MALACOLOGIA, 2013, 56(1–2): 333–338

GEOMALACUS AND *LETOURNEUXIA* (MOLLUSCA, PULMONATA): A CYTOGENETIC ASSESSMENT

Cláudia Patrão[1,2], Joana Teixeira de Sousa[3], Kurt Jordaens[4], Thierry Backeljau[2], Rita Castilho[1]& Alexandra Leitão[3*]

INTRODUCTION

The terrestrial malacofauna of the Iberian Peninsula is extremely rich and shows the highest diversity of arionid slug species in Europe (and probably worldwide), with 30 to 50 species, including several endemic ones (Castillejo, 1998). However, the taxonomic status of several of these species remains unclear due to the extreme variability in body size and color and the lack of reliable diagnostic morphological traits (Backeljau & De Bruyn, 1990). The taxonomy of terrestrial slugs is based almost entirely on the morphology of their reproductive apparatus, which varies according to developmental stage and sexual maturation, often preventing the correct identification of juveniles at the species level (Backeljau & De Bruyn, 1990, and references therein; Backeljau et al., 1996). As a consequence, the taxonomic status of supraspecific arionid groupings, such as (sub) genera, is still controversial. This applies to the genera *Geomalacus* Allman, 1843, and *Letourneuxia* Bourguignat, 1866, which have undergone several taxonomic changes since their original descriptions.

Geomalacus presently comprises four species grouped into two subgenera: *G.* (*Arrudia*) *anguiformis* (Morelet, 1845), *G.* (*A.*) *oliveirae* Simroth, 1891, *G.* (*A.*) *malagensis* Wiktor & Norris, 1991, and *G.* (*Geomalacus*) *maculosus* Allman, 1843. The first three species are endemic to the Iberian Peninsula, whereas *G. maculosus* is also found in southwestern Ireland. Although *G. maculosus* is easily identified by its unique color pattern, *G. anguiformis* and *G. oliveirae* are very similar in their external morphology, showing only subtle differences in their reproductive organs (Rodriguez et al., 1993). Often, these two species have been identified solely from the geographical origin of specimens (Castillejo & Rodriguez, 1991). Moreover, when Wiktor & Norris (1991) originally described *G. malagensis*, the set of existing features for the classification of Arionidae provided by Hesse (1926) was "considered unsatisfactory" (Wiktor & Norris, 1991), since this species could have been easily classified within any of the three genera of this family – *Arion*, *Geomalacus* or *Letourneuxia*. Therefore, Wiktor & Norris (1991) proposed additional diagnostic characters, and the new species was included in the genus *Geomalacus*.

The description of the genus *Letourneuxia* Bourguignat, 1866, was based on specimens from Algeria. This taxon is described as endemic from North Africa, and it has suffered successive changes in its taxonomic status, including being described as a subgenus of *Geomalacus* (Pollonera, 1890), a subgenus of *Arion* (Hesse, 1926), and, finally, acquiring generic status within the family Arionidae (Wiktor, 1983). The four nominal species, viz. *G.* (*L.) numidica* Bourguignat, 1866, *L. atlantica* Bourguignat, 1883, *L. maroccanus* Pollonera, 1916, and *G.* (*L.) turneri* Pollonera, 1890, together with *Arion moreleti* Hesse, 1884, have been synonymized with *L. numidica* by Wiktor (1983).

The debate as to whether *Letourneuxia* and *Geomalacus* should be kept in separate genera was fueled by the description of *G. malagensis*. However, even if *L. numidica* and *G. malagensis* are very similar in external morphology and color, they present two major differences in their reproductive organs: (1) *G. malagensis* has a large, thick epiphallus that is lacking in *L. numidica,* and (2) *L. numidica* has a voluminous atrium with a ligula inside, whereas the atrium of *G. malagensis* is slender and lacks a ligula (Wiktor & Norris, 1991). Because of the variability of these diagnostic features, and the fact

[1]Centro de Ciências do Mar (CCMAR, CIMAR-Associate Laboratory), Campus de Gambelas, 8005-139 Faro, Portugal.
[2]Royal Belgian Institute of Natural Sciences (Department of Invertebrates and JEMU), Vautierstraat 29, B-1000 Brussels, Belgium; Evolutionary Ecology Group, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium.
[3]Instituto Português do Mar e da Atmosfera (IPMA), Av. 5 de Outubro, 8700-305 Olhão, Portugal.
[4]Joint Experimental Molecular Unit, Royal Museum for Central Africa, Leuvensesteenweg 13, B-3080 Tervuren, Belgium.
*Corresponding author: aleitao@ipma.pt

that they mostly rely on fully mature individuals, additional evidence is needed to evaluate the degree of differentiation between *Letourneuxia* and *Geomalacus*.

