.

Individually, none of the nine characters used allows the differentiation of morphotypes; it is the combination of them which allows this separation. Characters three, eight and nine are not variable for the M1 to M9 morphotypes; hence, they only allow the discrimination of R1 morphotype.

COI data set

We obtained COI sequences from 34 specimens and nine morphotypes (Table 2). The trees obtained by ML and Bayesian inference show the same well-supported clades. The ML tree is shown in Fig. 3 with the bootstrap support (BS) values and posterior probabilities (PP). The phylogenetic results show the relationships among terricolan families to coincide with previous studies (Álvarez *et al*. 2008), Bipaliidae as the most basal group, followed by a clade constituted by Geoplaninae + (Caenoplaninae + Rhynchodeminae), and finally Microplaninae.

The terrestrial planarians studied in this paper clustered in nine well-differentiated clades. Two of these clades are made of a single individual (clade 5 and clade 10), while the rest are comprised of groups of between two and 17 individuals (clade 1, clade 2, clade 3, clade 4, clade 6, clade 7 and clade 8). All these groups are monophyletic and received high support both from bootstrap and PP. Clades 1–8 together with all *Microplana* sequences from GenBank constitute a monophyletically well-supported group (100% BS, 0.96 PP). Clade 10 is situated within the Geoplaninae + (Caenoplaninae + Rhynchodeminae) group, being the sister group to the two other Rhynchodeminae species (*Platydemus manowkari* and *Dolichoplana* sp.), also with high support (100% BS, 1.0 PP).

The molecularly defined clades (clades 1–9) were consistent with the morphotypes defined according to the external characters, with three exceptions that are mentioned below, and hence we named the clades according to morphotypes numbering (Fig. 3). We found the only M8 individual in clade 2, which was formed mainly by M2 individuals from localities in the North of the peninsula (L8, L9, L10, L11, L12); we also found the *Micoplana* sp. sequence from GenBank and *M. terrestris* sequences obtained recently by Jones *et al*. (2008) in this clade. The remaining two M2 individuals constitute another molecular clade (clade 8), which is genetically far apart from clade 2 and also presents high genetic divergence

