MORPHOLOGICAL AND DNA-BASED TAXONOMY OF *TUDORELLA* P. FISCHER, 1885 (CAENOGASTROPODA: POMATIIDAE)

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Abstract *The study of the shell size and shape, including microsculpture of protoconch and teleoconch, together with anatomical studies of the reproductive system allowed us to discriminate three different morphospecies within* Tudorella: T. ferruginea, T. mauretanica *and* T. sulcata. *DNA sequences of two mitochondrial genes, COI and 16S rRNA were used to understand the phylogenetic relationships within the genus. Both individual and combined analyses of these genes showed three main genetically unique lineages which were considered as different species. There was a complete congruence between morphology and phylogeny. The validity of the monophyletic* T. sulcata melitense *and* T. s. sulcata *were also evaluated on the basis of their genetic divergences and morphology.*

Key words *Pomatiidae,* Tudorella, *molecular analysis, morphology, Western mediterranean.*

INTRODUCTION

The genus *Tudorella* P. Fisher, 1805 is a W-Mediterranean genus living in limestone xerophilous habitats near the coast (Giusti, Manganelli & Schembri, 1995; Martínez-Ortí & Robles, 2003, 2005). Two species are currently considered within this genus: *T. ferruginea* (Lamark, 1822) [type species of the genus (Wenz, 1938-1944). Type locality: Balearic Islands (Mermod, 1952)] and *T. sulcata* (type locality: Cuges, Provence, France), although the latter is placed frequently within the genus *Pomatias* S. Studer, 1789. *Cyclostoma sulcatum* Draparnaud, 1805 was segregated from *Pomatias* and included in the genus *Tudorella* P. Fisher, 1805 (Falkner, Ripken & Falkner, 2002) on the evidence of differences in karyotype and chromosome number (Vitturi, Catalano & Macaluso, 1986). The two genera, *Pomatias* and *Tudorella* show also marked differences in the morphology of shell, radula and reproductive system and in the relative position of the distal portion of the digestive system and paleal oviduct (Venmans 1959; Ibáñez & Alonso, 1978, 1980). All these differences justify their consideration as different genera.

Other nominal species assigned to *Tudorella* include *Cyclostoma melitense* Sowerby, 1843 from Maltese Islands, *Cyclostoma mauretanicum* Pallary, 1898 from W-Algeria [type locality: Rhas-el-Madene in the Traras mountains], *Pomatias sulcatus multisulcatus* Potiez & Michaud, 1838 and *P.s. panormitanus* Sacchi, 1954 from Sicily, and *P.s. reticulatus* Kobelt, 1879 from Sardinia. All these names have been considered by some authors synonymous of *T. sulcata* (Germain, 1931; Giusti *et al.*, 1995; Pavón, 2005). Nevertheless, Giusti *et al.* (1995) pointed out that a revision, based in particular on genetic analysis, was needed to ascertain the real status of the western Mediterranean populations of *Tudorella*.

Concerning the Iberian Peninsula, most authors considered that all fossil and recent forms of *Tudorella* from this region should be classified as *Tudorella sulcata* (Gasull, 1972; Ibáñez & Alonso, 1978; 1980; Martínez-Ortí, 1999; Robles & Martínez-Ortí, 1995; Martínez-Ortí & Robles, 2003, 2005). Conversely, Pallary (1898) indicated fossil forms of *Cyclostoma mauretanicum* from the Ibero-Mediterranean area. The aim of the present work was to understand the taxonomical identity of the Iberian populations of *Tudorella* and to give a first draft of the phylogenetic relationships of the main lineages of the genus, by means of shell and reproductive system morphology together with mtDNA sequenciation.

MATERIAL AND METHODS

Morphological studies

Species identification was based on the morphology of shell and reproductive system. Shell meas-

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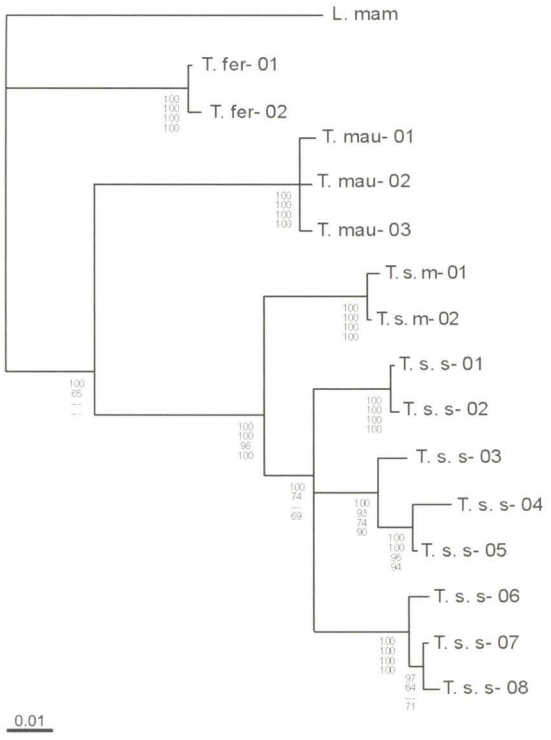

Fig. 1 Molecular phylogeny of *Tudorella* genus was produced by MrBayes analysis of combined sequence data set from COI and 16S rRNA genes. Bootstrap values under MrBayes (2000000 generations), neighbour-joining (1000 replicates), maximum likelihood (1000 replicates) and maximum parsimony (1000 replicates) criteria are shown at each node (MB/NJ/ML/MP) (>65%). Abbreviations as in Table 1.

ures were based on the study of 198 specimens of *Tudorella* (including four specimens of the Siro de Fez collection, n°98). Detail sculpture of the protoconch, teleoconch and operculum were studied by SEM, using a Hitachi S-4100 Scanning Electron Microscope over solid structures coated with gold-palladium. Specimens used for anatomical investigations were dissected after preservation in 70% ethanol and reproductive systems removed under stereomicroscope.

DNA Extraction, PCR amplification and sequencing

A total of 16 samples were used for molecular studies using *Leonia mamillaris* (Lamarck, 1822) as outgroup. Fresh specimens were collected alive, killed by freezing and preserved in absolute ethanol for the analyses. One old specimen (Taourirt, Morocco) was taken from the Siro de Fez collection and dry tissues removed for molecular analyses. Sample localities are included in Table 1. All specimens are stored in the Museu Valencià d'Història Natural in Valencia (Table 1).

Total DNA extraction, from the muscle of each animal was performed using the DNAeasy Tissue Kit (Qiagen). DNA was amplified by the polymerase chain reaction (PCR) with the universal primers: 16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3'), 16Sbr-H (5'-CCGGTCTGAACTCAGATCACGT-3') for

Table 1 Code, localities, geographical coordinates (using Spanish grid referentes, UTM) and GenBank accession numbers of *Tudorella* specimens and *Leonia mamillaris*. Abbreviations: n°coll: collection number of MVHN; T.s.m.: *T. sulcata melitense*; T.s.s.: *T. sulcata sulcata*; T.mau: *T. mauretanica*; T.fer: *T. ferruginea*; L.mam: *Leonia mamillaris*.

Code	Localities	n°coll	UTM	COI	16S RNA
T.s.m-01	Red Tower, Cirkewwa (Malta)	1083	33SVV48	EF053236	EF053251
T.s.m-02	" "	"	"	EF053237	EF053252
T.s.s-01	Palermo: Monte Gallo, La Fossa (Sicily, Italy)	1416	33SUC52	EF053238	EF053253
T.s.s-02	" "	"	"	EF053239	EF053254
T.s.s-03	Alghero: Capo da Caccia (Sardinia, Italy)	1344	32TMK29	EF053240	EF053255
T.s.s-04	Rass el Hamra, Annaba (= Bone) (Algeria)	1086	32SLF98	EF053241	EF053256
T.s.s-05	" "	"	"	EF053242	EF053257
T.s.s-06	Massif des Calanques, Sugiton Marseille (France)	1096	31TFH99	EF053243	EF053258
T.s.s-07	" "	"	"	EF053244	EF053259
T.s.s-08	Monte Albo, cuestas de la Punta Catirina (Sardinia, Italy)	1088	32TMK48	EF053245	EF053260
T.mau-01	Motril: El Tajo del Escalate (Granada, Spain)	1094	30SVF57	EF053234	EF053249
T.mau-02	Orihuela: the gully north of the gully of la Cañada de la Estaca (Alicante, Spain)	1162	30SXH99	EF053235	EF053250
T.mau-03	Taourirt (Morocco)	98	30SWD00	EF215453	EF215452
T.fer-01	Ciutadella de Menorca: Beach d'Es Tancats. Algaiarens (Minorca island, Spain)	1202	31TEE73	EF053232	EF053247
T.fer-02	" "	"	"	EF053233	EF053248
L.mam	Pilar de la Horadada: Village (Alicante, Spain)	862	30SXG99	EF053231	EF053246

Table 2 p genetic distances (16S rRNA above diagonal, COI below diagonal) for all pairwise comparisons among *Tudorella* specimens and *Leonia mamillaris*. Abbreviations as in Table 1.