Cytogenetic studies in slugs and snails have yielded important taxonomic insights (e.g., Vitturi et al., 2005; Colomba et al., 2009; Kongim et al., 2009, 2010). However, for slugs, these reports are exclusively based on the use of gonadal tissue for chromosome preparations. This constitutes a serious drawback as it excludes juvenile individuals in which the ovotestis is not yet fully developed. Juvenile terrestrial slugs are particularly difficult to identify considering that the taxonomy is based on the morphology of their highly variable reproductive apparatus. So, if other organs provide reliable cytogenetic results, then this traditional disadvantage would be overcome.

In the past, chromosome studies of slugs (Beeson, 1960) and of euthyneuran gastropods in general (Burch, 1965) suggested that karyological data might be useful to distinguish (sub) genus level taxa in limacid and arionid slugs. There seems to be a suggestive correspondence between haploid chromosome numbers and subgeneric groupings in the genus *Arion*: n = 25 in *Mesarion* Hesse, 1926, n = 26 in *Arion* Férussac, 1819, n = 28 in *Kobeltia* Seibert, 1873 and *Microarion* Hesse, 1926*,* and n = 29 in *Carinarion* Hesse, 1926 (Beeson, 1960). This observation was, amongst others, used to include *Microarion* in the subgenus *Kobeltia* (Backeljau & De Bruyn, 1990). However, no karyotypes are available for any *Arion,* and no cytogenetic study (chromosome number and karyotypic formula) has been conducted in *Letouneuxia* or *Geomalacus.*

Here we present karyotypes and a comparative karyological study of the four *Geomalacus* species and *L. numidica* after testing different somatic tissues (mouth and both optical and sensory tentacles) to evaluate their suitability for karyological studies. We also assess the contribution of cytogenetics to provide additional evidence to resolve the taxonomy of these slugs.

MATERIALS AND METHODS

Biological Material

Specimens of the five nominal species were collected in the Iberian Peninsula and Morocco as detailed in Table 1 and identified following Castillejo et al. (1994). Animals were kept alive at 4°C and fed with lettuce. Prior to the experiments (48 h), the slugs were kept at room temperature.

TABLE 1. Localities, number of specimens (n), diploid chromosome number and karyotypic formulae for the four *Geomalacus* and the *Letourneuxia* species used in this study (Sp = Spain; Pt = Portugal).

Genus/ species	Locality	n	Geographical coordinates	Chromosome number	Karyotypic formula
G. oliveirae	Gredos, Sp	1	40.3217°N, 5.0135°W	2n = 62	15m + 13sm + 3st
G. oliveirae	Gredos, Sp	3	40.3151°N, 5.0090°W	2n = 62	15m + 13sm + 3st
G. oliveirae	Pena de Francia, Sp	3	40.5144°N, 6.1567°W	2n = 62	15m + 13sm + 3st
G. maculosus	Chãos, Serra Estrela, Pt	1	40.5386°N, 7.3125°W	2n = 62	14m + 12sm + 5st
G. maculosus	Caldas Manteigas, Serra Estrela, Pt	8	40.3825°N, 7.5442°W	2n = 62	14m + 12sm + 5st
G. maculosus	Viana do Castelo, Minho, Pt	5	41. 7739°N, 8.6186°W	2n = 62	14m + 12sm + 5st
G. anguiformis	São Brás de Alportel, Algarve, Pt	4	37.2728°N, 7.8753°W	2n = 62	14m + 10sm + 7st
G. malagensis	Fonte Sesimbra, Setúbal, Pt	2	38.4761°N, 9.1143°W	2n = 62	10m + 12sm + 9st
G. malagensis	Guilhim, Algarve, Pt	15	37.1016°N, 7.9279°W	2n = 62	10m + 12sm + 9st
L. numidica	Tanger, Morocco	7	35.7844°N, 5.9011°W	2n = 62	10m + 12sm + 9st
L. numidica	Tanger, Morocco	2	35.7827°N, 5.8506°W	2n = 62	10m + 12sm + 9st

Chromosome Preparation

Whole individuals were submerged for 75 min in a 0.01% solution of colchicine at room temperature. Then ovotestis, mouth, and optical and sensory tentacles were dissected. Somatic tissues were chosen as representative structures with high mitotic rates: the mouth for the constant renewal of the radula by odontoblastic and membranoblastic cells and the tentacles for their ability to regenerate (Barker, 2001).