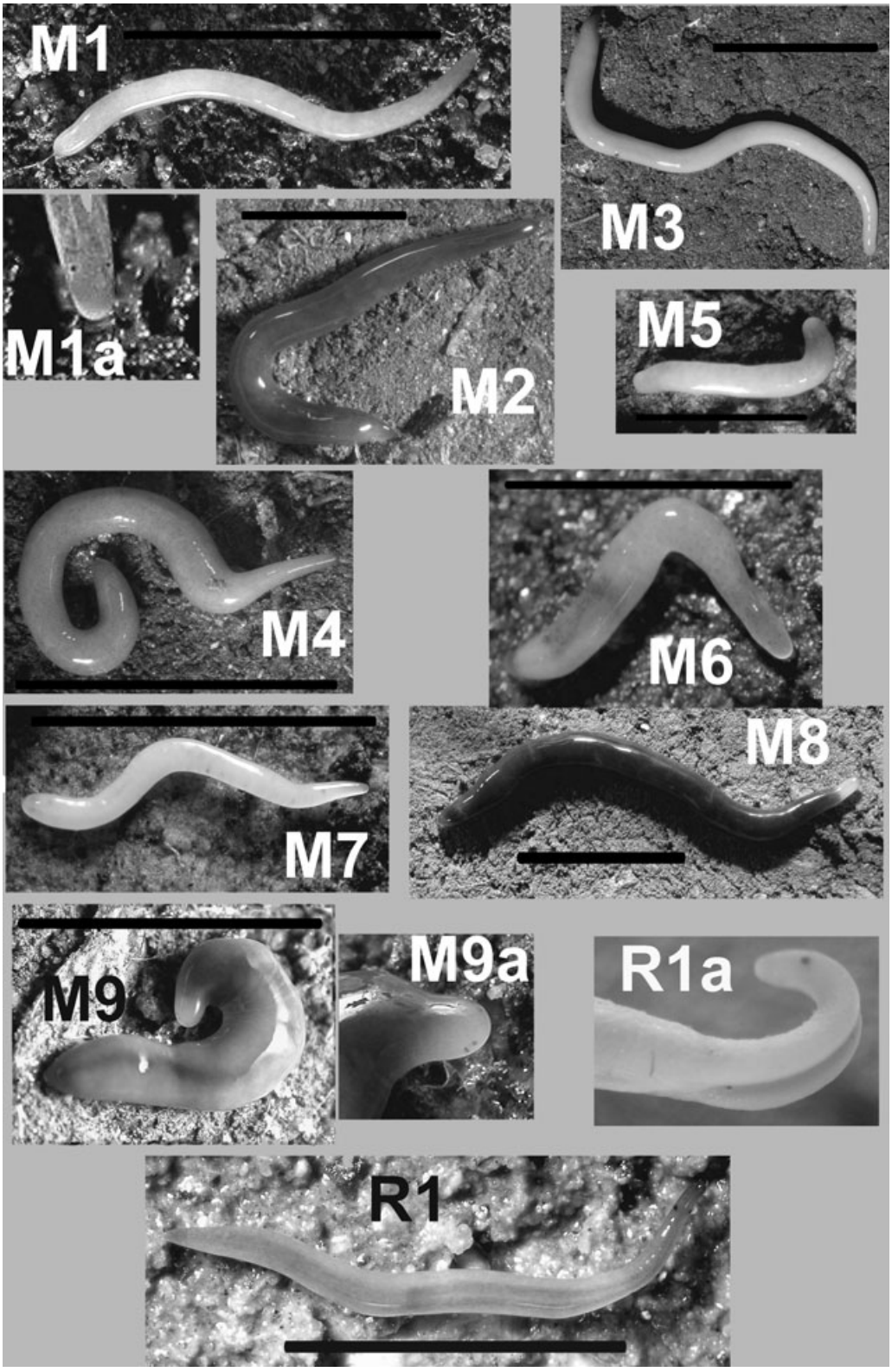

Fig. 2 Morphotypes. R1, M1, M2, M3, M4, M7, M8 and M9 scale bar = 10 mm; M5 and M6 scale bar = 5 mm. M1a, M9a and R1a (without scale bar) illustrates the anterior end of the corresponding morphotype. All pictures by E. Mateos.