Samples	T. s.s- 01	T. s.s- 02	T. s.s- 03	T. s.s- 04	T. s.s- 05	T. s.s- 06	T. s.s- 07	T. s.s- 08	T. s.m- 01	T. s.m- 02	T. mau- 01	T. mau- 02	T. mau- 03	T. fer- 01	T. fer- 02	L- mam
T. s.s- 01		0 %	2,87 %	3,09 %	3,09 %	2,2 %	1,98 %	1,98 %	4,84 %	4,84 %	10,81 %	10,59 %	10,84 %	9,13 %	9,76 %	12,75 %
T. s.s- 02	0,15 %		2,87 %	3,09 %	3,09 %	2,2 %	1,98 %	1,98 %	4,84 %	4,84 %	10,81 %	10,59 %	10,84 %	9,13 %	9,76 %	12,75 %
T. s.s- 03	3,64 %	3,79 %		0,88 %	0,88 %	1,32 %	1,54 %	1,54 %	3,08 %	3,08 %	10,17 %	10,39 %	10,64 %	8,23 %	8,86 %	12,12 %
T. s.s- 04	4,7 %	4,86 %	2,58 %		0 %	1,32 %	1,54 %	1,54 %	3,08 %	3,08 %	10,83 %	11,05 %	11,3 %	8,24 %	8,87 %	12,11 %
T. s.s- 05	3,79 %	3,95 %	1,37 %	1,21 %		1,32 %	1,54 %	1,54 %	3,08 %	3,08 %	10,83 %	11,05 %	11,3 %	8,24 %	8,87 %	12,11 %
T. s.s- 06	4,7 %	4,86 %	4,4 %	4,86 %	4,55 %		0,2 %	0,2 %	3,51 %	3,51 %	10,13 %	10,35 %	10,60 %	8,21 %	8,84 %	11,60 %
T. s.s- 07	4,55 %	4,7 %	3,95 %	5,31 %	4,4 %	0,76 %		0 %	3,29 %	3,29 %	10,35 %	10,57 %	10,82 %	7,99 %	8,62 %	11,82 %
T. s.s- 08	4,4 %	4,55 %	4,1 %	5,16 %	4,25 %	1,21 %	0,46 %		3,29 %	3,29 %	10,35 %	10,57 %	10,82 %	7,99 %	8,62 %	11,83 %
T. s.m- 01	5,01 %	5,16 %	4,7 %	6,07 %	5,46 %	5,77 %	5,92 %	6,07 %		0 %	10,8 %	11,02 %	11,27 %	8,88 %	9,51 %	13,15 %
T. s.m- 02	4,7 %	4,86 %	4,4 %	6,07 %	5,16 %	5,77 %	5,61 %	5,77 %	0,3 %		10,8 %	11,02 %	11,27 %	8,88 %	9,51 %	13,15 %
T. mau- 01	7,59 %	7,74 %	7,13 %	8,35 %	7,59 %	8,05 %	8,2 %	8,65 %	8,04 %	7,74 %		0,22 %	0,66 %	8,46 %	8,91 %	12,34 %
T. mau- 02	7,59 %	7,74 %	7,13 %	8,65 %	7,89 %	8,04 %	8,19 %	8,65 %	8,04 %	7,74 %	0,61 %		0,44 %	8,24 %	8,68 %	12,12 %
T. mau- 03	7,28 %	7,44 %	6,83 %	8,35 %	7,59 %	7,74 %	7,89 %	8,35 %	7,74 %	7,44 %	0,3 %	0,3 %		8,48 %	8,04 %	12,59 %
T. fer- 01	8,35 %	8,5 %	8,65 %	9,41 %	8,5 %	9,56 %	9,71 %	9,56 %	9,56 %	9,26 %	8,8 %	9,1 %	8,8 %		0,44 %	10,06 %
T. fer- 02	8,35 %	8,5 %	8,65 %	9,41 %	8,5 %	9,56 %	9,71 %	9,56 %	9,56 %	9,26 %	8,8 %	9,1 %	8,8 %	0 %		10,73 %
L. mam	9,41 %	9,56 %	10,32 %	10,47 %	9,71 %	10,77 %	10,93 %	10,77 %	9,41 %	9,41 %	9,41 %	9,56 %	9,26 %	9,26 %		

16S gene fragment (Palumbi, Martin, Romano, McMillan, Stice & Grabowski, 1991) and LCO 1490 (5′-GGTCAACAAATCATAAAGATATTG-3′), HCO 2198 (5′-TAAACTTCAGGGTGACCA AAAAATCA-3′) for COI gene fragment (Folmer, Black, Hoeh, Lutz & Vrijenhoek, 1994). The PCR conditions were an initial denaturation step of 93° C (2 min), followed by 40 cycles of 93° C (45 s), 54° C and 57.7°C (1 min) (54° C for COI and 57.7°C for 16S rRNA) and 72° C (1 min). The cycling ended in an extension phase at 72° C for 7 min. The products of reaction were run in 1.5% agarose gels and stained with ethidium bromide to verify the amplifications of both fragments. Amplicons were sequenced using the dRhodamine TerminatorCycle Sequencing Ready Reaction Kit (AppliedBiosystems), in an ABI PRISM Model 3100 Avant Genetic Analyzer. The sequences were deposited in GenBank (Table 1).

Phylogenetic analyses

All sequences were aligned prior to phylogenetic analyses with Clustal X (Thompson, Gibson, Plewniak, Jeanmougin F & Higgins 1997) and manually adjusted. The levels of saturation were calculated in DAMBE version 4.2.13 (Xia & Xie 2001). There was no saturation, therefore third codon position data for COI were included in the analyses. Gaps were treated as missing data. The sequence data were partitioned into two data sets: the COI and the 16S rRNA. Subsequently these two data sets were concatenated into a combined matrix. These three data sets were subjected to MrBayes (MB) (Huelsenbeck & Ronquist, 2001), neighbour-joining (NJ) (Saitou & Nei, 1987), maximum likelihood (ML) (Felsenstein, 1981) and maximum parsimony (MP) (Fitch, 1971) analyses. The models of evolution were calculated using Modeltest v.3.06 (Posada & Crandall, 1998) imported into PAUP* 4.0b10 (PPC) (Swofford, 2002). The Akaike information criterion (AIC) was used to determine the appropriate models in each dataset. The models obtained were GTR + G (Rodríguez, Oliver, Marín & Medina, 1990) for COI, GTR + I (Rodríguez *et al.*, 1990) for 16S rRNA and GTR + G (Rodríguez *et al.*, 1990) for the combined matrix. Bayesian analyses (MB) were performed using the MrBayes v3.0 package. The GTR model was used in the three data matrix and rate variation across sites was modelled using gamma distribution, with a proportion

Table 3 Morphometrical data of the shells of *Tudorella* spp. (n°spm= number of specimens; Huv= height of last whorl; Hmx: maximum height of the aperture; Wmx: maximum width of the aperture; Mx= maximum number of spire whorls for each species/population; Pr= Number of protoconch whorls; A/H (%)= Relation between the aperture height and the total height. TABLE 2