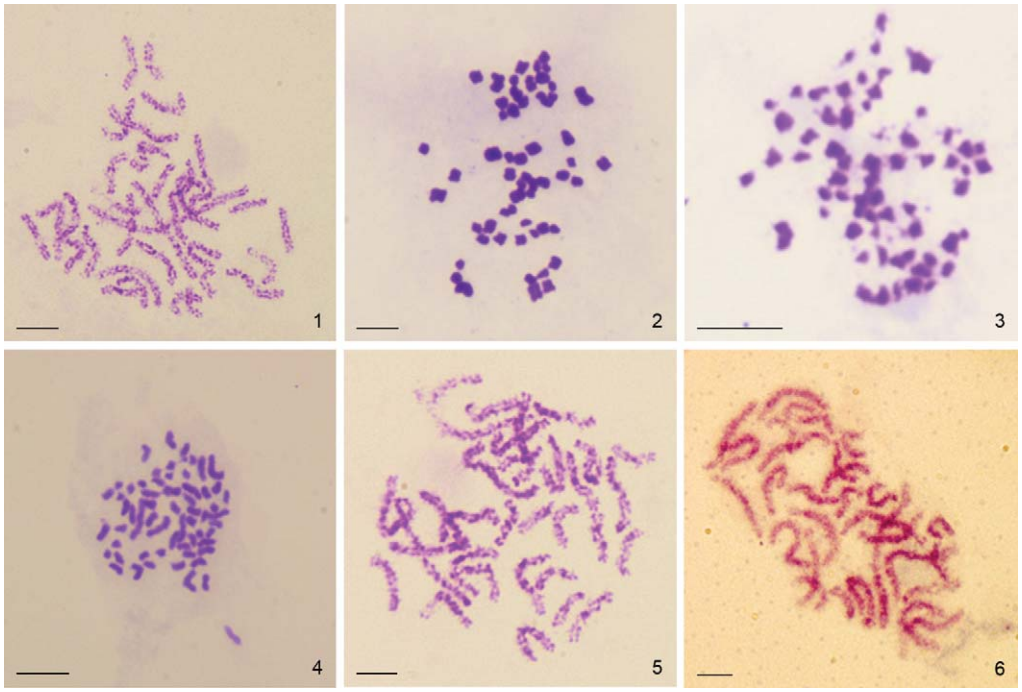
All structures were subjected to a hypotonic treatment for 45 min in 0.9% sodium citrate and fixed in a freshly prepared mixture of absolute ethanol and glacial acetic acid (3:1). Fixed pieces of ovotestis, mouth and tentacles were dissociated in 50% glacial acetic acid and distilled water. Slides were prepared following an air-drying technique (Thiriot-Quiévreux & Ayraud, 1982). Slides were stained with Giemsa (4%, pH 6.8) for 10 min.

Karyotyping

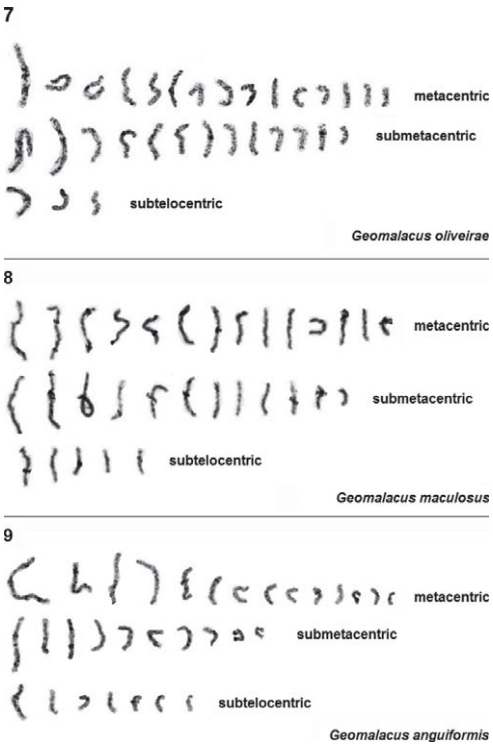
Images of Giemsa stained metaphases were acquired with a digital camera (Nikon DSFi 1) coupled to a light microscope (Nikon Eclipse 80i). Digital images were processed with Adobe Photoshop (edition CS3) using functions only affecting the whole image. Ten karyotypes per species were performed. Chromosomes were organized based on relative length and centromeric position; terminology followed Levan et al. (1964).