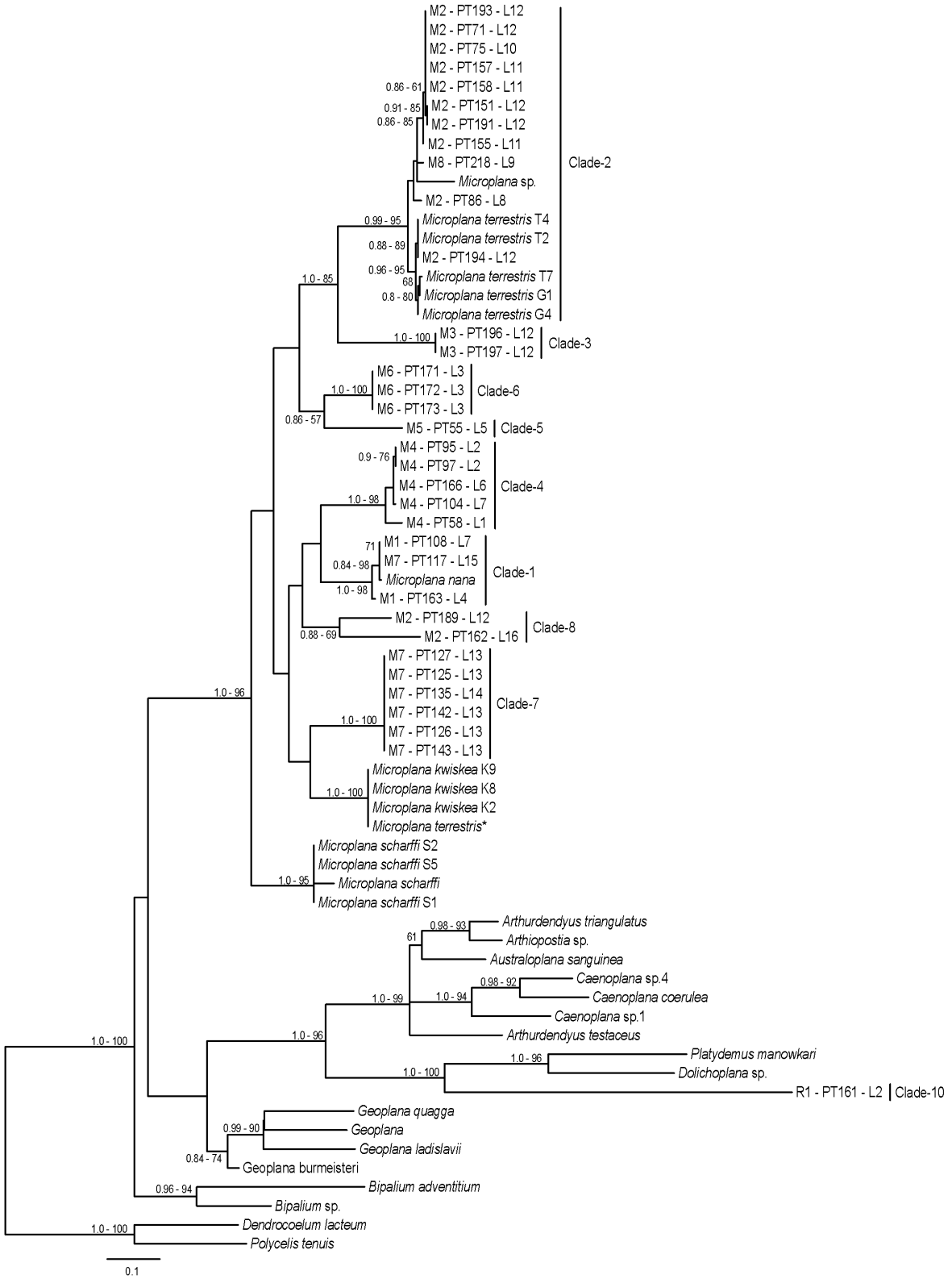

Fig. 3 Phylogenetic analysis of COI gene sequences. ML tree with bootstrap support and posterior probability values indicated at the nodes. Scale bar indicates number of changes per position.

between its two members, both from western localities (L12, L16). The third exception was morphotype 7, constituting clade 7, the majority of individuals of which were identical, and came from two close southern populations (L13 and L14); however, individual M7-PT117 is genetically different and clusters within clade 1, which also includes *Microplana nana* sequence (from the type locality) and the two M1 individuals. Some relationships between clades were well supported: clades 2 and 3 (1.0 PP, 85% BS), clade 6 and clade 5 (0.86 PP, 57% BS), but for the others the support was not good.

Genetic divergence between clades. The genetic distances between clades calculated from the COI fragment alignment are shown in supplementary data Table 1. In that table, we have shaded the values corresponding to the comparisons between individuals belonging to the same clade in blue. The values vary from 0% to 4.1% of divergence. The values found for the interclade relationships range from 12% and upper.

18S rRNA data set

We obtained 18S type II sequences from 24 new specimens (10 morphotypes) and also from *Microplana nana* (DNA extracted from an individual from the type locality) (Table 2). The trees obtained by ML and Bayesian inference show the same clades. The ML tree is shown in Fig. 4 with the BS values and PP. The phylogenetic results show a lack of resolution for the most basal nodes in the tree — those corresponding to the relationships between families and subfamilies — clearly a consequence of the short fragment used for this study; however, these relationships are outside the scope of the present analysis. As for the situation and relationships between the new individuals, individual PT161 grouped with *Dolichoplana* and *Platydemus* as in the COI tree. The 24 new *Microplana* sequences grouped in the same nine clades found for COI, grouping with all *Microplana* species sequences from GenBank in a monophyletic group. On this occasion, there were three single-individual clades (clades 5, 7 and 9) while the rest of the clades were constituted by a variable number of sequences, all of them receiving high or moderate support from the bootstrap and posterior probability. The relationships between these clades did not receive high support and were not consistent with the relationships found with COI sequences. For 18S there was a sequence from morphotype 9 that was lacking in COI and that was clearly differentiated from the rest of the sequences, thus constituting clade 9.