Taxa	Localities		n° spm	Height	Width	Huv	Aperture			n° Whorls	
							A/H (%)	Hmx	Wmx	Mx	Pr
T. ferruginea	Balearic Islands		♂/20	15.7-18.5	8.8-10.5	10.4-11.3	35–40	7.1	6.5	6	2⅛
	Ibáñez &Alonso (1978) + own data		♀/21	17.3-22.4	9.6-12.2	11.2-13.2		8.1	6.9		
	Orihuela (Spain)		♂/43	15.55-18.98	11.42-14.65	12.6-15.1		9.1	8.7	5¼	2
			♀/31	17.75-25.0	12.61-18.1	14.7-18.9		12.1	10.4		
	Motril (Spain)		♂/23	17.6-19.9	12.5-14.65	14.2		9.1	7.8	5¼	2⅛
	Ibáñez &Alonso (1978) + own data		♀/23	21.6-23.8	14.4-17.2	17.5		10.9	9.3		
T. mauretanica	Morocco-W-Algeria	Pallary 1898	-	22.0	16.0	12.0	43.2–52	10.0	9.0	-	-
		Own data	♂/3	19.2	13.9	14.0-14.4		9.1	8.4	5	2
			♀/1	20.4	15.0	15.1		9.8	8.2		
T. s. sulcata	Cassis (Fr) (Venmans, 1959)		2	16.8	12.9-13.3	-	42–50	8.0	7.4	5¼	-
	Marseille- Sugiton (France)		♂/5	15.7-17.1	11.2-13.3	11.0-12.4		8.0	7.0	5¼	2
			♀/5	17.5-19.6	13.0-15.1	12.1-13.5		8.9	8.0		
	Sardinia (Italy)		♂/3	13.8-15.0	10.6-11.4	9.9-10.6		6.8	5.8	5⅛	2
			♀/4	15.9-17.7	11.4-12.7	11.4-12.0		7.4	6.7		
	Sicily (Italy)		♂/2	15.7-16,4	11.8-11.9	11.7-11.9		7.6	6.9	5	2⅛
			♀/2	15.8-17,6	11.8-12,7	11.6-12.4		7.9	7.2		
	Annaba + Bejaia (Algeria)		♂/2	14.9-16.8	10.8-12.6	10.0-12.3		8.0	7.1	5 3/8	2⅛
			♀/4	16.0-18.3	10.9-13.3	10.8-13.1		8.6	7.6		
	(Venmans, 1959)		4	12.8-16.4	9.0-10.9	-		6.8	6.7	5½	-
T. s. melitense	Red Tower + Ghajn Tuffieha (Malta)		♂/ 13	12.8-14.6	8.5-10.3	9.1-10.7	39.5–45.3	6.8	5.0	5 ¾	2
			♀/ 13	14.7-18.0	9.7-12.2	10.3-12.3		7.5	7.0		

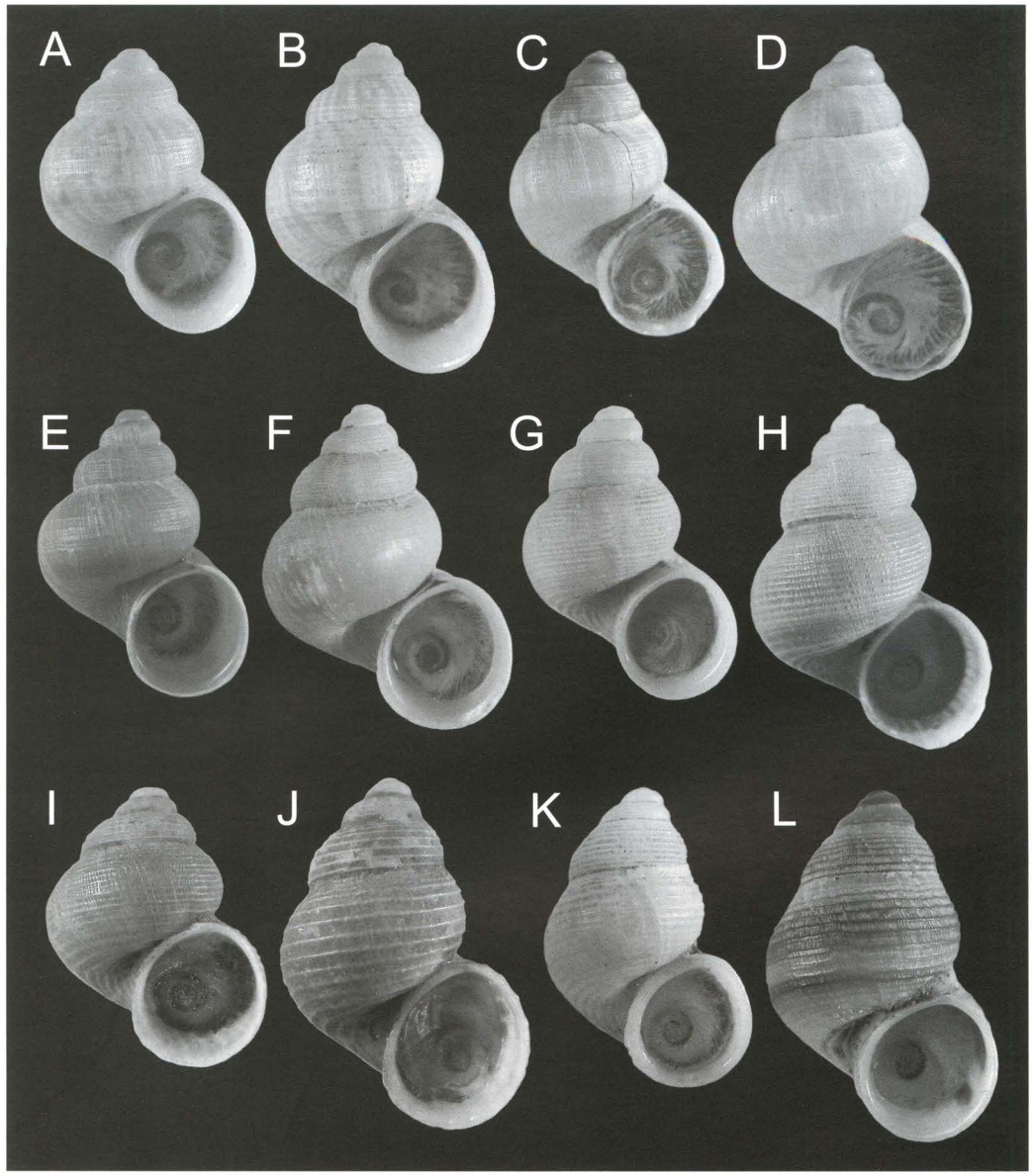

Fig. 2 Some *Tudorella*'s shells of the studied localities. **A-B** *Tudorella mauretanica* (Orihuela, Spain) **A** Male (12.6 mm Ø; 16.0 mm H). **B** Female (16.2 mm Ø; 22.5 mm H). **C-D** *T. mauretanica* (Taourirt, Morocco). **C** Male (13.6 mm Ø; 18.8 mm H) **D** Female (14.8 mm Ø; 20.5 mm H). **E-F** *T. sulcata sulcata* (Marseille, France). **E** Male (11.8 mm Ø; 16.0 mm H). **F** Female (15.4 mm Ø; 19.4 mm H). **G-H** *T. s. sulcata* (Capo da Caccia, Sardinia, Italy). **G** Male (11.3 mm Ø; 14.7 mm H). **H** Female (12.0 mm Ø; 17.9 mm H). **I-J** *T. s. sulcata* (Bejaia, Algeria). **I** Male (12.5 mm Ø; 16.1 mm h). **J** Female (13.0 mm Ø; 18.2 mm H). **K-L** *T. s. melitense* (Red Tower, Malta). **K** Male (9.7 mm Ø; 14.1 mm H). **L** Female (10.8 mm Ø; 16.2 mm H).

of the sites being variants. The Markov Chain Monte Carlo (MCMC) search was run with four chains for 2 million generations, with trees being sampled every 100 generations (the first 2000 trees were discarded as "burnin"). In the combined analyses, variation was partitioned among genes. Another hypothesis of the phylogenetic relationships was obtained with neighbor-joining analyses (NJ) imported in PAUP*. The trees were constructed using respectively the model of evolution in each data set. The uncorrected "p" pairwise distances were also calculated for COI and 16S rRNA (Table 2). Bootstrap confidence estimates for each node were based on 1000 replicates. In the maximum parsimony (MP) analyses an heuristic search was used, with 10 random addition replicates, using the tree bisection reconnection (TBR) option and saving