RESULTS

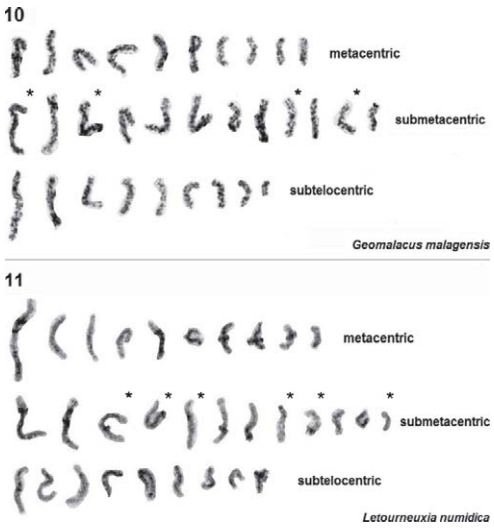
To test the suitability of different organs for producing usable chromosome images, we performed a number of trials with different individuals from the five species. Chromosomes were not obtained from preparations of



FIGS. 1–6. Giemsa stained metaphases of *Geomalacus* and *Letourneuxia*. FIG. 1: *G. oliveirae* meiotic metaphase II (n = 31); FIG. 2: *G. oliveirae* mitotic metaphase (2n = 62); FIG. 3: *G. maculosus* mitotic metaphase (2n = 62); FIG. 4: *G. anguiformis* mitotic metaphase (2n = 62); FIG. 5: *G. malagensis* meiotic metaphase II (n = 31); FIG. 6: *L. numidica* meiotic metaphase II (n = 31). Scale bar = 4 µm.



FIGS. 7–9. Giemsa stained karyotypes of *Geomalacus*. FIG. 7: *G. oliveirae* (15m + 13sm + 3st); FIG. 8: *G. maculosus* (14m + 12sm + 5st); FIG. 9: *G. anguiformis* (14m + 10sm + 7st). m - metacentric chromosomes, sm - submetacentric chromosomes and st – subtelocentric chromosomes.



FIGS. 10, 11. Giemsa stained karyotypes of *Geomalacus* and *Letourneuxia*. FIG. 10: *G. malagensis* (10m + 12sm + 9st); FIG. 11: *L. numidica* (10m + 12sm + 9st). m - metacentric chromosomes, sm - submetacentric chromosomes and st – subtelocentric chromosomes); asterisks (*) indicate submetacentric chromosomes that present a submetacentric/subtelocentric tendency.

ovotestis from juvenile individuals, regardless the analyzed species, but only from specimens where the ovotestis was well differentiated. However, using mouth and both optical and sensory tentacles, it was possible to obtain diploid chromosome preparations independently of the individual stage of development (Figs. 2–4). Concerning the number of chromosomes, the five species presented the same diploid chromosome number of 2n = 62 (n = 31). However, karyotypic formulae are different and unique to each *Geomalacus* species, with karyotypes mainly consisting of metacentric (m) and submetacentric (sm) chromosomes; with few subtelocentric (st) and lacking telocentric chromosomes (*G. oliveirae* 15m + 13sm + 3st; *G. maculosus* 14m + 12sm + 5st, *G. anguiformis* 14m + 10sm + 7st, *G. malagensis* 10m + 12sm + 9st) (Figs. 7–11, Table 1). *Geomalacus malagensis* and *L. numidica* share the same karyotypic formula, yet, in *L. numidica* six of the 12 submetacentric chromosome pairs presented a submetacentric/subtelocentric tendency, while in *G. malagensis* only four chromosome pairs show this trend (chromosomes marked with an * in Figs. 10 and 11).