Discussion

Morphotype recovery by molecular data

The molecules used in this study, COI and the 18S rRNA gene, reproduced the same monophyletic clades for the individuals collected. This has some important implications, as the consistency between a nuclear and a mitochondrial marker, both with high support for all the clades, indicates a congruent history for both genomes in this group. As a result, both types of data would be of use to detect clades and to situate new individuals. However, the mitochondrial gene showed better discrimination among clades, as shown by the genetic uniformity within clades vs. the long distances separating them (Fig. 3), hence making this molecule a better choice for this kind of analysis. In addition, the molecular and morphological data were congruent, with only a few exceptions that may be due to the morphological plasticity of some of the morphological characters analysed here. The first exception corresponded to morphotype M2, which was not monophyletic in the molecular analyses but was divided into two monophyletic groups (clade 2 and clade 8) that were quite distinct at the molecular level for both molecules. On the other hand, clade 2 included the only individual of morphotype M8, which was considered to be a different morphotype due to the presence of an unpigmented anterior end. However, close inspection of the individual (Fig. 2, M8) and the molecular results led us to hypothesize that the lack of pigment in the anterior part of that animal was a consequence of it having recently lost its head and thus it was in the process of regeneration. Although the regeneration capacity of terrestrial planarians has not been studied it has been described (Darwin 1844), and it is known that their phylogenetic sister group of freshwater planarians, the family Dugesiidae, have ample capacity for regeneration, even using it as a method of reproduction in some cases (Saló & Baguñà 2002). The final exception was morphotype M7; in this case one individual, PT117, was misclassified and the molecular data clearly showed it to belong to clade 1.

The general good-fit among the morphotypes defined and the molecular outcome indicates the suitability of the combination of morphological characters used to define them. Nonetheless, caution should be used when considering each of the characters alone, as it is also important to take into account the state of the individuals considered, such as whether they are juvenile or regenerating.

Estimated number of terrestrial planarian species in the Iberian Peninsula

Out of the 35 animals studied, only one individual (PT161, clade 10) grouped within the subfamily Rhynchodeminae, indicating that this group is probably poorly represented in the Iberian Peninsula. The external morphology of the individual PT161, morphotype R1, suggested that the specimen may belong to the species *Rhynchodemus sylvaticus* (see Ball & Reynoldson 1981). For subfamily Microplaninae the situation was totally different. As shown by the phylogenetic analyses of both genes, the rest of the new individuals analysed clustered within a group that includes all the *Microplana* sequences downloaded from GenBank, and within this group we found a number of clearly defined clusters (all showing high support).