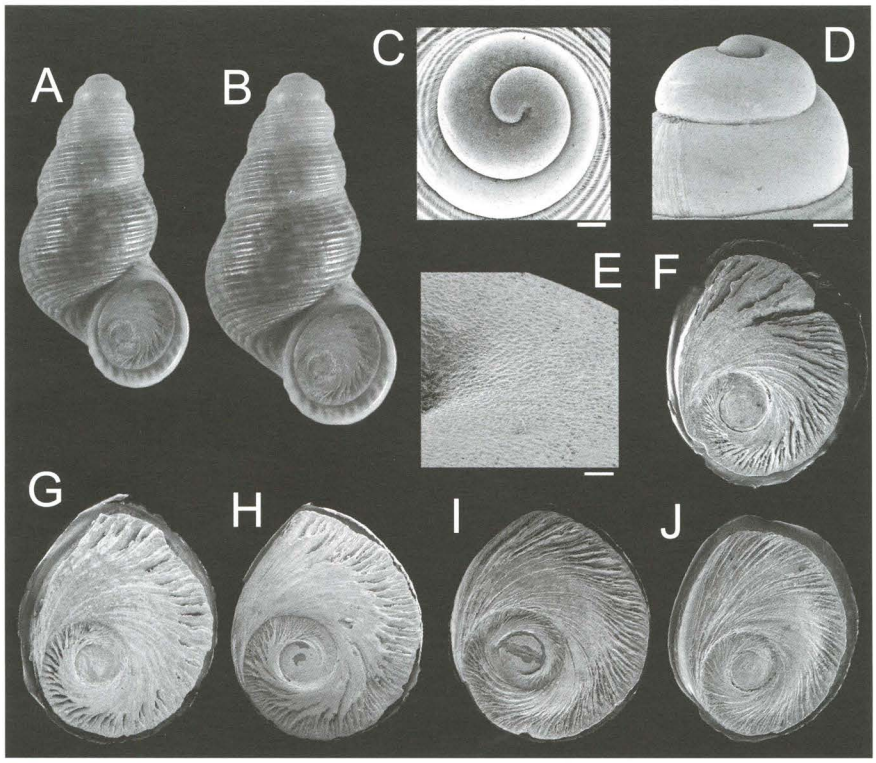

Fig. 3 A-F *T. ferruginea* (Ciutadella, Minorca island, Spain). **A** Male (Ciutadella, Minorca, Spain) (9.3 mm Ø; 16.9 mm H). **B** Female (Ciutadella, Minorca) (12.2 mm Ø; 21.1 mm H). **C** Protoconch (Sant Vicenç, Majorca) (b= 500 µm). **D** Protoconch (Sant Vicenç, Majorca) (b= 500 µm). **E** Protoconch sculpture (Sant Vicenç, Majorca) (b= 50 µm). **F** Operculum (Ciutadella, Minorca) (H=6.3 mm). **G** Operculum of *T. mauretanica* (Orihuela, Spain) (H=7.4 mm). **H** Operculum of *T. mauretanica* (Taourirt, Morocco) (H= 7.8 mm). **I** Operculum of *T. sulcata sulcata* (Marseille, France) (H=6.5 mm). **J** Operculum of *T. sulcata melitense* (Red Tower, Malta) (H= 6.0 mm).

multiples trees to find the most parsimonious ones. Parsimony bootstrap support values were calculated through 1000 bootstrap replicates. The weight of transversions (Tv) and transitions (Ts) was varied depending of the fragments analysed and it was estimated by maximum likelihood. The weighting scheme was 5:1 for COI fragment and 7:1 for the 16S rRNA.

Using maximum likelihood (ML) analyses, there were constructed three trees for COI, 16S rRNA and the concatenated data sets, with an heuristic search strategy using random sequence addition and 1000 replicates implanted in PAUP*.

Results

Morpho-anatomical studies

Three different morphological patterns were evident in *Tudorella* after the morphological study of shell and reproductive system. *T. ferruginea* joined the specimens from the Balearic Islands. Populations from Sardinia, Sicily and N-Algerie were very similar to topotype specimens of *T. sulcata* from Marseille and were classified as *T. sulcata sulcata*. Specimens from Malta showed differences in morphology and were classified as *T. sulcata melitense*. The populations of the ibero-mediterranean area (Motril and Orihuela) were morphologically similar to the specimens collected from Taourirt (Morocco), a locality placed near the type locality of *T. mauretanica*, and were thus classified as *T. mauretanica*. Identification of the four taxa fully agreed with the original descriptions (Pallary, 1898; Ibáñez & Alonso, 1978, 1979, 1980; Giusti *et al.*, 1995; Martínez-Ortí & Robles, 2003, 2005).

Shell (Figs 2–6, Table 3) For this section we have assigned to *T. mauretanica* the data published by Ibáñez & Alonso (1978, 1980) for *T. sulcata* (collected from Motril, Granada). Shell measures and maximum number of protoconch and teleoconch worls are summarized in Table 3. All the species of the genus *Tudorella* show a marked sexual

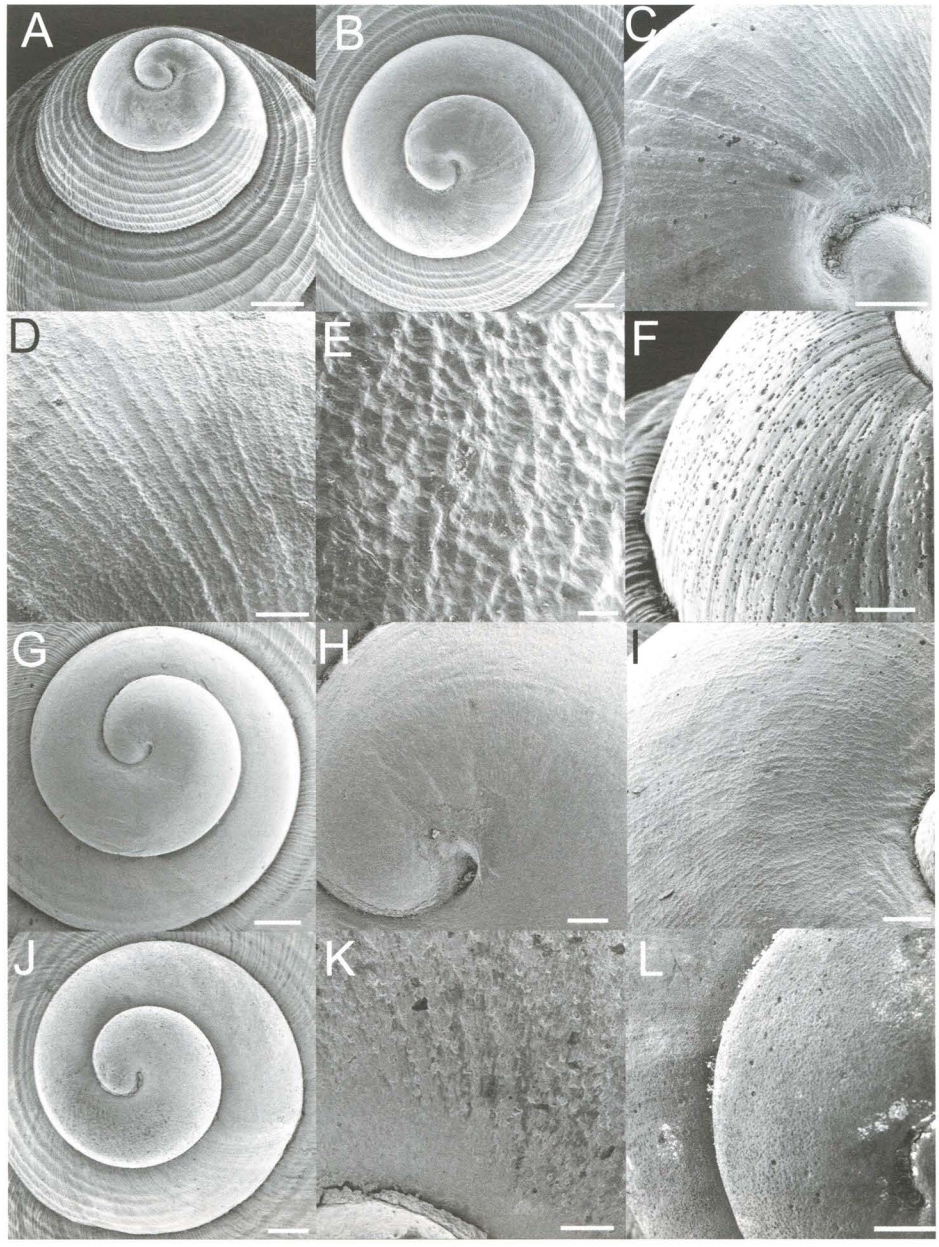

Fig. 4 Shell morphological details of *T. mauretanica*. **A-F** Orihuela (Spain). **A** Apex [bar (b)= 1000 µm]. **B** Protoconch (b= 500 µm). **C-F** Sculpture protoconch details. **C** Detail of protoconch (b= 250 µm). **D** Detail of the thin protoconch lines (b= 100 µm). **E** Detail of the sculpture of the protoconch surface (b= 25 µm). **F** Sculpture of the protoconch last lap (b= 250 µm). **G-I** Motril (Spain). **G** Protoconch (b= 500 µm). **H** Detail without lines (b= 100 µm). **I** Details of the protoconch lines (b= 100 µm). **J-L** Taourirt (Morocco). **J** Protoconch (b= 500 µm). **K** Details of the thin lines on the protonch (b= 100 µm). **L** Sculpture detail protoconch of another specimen (b= 250 µm).

dimorphism with female shells being bigger than male ones (Ibáñez & Alonso, 1978, 1980; Martínez-Ortí, 1999; Martínez-Ortí & Robles, 2003, 2005).

– *T. ferruginea* (Figs 3a–f; 6f): Shell is more slender, with the body whorl being higher and narrower than in other species of the genus (Table 3). The last whorl is 5/8 of the whole shell length, with diameter smaller than height. Shell whorls are slightly convex and, consequently, sutures are shallow. Teleoconch spiral costulae are prominent, but narrow, close and tenuous radial striae can be distinguished only with microscope magnification. Protoconch is nearly smooth with very small depressions. The first whorls are yellow-orange coloured but the rest is brown-red-

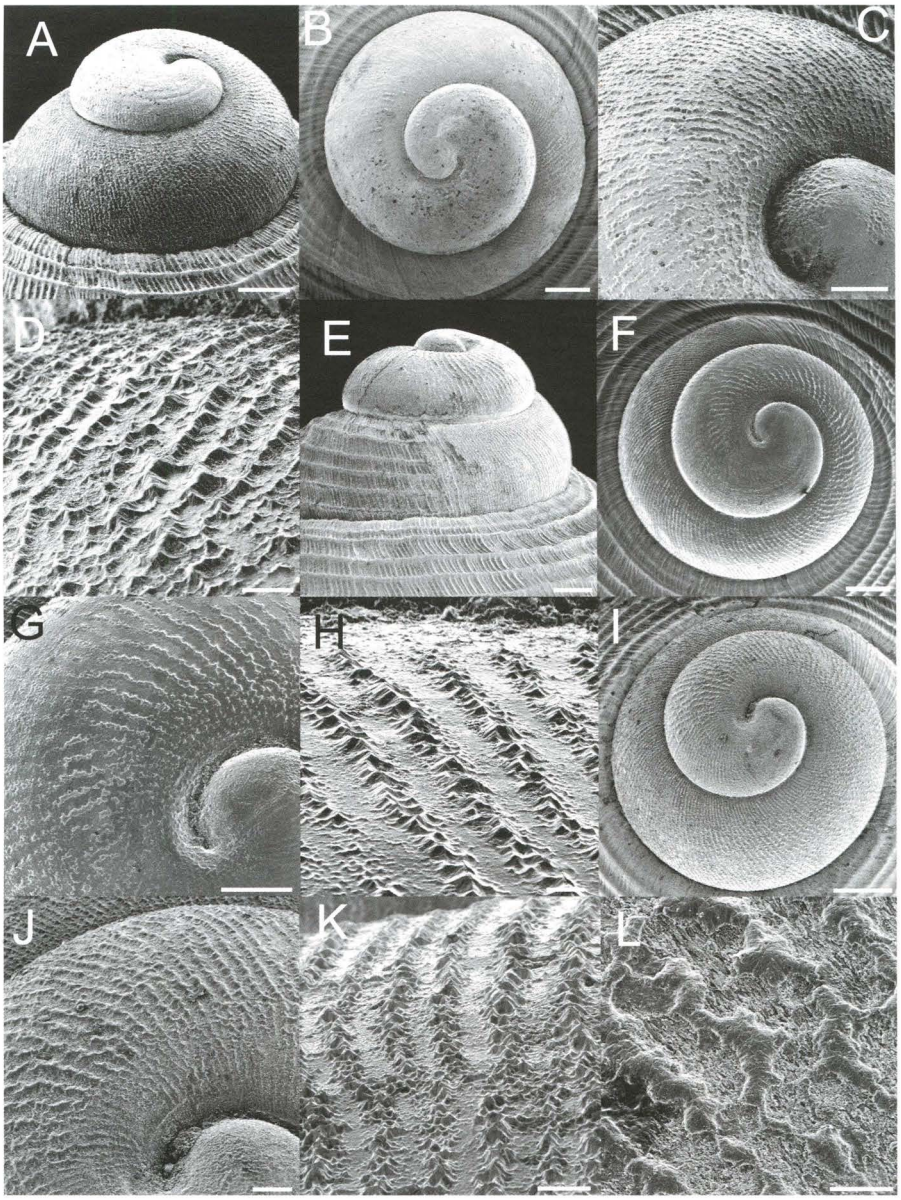

Fig. 5 **A-D** Shell morphological details of *T. sulcata sulcata* (Marseille, France). **A** Apex (b= 500 µm). **B** Protoconch (b= 500 µm). **C** Detail of the first whorl of the protoconch (b= 250 µm). **D** Detail of the protoconch sculpture (b= 50 µm). **E-H** *T. s. sulcata* (Bejaia, Algeria). **E** Apex (b= 500 µm). **F** Protoconch (b= 500 µm). **G** First whorl detail of the protoconch (b= 250 µm). **H** Detail of the lines on the protoconch (b= 25 µm). **I-L** *T. s. melitense* (Red Tower, Malta). **I** Protoconch (b= 500 µm.). **K** (b= 25 µm). **L** Detail of the sculpture protoconch at the beginning of the first whorl (b= 25 µm).

dish with light flammulae. The operculum is not deeply sunk within the aperture and the nucleus has 1¼-1⅜ whorls, with the upper corner acute.
– *T. mauretanica* (Figs 2a–d; 3g–h; 4; 6a–b): Shells can reach the biggest size within the genus; they are more globose because of the greater wideness of the last whorl. Last whorl is big, similar in length and diameter, and equal or greater than ¾ of total shell length (Table 3). It has more convex whorls and deeper sutures. The colour is yellow-orange, homogeneous in the first whorls but with radial, parallel and irregular darker stripes. Teleoconch sculpture is reticulated being less marked, with the spiral costulae less elevated, less concave and more separated than in the other *Tudorella* species. Protoconch is nearly smooth but it can show shallow radial, parallel, ribs. The operculum is flattened, elongated and with not more convex margins and deeply sunk within the aperture. The nucleus of operculum is deeper and has 2 whorls and occasionally a little more than 2 whorls. The upper corner of

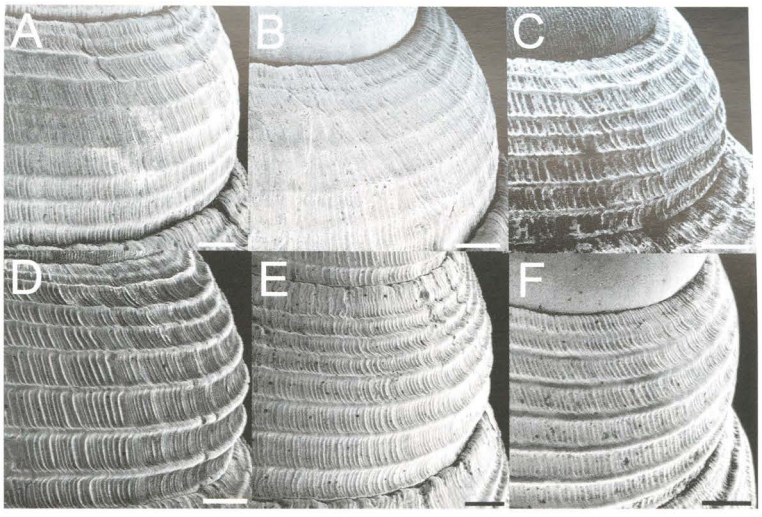

Fig. 6 Teleoconch sculpture of: **A** *T. mauretanica* (Orihuela, Spain). **B** *T. mauretanica* (Taourirt, Morocco). **C** *T. sulcata sulcata* (Marseille, France). **D** *T. s. sulcata* (Bejaia, Algeria). **E** *T. s. melitense* (Red tower, Malta Island). **F** *T. ferruginea* (Sant Vicenç, Majorca).

the operculum is the most acute of all *Tudorella* species.