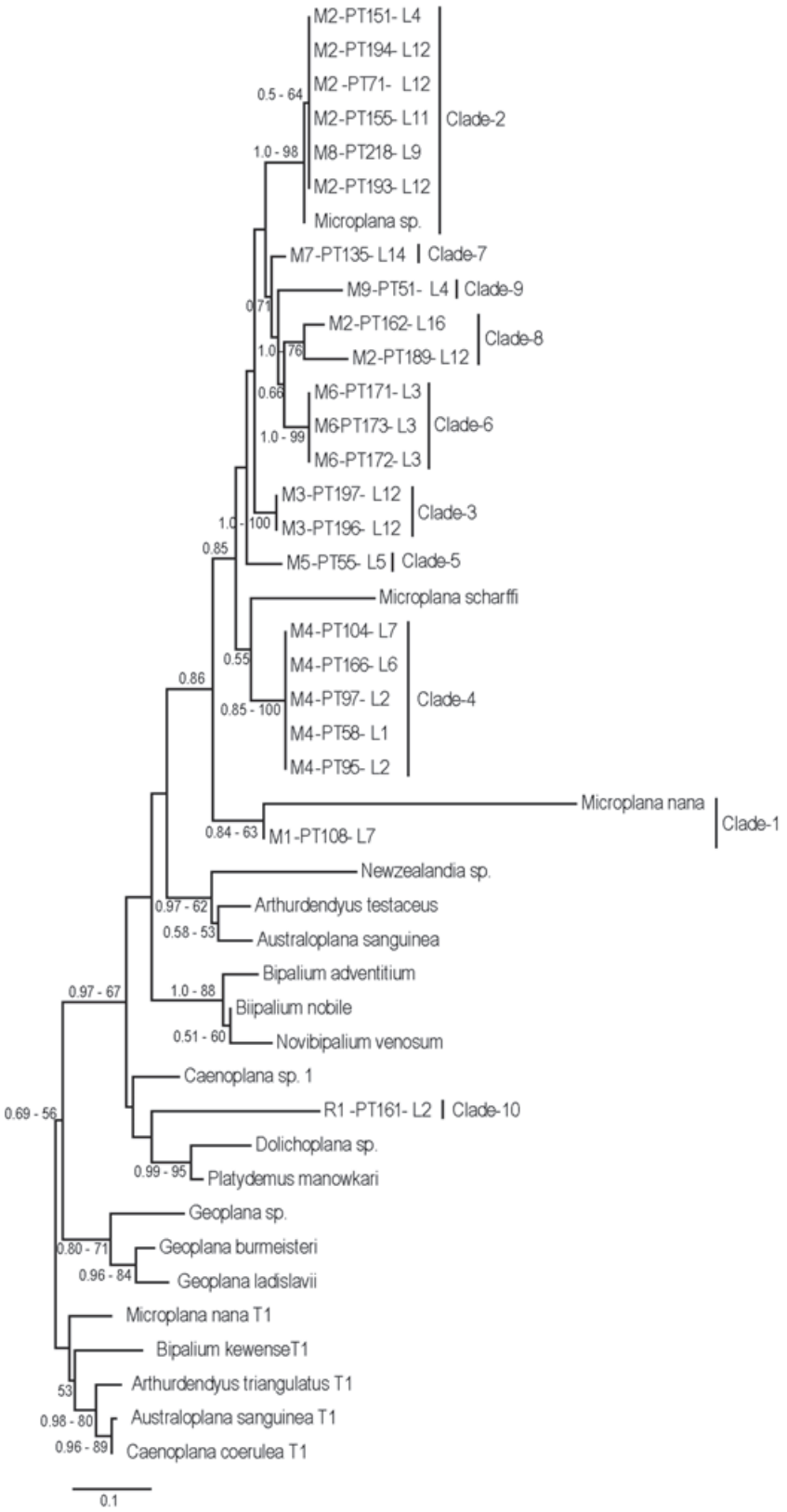

Fig. 4 Phylogenetic analysis of 18S gene fragments. ML tree with bootstrap support and posterior probability values indicated at the nodes. T1 means 18S type-I sequences, the rest of the sequences are 18S type-II. Scale bar indicates number of changes per position.

In the case of COI sequences, we found 10 well-differentiated clades, numbered 1–8, plus the clades constituted by the species *M. kwiskea* (recently described by Jones *et al*. 2008) and *M. scharffi* (cited in Spain and Portugal by Faubel 2004). In the case of 18S we found the same 1–8 clades plus a clade 9 corresponding to the only individual of morphotype M9, for which it was not possible to amplify the mitochondrial gene. The distance data calculated from the COI sequences (supplementary Table 1) showed a clear gap between the intra (maximum 4.1%) and inter (minimum 12%) clade distances, similar to those found for other groups of animals (Hebert *et al*. 2004). The presence of this gap, added to the fact that some of the clades are constituted by or include individuals belonging to a morphologically defined species, supports the lack of gene flow between these groups and hence their species status. Although an extensive morphological analysis of the internal anatomy should be undertaken to corroborate their species status, we think that we can risk saying that at least six undescribed species of *Microplana* are present in the Iberian Peninsula, corresponding to clades 3, 4, 5, 6, 8 and 9. Clade 2, constituted by mostly M2 individuals plus the only M8, also includes all the *M. terrestris* sequences from a recent study (Jones *et al*. 2008), hence pointing to the presence of this species in Spain. The COI sequence of specimen *M. terrestris** (Mateos *et al*. 1998) did not cluster in clade 2, instead being identical to the new *M. kwiskea* sequences (we will comment on the problems raised by the Collserola population below). The other previously described species is *Microplana nana*, corresponding to clade 1, which includes one M7, PT-117, and all M1 individuals, the former probably being a juvenile individual. Finally, the rest of the individuals belonging to morphotype M7 constitute clade 7; the external morphology of the specimens of this clade is very similar to the species *Microplana humicola* Vejdovsky, 1889 (which might be present in the Iberian Peninsula) but also to the recently described *M. aixandrei*. We believe they belong to the latter species since we collected them at the same location and the same date as the holotype (L13) and paratypes (L14) of this species (Vila-Farré *et al*. 2008).

Taken together, these data suggest the presence of, at least, 15 Rhynchodemid species in the Iberian Peninsula. Four of those included in the present study can be assigned a species name: *Rhynchodemus sylvaticus* (R1), *Microplana terrestris* (clade 2, M2 except PT189 and PT162, and M8), *Microplana nana* (M1 + PT117), and *Microplana aixandrei* (M7). To these four species we can add *Microplana scharffi*, *Microplana groga*, possibly *Microplana kwiskea*, and two more species recently described on morphological grounds but for which no molecular data are available: *Microplana grazalemica* and *Microplana gadesensis* (Vila-Farré *et al*. 2008). The remaining six clades that were defined on molecular grounds here require a study of their internal anatomy before receiving a formal description.