– *T. sulcata sulcata* (Figs 2e–j; 3i; 5a–h; 6c–d) and *T. sulcata melitense* (Figs 2k–l; 3j; 5–l; 6e): Shell measures of the two subspecies are the smallest of the genus (Table 3). Shell whorls in *T.s. melitense* are slightly convex and, consequently, sutures are shallow. On the contrary, *T.s. sulcata* have more convex whorls and deeper sutures. The protoconch of both subspecies is similar, with microsculpture of radial, parallel ribs which are very apparent being more prominent far from the nucleus with very irregular margins. Interstices are slightly wider than ribs. Shell colour of *T.s. sulcata* is more constant; commonly, it is uniformly white-yellowish to orange, or it can have light-brown to red spiral stripes; in the more intensely coloured shells, these brown-red stripes can cover the whole whorls with only one thin light band near the suture. Shell colour in *T.s. melitense* is similar to that described for *T.s. sulcata*, but the last pattern is the most common. The operculum in both taxa is deeply sunk within the aperture, flattened, elongated and with convex margins, but it is nearly rounded in *T.s. melitense*. The nucleus of operculum has from 1½ to 2 whorls in *T.s. sulcata* and to 1½ in *T.s. melitense*.

Reproductive system (Figs 7–8) In all the dissected females, the relative position of the distal portion of the digestive system and paleal oviduct corresponds to that defined by Ibáñez & Alonso (1980: Fig. 5B) as characteristic for *Tudorella*. The same position can be seen in *T.s. melitense* (Giusti *et al.*, 1995: Fig. 38) (Figs 7c, 8b, 8e, 8g). The relative position between both organs is different in *Pomatias* and *Leonia* Gray, 1850.

We can distinguish two different penis forms. One is the penis of *T. ferruginea* and *T. mauretanica* wider in the middle of the distal portion of penis than in the proximal half, with the penial apex shorter and conical (Figs 7a, 7e– f) (Ibáñez & Alonso, 1978, 1979, 1980; Martínez-Ortí, 1999; Martínez-Ortí & Robles, 2005). The second corresponds to the penis shape of *T. sulcata* which is more cylindrical, with the penial apex slender and longer (Figs 8a, 8c–d, 8f) (Giusti *et al.*, 1995). The prostatic gland shows a great variability, from rectangular to round or oval, in *T. ferruginea* and *T. mauretanica* (Figs 7a–b, 7e–f). It is oval elongated in *T. sulcata* (Figs 8a, 8c–d, 8f). The connection of the renal vas deferens to the prostatic gland is very different in *T. sulcata* with respect to *T. ferruginea* and *T. mauretanica*. The junction of both organs is subterminal, near the left proximal end of the prostatic gland, in *T. ferruginea* and *T. mauretanica* (Figs 7a–b, 7e–f) (Ibáñez & Alonso, 1978, 1979, 1980; Martínez-Ortí, 1999; Martínez-Ortí & Robles, 2005), while the renal vas deferens joins laterally to the prostatic gland in *T. sulcata* (Figs 8a, 8c–d, 8f). This lateral displacement of the vas deferens insertion to prostatic gland is more evident in the Algerian

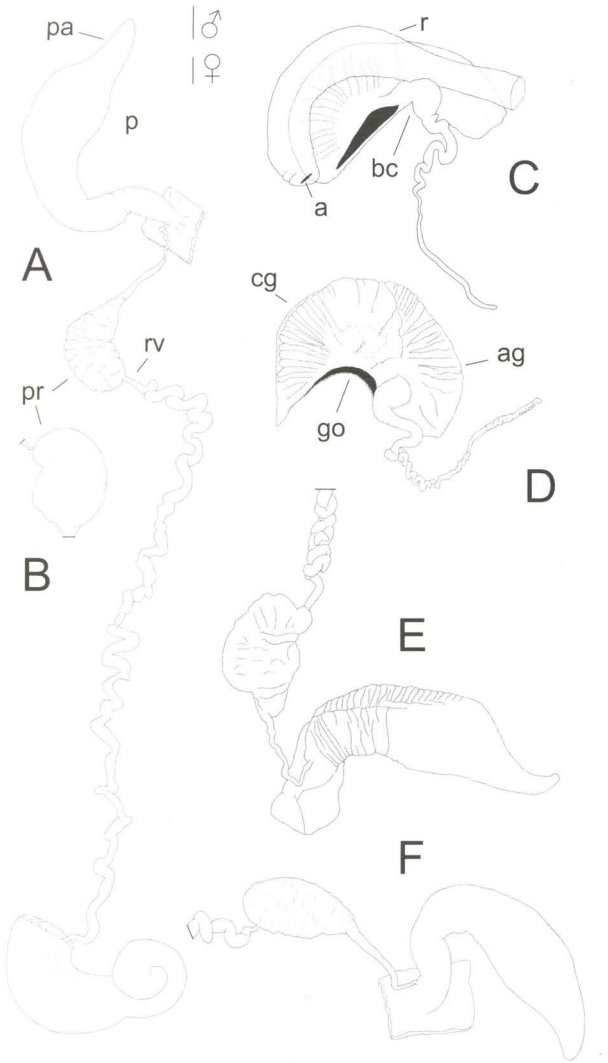

Fig. 7 Reproductive's system of *Tudorella*'s species. **A-B-C** *T. ferruginea* (Sant Vicenç, Mallorca). **A** Male. **B** Prostatic gland. **C** Female. **D-E-F** *T. mauretanica* (Orihuela, Spain). **D** Female. **E-F** Male. (Abbreviations: a: anus; ag= albumen gland; bc= bursa copulatrix; cg= capsule gland; go= genital opening; p= penis; pa= penial apex; pr= prostatic gland; r= rectum; rv= renal vas deferens). (b= 1 mm).

and Maltese populations of *T. sulcata*. The bursa copulatrix is smaller in *T. sulcata* than in *T. ferruginea* and *T mauretanica* (Figs 7c-d). This organ in *T.s. melitense* can be located next to the albumen gland, farther away from the paleal oviduct opening than in the other three taxa (Fig. 8g).

MOLECULAR STUDIES

Phylogenetic analyses were based on the three different sequence data sets: COI, 16S rRNA and the concatenated matrix. Two fragments of 658 bp (after removing primers) from COI and 478 bp from the 16S rRNA were sequenced. The combined length of the two aligned fragments was 1136 bp. Nine hundred and fifteen (80.54%) characters were constant, 44 (3.87%) variable characters were parsimony uninformative and 177 (15.58%) characters were parsimony informative. The four methods of phylogenetic inference (MB, NJ, ML and MP) recovered nearly identical trees for COI and concatenated data sets (Fig. 1). The phylogenetic tree showed three major monophyletic groups supported by very high bootstrap values. *T. ferruginea* samples were joined in a clade and appeared as the basal group of the rest of *Tudorella* populations. The two Iberian populations of *Tudorella* were grouped together with the sample from Morocco in a single clade that we called *T. mauretanica*, which constituted the sister group of *T. sulcata* populations. The phylogenetic analysis of 16S rRNA recovered the same three main monophyletic clades, but *T. ferruginea* and *T. mauretanica* constituted a monophyletic clade as the sister group of *T. sulcata*. The different rearrangement of the three main phylogroups for the two mtDNA fragments was the cause of the low statistical support (except for MB analysis) of the clade joining *T. mauretanica* and *T. sulcata* in the concatenated analysis. The genetic divergences of these clades are summarized in Table 2. Excluding the Malta specimens, all the populations of *T. sulcata* (coming from Sicily, Sardinia, Algeria and Marseille) were grouped in the same clade joined by a common polytomy. Thus, their phylogenetic relationships were not resolved indicating that more populations should be analysed to know their phylogeny. *T. sulcata* populations from Malta were grouped as the sister group of the clade that joined the other populations of *T. sulcata*. The subdivision of two different subspecies in *T. sulcata* (*T.s. melitense* and *T.s. sulcata*) which were described by Picard (1949) based on the morphological differences and biogeographical distribution, seemed to be well supported by the phylogenetic analyses of the mtDNA sequences. Genetic distances between *T.s. sulcata* and *T.s. melitense* ranged from 4.4% to 6.07% in COI and from 3.08% to 4.84% in 16S rRNA (Table 2). Within the clade which joined all the different populations of *T. s. sulcata* the pairwise genetic distances were 5.31% for COI and 3.09% for 16S rRNA.

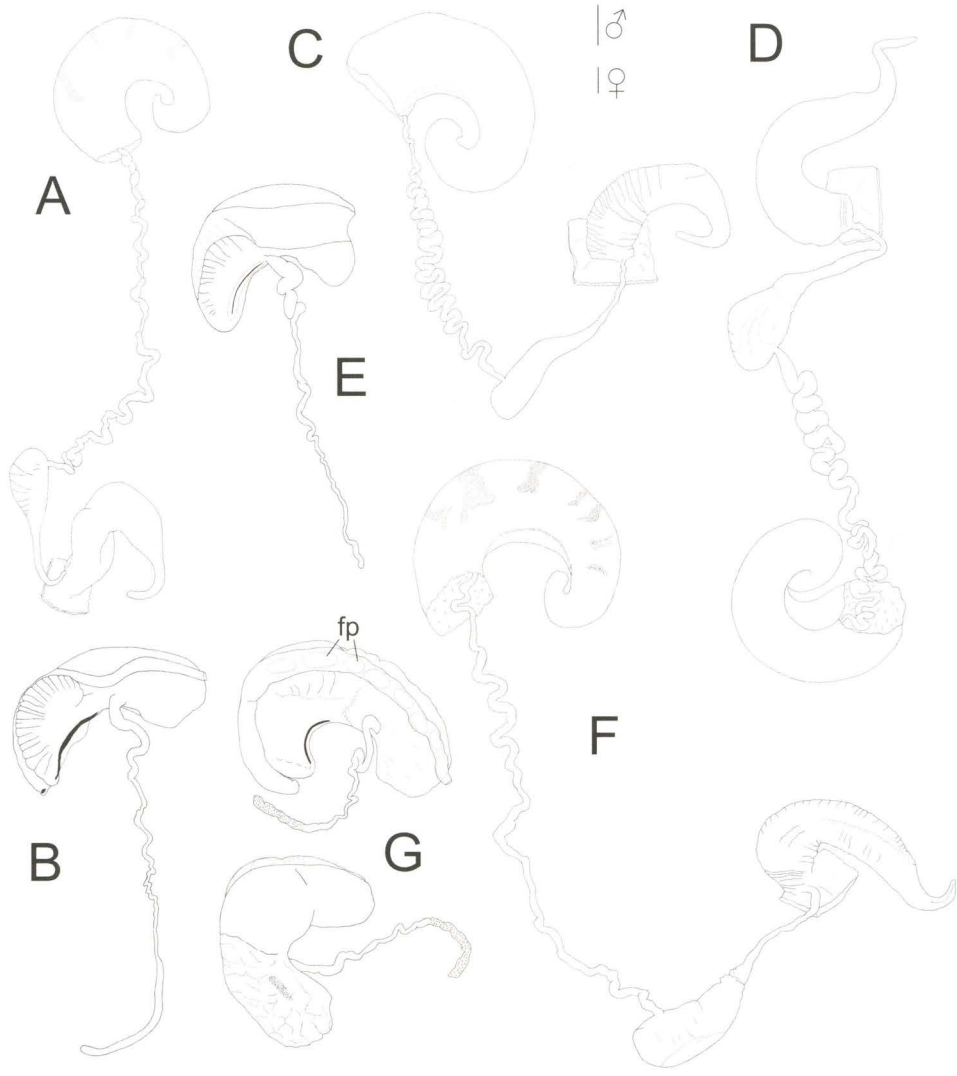

Fig. 8 A-B *T. sulcata sulcata* from Marseille (France). **A** Male. **B** Female. **C-D-E** *T. sulcata sulcata* from Algeria. **C** Male (Bejaia). **D** Male (Annaba). **E** Female (Annaba). **F-G** *T. sulcata melitense* (Red Tower, Malta). **F** Male. **G** Female. (Abbreviations: fp= faecal pellets). (b= 1 mm).

Geographical distribution (Fig. 9) The whole genus is distributed along the W-Mediterranean area. *T. ferruginea* is endemic of the Balearic Islands (Majorca, Minorca and Cabrera) (Ibáñez & Alonso, 1979). *T. mauretanica* is present at the SE-Iberian Peninsula, in two localities in the Alicante and Granada provinces, and NW-Africa [near the line coast of W-Algeria and E-Morocco from Oran to Melilla (Spain), up to 80 km inland Morocco]. Fossil forms of *T. mauretanica* dated at the end of the Pliocene are known from the Iberian Peninsula (Ibáñez & Alonso, 1978, 1980; Robles & Martínez-Ortí, 1995; Martínez-Ortí, 1999; Martínez-Ortí & Robles, 2005) and N-Africa (Pallary 1898, 1901). Besides, Robles (pers. comm.) collected one Pleistocene fossil shell from Al Hoceima (Morocco), the westernmost known locality of *T. mauretanica*. We have been searching for *T. mauretanica* in November, 2006 along the north of Morocco from Melilla to the Algerian border, but we did not see any specimen or shell of the genus. *T. sulcata sulcata* is present in E-Algeria, Sardinia, Sicily and SE-France (Drapanaud, 1805; Potiez & Michaud, 1838; Kobelt, 1879; Sacchi, 1954; Pavón, 2005). Recently Abbes & Nouira (2007) have reported alive specimens of *T. sulcata* from Tunisia. Finally, *T. sulcata melitense* is endemic of the Maltese Islands (Giusti *et al.*, 1995).

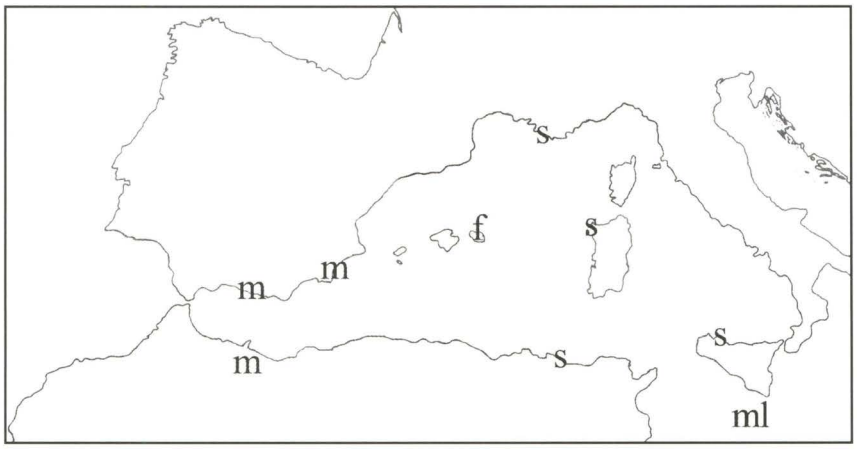

Fig. 9 Location of the material studied *Tudorella*'s species (f= *T. ferruginea*; m= *T. mauretanica*; ml= *T. sulcata melitense*; s= *T. sulcata sulcata*).