M. terrestris/M. kwiskea/M. groga conundrum. The sequences for *Microplana terrestris** (AF178318) were obtained from one specimen from Collserola (Barcelona, Spain) that was identified by Mateos, Giribert & Carranza (1998) as *M. terrestris*, based on histological analysis of other individuals from the same locality. According to Jones *et al*. (2008), specimens from this locality that are deposited in the NMH of London are morphologically distinct from *M. terrestris* and from any other known species and they described it as *M. groga*. On the other hand, the *M. terrestris** sequence was identical to the sequences from British specimens identified as *Microplana kwiskea* (see Mcdonald & Jones 2007) described by Jones *et al*. (2008). This strange situation could be explained if that locality harbours two species (*M. groga* and *M. kwiskea*) and by chance the individual sequenced was from one species and the histological slides (now used to describe the new species) from another. To solve this riddle it would be necessary to collect animals from the same point and obtain new sequences and histological sections from each individual.

Biogeography and distribution of terrestrial planarians in the Iberian Peninsula

Microplana nana (clade 1) showed the widest distribution in our analyses, being found along a North–South transect from the Spanish–French border (locality A in Fig. 1) to Cádiz (L15) (960 km). *Microplana terrestris* (clade 2) was distributed along the northern part of the peninsula, from Coruña (L12) to Navarra (L8, L9) (560 km). Clade 8 individuals were from two populations from the north-west (L12 and L16), only 360 km apart, and they were surprisingly distant from a genetic point of view, suggesting that they may belong to two different species. Clade 4 was distributed in the north-east of the peninsula in four populations (L1, L2, L6, L7) with a maximum distance between them of 85 km. *Microplana aixandrei* (clade 7) seemed to be endemic in the Sierra de Grazalema in southern Spain. The rest of the clades were only found in one population.

The species *Rhynchodemus sylvaticus*, from which we obtained only one specimen in the present study (clade 10), is widely distributed in the British Isles and continental Europe including northern Spain (see Minelli 1977; Vila-Farré *et al*. 2008). The species *Microplana grazalemica* and *Microplana gadesensis* (along with *Microplana aixandrei*) are now endemic in the Sierra de Grazalema on the southern tip of the Iberian Peninsula (Vila-Farré *et al*. 2008). In addition, *Microplana groga* is now endemic in the Serra de Collserola (Barcelona province). Faubel (2004) cited *Microplana scharffi* in Spain and Portugal, but without specifying any locality.

General conclusions

DNA barcode techniques are of special interest in groups like this where the taxonomy is difficult and species identification

very complex, and, as shown, external morphological data should be used with caution. COI was shown to be a good marker in this study; regarding 18S, although showing the same groups, its lower variability could result in a loss of resolution when close species are analysed. Our results show that the comparison of molecular sequences will help to identify genetically congruent groups that would have to be analysed morphologically to definitely identify possible new species. These data can help assign any new individual to the existing clades or determine the presence of new species. The molecular markers used, however, showed a lack of resolution at the basal levels, precluding the inference of a good interspecies phylogeny. New molecules are being tested in order to solve this problem (nuclear protein coding genes such as ATPase and EF-1 alpha).

In continental Europe there are currently 28 valid species of Rhynchodemidae (Mateos *et al*. 1998; Faubel 2004; Jones *et al*. 2008; Vila-Farré *et al*. 2008) in 18 countries, with the following distribution: Spain (8 species), Austria, Britain and France (6); Germany (5); Ireland and Italy (4); Belgium, Bosnia-Herzegovina, Czech Republic and Switzerland (3); Monaco and the Netherlands (2), Bulgaria, Denmark, Norway, Poland and Portugal (1). With our results the Iberian Peninsula (with at least 15 species) is the most species-rich area. In other regions of the world more intensive surveys have shown this group of organisms to be much more rich in species than previously thought (Leal-Zanchet & Carbayo 2000). Also, ongoing studies in the Atlantic Forest in Brazil (Carbayo, Riutort unpublished data) and in Australia (Sunnucks *et al*. 2006) are unveiling high molecular diversity among and within species and the potential usefulness of this group of animals as a model in phylogeographical analyses intended for habitat conservation and as a biodiversity indicator taxon, as proposed by Sluys (1999). These results point to the need for a re-evaluation of species richness in Europe, both from a morphological and a molecular point of view, and also the consideration of their use as a biodiversity marker in the Mediterranean region.

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