DISCUSSION

The morphological and molecular studies revealed tour different taxa in *Tudorella*: *T. ferruginea*, *T. mauretanica*, *T. sulcata sulcata* and *T.s. melitense*. Shell differences include shell size, shape and colour, sculpture of protoconch and teleoconch, last worl and shell aperture height, and opercula.

The shell of *T. ferruginea* is slender, being more globose in *T. mauretanica*, *T.s. sulcata* and *T.s. melitense*. *T. mauretanica* shell is the biggest of the genus. Pallary (1898) indicated that the main difference between *T. mauretanica* and *T. sulcata* was the shell size that in the former could get up to 22 mm height and 16 mm width (without sex specification). Germain (1931) indicated a range of 12 – 18 mm height and 10 – 15 mm width for *T.s. sulcata.* Picard (1949) indicated that *T. sulcata melitense* had the smallest shell of the genus. Nevertheless, our results indicate that specimens of *T.s. sulcata* can be smaller than *T.s melitense* (Table 3). Giusti *et al.* (1995) indicated 16.5 mm height and 11.0 mm width for *T.s. melitense*, without sex specification. *T. mauretanica, T. s. sulcata* and *T. s. melitense* get up to 5 to 5¾ shell whorls while *T. ferruginea* gets as many as 6 whorls (Table 3). Last whorl in *T. ferruginea* is ⅝ of the whole shell length, in *T. mauretanica* it is equal or greater than ¾ of total shell length while last worl in *T.s. sulcata* and *T.s. melitense* is below ¾ of the whole shell height. These results agreed with Pallary (1898) who indicated that the body whorl was greater in *T. mauretanica* than in *T. sulcata*. Germain (1931) for *T. sulcata* and Ibáñez & Alonso (1978, 1980) for *T. mauretanica* pointed out that last whorl of these species nearly represented the half part of the total length, but Giusti *et al.* (1995) said that it was ⅔ of the whole length. Teleoconch sculpture is reticulated in the three species of *Tudorella*, being less marked in *T. mauretanica* (Pallary, 1898; Picard, 1949; Ibáñez & Alonso, 1980) (Fig. 6). Specimens of *T.s. sulcata* from Marseille show rather shallow sculpture in the body whorl (Fig. 2f), being deeper in the rest of the whorls (Fig. 6c). Venmans (1959) pointed out that Maltese specimen shells had less number of longitudinal striae (about 20 on the last whorl) than shells from Cassis (France) (about 30). In opposition to Venmans (1959), we have not found differences in the number of striae after the study of two samples from Marseille and several from Malta. Traditionally, it has been considered that *Tudorella* protoconch was smooth (Germain, 1931; Ibáñez & Alonso, 1978, 1979, 1980). Nevertheless, a detailed study with SEM revealed a clear microsculpture in all species (Figs 3c–e, 4, 5). Shell colour is varied in the three species, although Pallary (1898) indicated that *T. mauretanica* was lighter than *T. sulcata*. Shell aperture in *Tudorella* is slightly oval, rounded in the base and subangular in the upper corner. According to Ibáñez & Alonso (1979, 1980) shell aperture in *T. ferruginea* is slightly higher than ⅓ of the total shell height (35%–40% of total length in the shells studied in the present work). The shell aperture of *T. mauretanica* is the largest of the genus. It is wider in *T. sulcata sulcata* than in *T.s. melitense* being more rounded in the latter than in the other taxa of the genus. The size and shape of the aperture determine that of the operculum. Opercula are paucispiral, calcareous, oval, with lateral eccen-

tric nucleus, and having in general up to two whorls, of counter-clockwise growth, is deeply sunk within the aperture in *T. mauretanica*, *T. sulcata sulcata* and *T.s. melitense*, less in *T. ferruginea*. There are many clockwise subparallel spiral lines running from sutures to the operculum margin, where they give rise to wide and deep grooves (Figs 3f–j). According to Pallary (1989) the operculum of *T. mauretanica* is more flattened, elongated and with less convex margins than in *T. sulcata*. Germain (1931) indicated that in *T. sulcata* the operculum is thick, with very deep radial striae and with 4 – 5 apparent spiral striae. Picard (1949) differentiated the operculum of *T. mauretanica* from that of *T. sulcata* by the presence of marked transversal grooves in the former, and indicated that operculum had 3 whorls in both *T. sulcata* and *T. ferruginea*. Nevertheless, our results show that the number of opercular nucleus whorls in *T. ferruginea* is the smallest (1¼–1⅜) being the greatest in *T. mauretanica* (2 or >2).

Concerning the reproductive system we have found four differences between the taxa of *Tudorella*. They include, the penis morphology, the connection of the renal vas deferens to the prostatic gland, the morphology of the prostatic gland, and the size and position of the bursa copulatrix. We have not found significant differences in the morphology of male and female reproductive system between *T. ferruginea* and *T. mauretanica*. Ibáñez & Alonso (1978, 1980) working on both species indicated that in the population from Granada the length of the male reproductive system does not reach two times the female reproductive system length. These authors also stated that the proximal portion of penis is more than two times shorter than the distal one. We consider that both indications are valid for the two species and can not discriminate them. Ibáñez & Alonso (1978, 1980) said that pallial oviduct in females of *T. ferruginea* is half moon shaped and pointed, while it is less curved and with rounded end in *T. mauretanica*. Figures 7c (*T. ferruginea*) and 7d (*T. mauretanica*) of the present work do not support this consideration. Besides, we have observed that the capsule gland can be pointed or rounded in different specimens of *T. mauretanica*. The only difference between *T. ferruginea* and *T. mauretanica* with respect to *T. sulcata* could be the size of the bursa copulatrix, which is smaller in *T. sulcata*

Genetic divergences obtained for *T. mauretanica* with respect to *T. sulcata* and *T. ferruginea* were high (above 6.83% and 8.8% in COI, and above 10.13% and 8.04% in 16S rRNA, respectively, see Table 2). Delimiting the taxonomic status of the phylogenetic lineages identified based on their differences in DNA sequences is very dificult. Interspecific mtDNA distances in the same snail group can fluctuate broadly (Holland & Hadfield, 2002; Rundel, Holland & Cowie 2004). Besides, maximal intraspecific mtDNA gene sequences above 10% have been reported several times in terrestrial snails (Thomaz, Guiller & Clarke, 1996; Watanabe & Chiba, 2001; Parmakelis, Spanos, Papagiannkis & Mylonas, 2003; Pinceel, Jordaens & Backeljau, 2005). Nevertheless, genetic divergences of *T. mauretanica* are of the same magnitude than the divergences obtained between *T. sulcata* and *T. ferruginea*, and they are great enough to consider the three taxa as a valid species, under the evolutionary species concept: single lineage of ancestral descendant populations, which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate (Wiley, 1978). These molecular results are highly congruent with the morphological analysis, supporting the specific differentiation of the three species as monophyletic entities separated by high genetic distances. Molecular analysis showed that the population of *T. sulcata* from Malta is a monophyletic group closely related to *T. sulcata*. Differences in morphology, together with the genetic distances, supported the consideration of *T.s. melitense* as a valid subspecies.

It is noteworthy that genetic divergences found between the Iberian and Morocco populations of *T. mauretanica* are very low (0.6% for both mtDNA fragments). There are known fossil records (from the Pliocene-Pleistocene transition) of *T. mauretanica* in both areas, Iberian Peninsula and Morocco (Pallary, 1898, 1901; Picard, 1949; Gasull, 1972; Ibáñez & Alonso, 1978, 1980; Robles & Martínez-Ortí, 1995; Martínez-Ortí & Robles, 2003, 2005), but with the present data we can not exclude recent human-aided dispersal or aerial dispersal by birds of *T. mauretanica* across the Mediterranean. Passive dispersal has been indicated previously in other terrestrial molluscs (Uit de Weerd, Scheneider & Gittenberger, 2005; Gittenberger, Groenenberg, Kokshoorn & Preece,

2006). *Tudorella* species live in areas of limestone substrate near the coast line and they could have easily been transported by humans through commercial trade, as it has been indicated for the clausilid *Isabellaria pharsalica* (Uit de Weerd *et al.*, 2005). Moreover, SE-Iberian Peninsula is a natural bird migratory route between Europe and N-Africa in both directions. Currently, we have not enough information to test these two hypotheses and, consequently, more studies should be done to solve these questions.

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