DISCUSSION

The use of somatic organs for cytogenetic studies instead of ovotestis proved to be effective. Mouth and both optical and sensory tentacles yielded several mitotic metaphases and hence were successfully used to determine diploid chromosome numbers. In previous cytogenetic studies of terrestrial slugs (Beeson, 1960; Burch, 1965; Patterson, 1969; Colomba et al., 2009), only well-developed ovotestis were used for karyotyping. The new approach presented here using somatic tissues allows karyological studies to be performed regardless of the sexual developmental stage of the specimens.

The five species studied displayed an invariant chromosome number (n = 31) so that the observed interspecific karyotypic differences only involved structural chromosomal rearrangements without affecting chromosome number. Such patterns of chromosomal change have been previously observed in the neogastropod family Muricidae (Leitão et al., 2009). Unlike the genus *Arion*, in which chromosome numbers were useful to distinguish between subgenera (Beeson, 1960), it is clear that it is not possible to distinguish between these five species based on the chromosome number alone. The haploid chromosome number in *Geomalacus* and *Letourneuxia* is the highest observed within the Arionidae (with n = 25–29) (Beeson, 1960). Also, this chromosome number is among the highest of all terrestrial pulmonate gastropod mollusks (order Stylommatophora). Haploid chromosome numbers within this group vary between n = 18 and n = 34 (Park & Kim, 1997; Thiriot-Quiévreux, 2003; Colomba et al., 2009), with Athoracophoridae being an outlier with n = 44 (Patterson, 1969; Burch & Patterson, 1971).

Ancestral character state reconstruction tracing chromosome numbers in *Geomalacus* and *Letourneuxia* on a molecular phylogenetic tree, would possibly allow the inference of a chromosome number evolutionary trend for the family Arionidae. However, currently too few karyotypic data are available to conduct such analysis.

The karyotypes of the species in this study showed a prevalence of metacentric and submetacentric chromosomes, which follows the general trend in gastropod karyotypes (Thiriot-Quiévreux, 2003). Despite presenting the same chromosome number, each *Geomalacus* species displays different and diagnostic karyotypes (Figs. 1–5, Table 1). *Geomalacus oliveirae* has the most symmetric karyotype of the studied species, with the highest number of metacentric and submetacentric chromosomes, while *G. malagensis* and *L. numidica* present the more asymmetrical ones. Symmetrical karyotypes are often considered plesiomorphic, since a higher proportion of metacentric pairs may point to relative chromosomal evolutionary stability (White, 1978).

It is taxonomically relevant that *G. malagensis* is karyotypically different from its congeners, but shares the same karyotypic formula and similar chromosome morphology with *L. numidica.* The difference between *G. malagensis* and *L. numidica* resides solely in the numbers of chromosome pairs showing a submetacentric/subtelocentric tendency, that is, respectively, 4 and 6. Even if the present chromosomal data alone is not conclusive to establish the taxonomic status of *Geomalacus* and *Letourneuxia*, this finding suggests that both genera may be closely related and perhaps should be merged in a single genus-level taxon. Similarly, Backeljau & De Bruyn (1990) used chromosome numbers, together with morphology and allozyme data, to merge the arionid subgenera *Microarion* and *Kobeltia*. Yet, whether such conclusion is also warranted for *Geomalacus* and *Letourneuxia* requires further corroboration.

In conclusion, our work showed that somatic tissues are perfectly suitable for cytogenetic studies and that the chromosome number of the genera *Geomalacus* and *Letourneuxia* is n = 31, which is among the highest of all Stylommatophora. The five described karyotypes constitute the first record for Arionidae, with *G. malagensis* and *L. numidica* presenting similar chromosome morphologies and karyotypic formulae. Cytogenetic studies may significantly contribute to clarify the taxonomy of these, and other, pulmonate gastropods.

ACKNOWLEDGEMENTS

The authors wish to thank K. Erzini, A. Patrão, T. Pereira, D. Reis and G. Silva for collecting specimens in the field and T. Rodriguez for assistance with the identification of the specimens. The authors are also grateful to S. Natale, for revising the English. This work was partially funded by “Fundação para a Ciência e Tecnologia” (Portugal): doctoral grant SFRH/BD/30024/2006 (CP), project “Pillars of Hercules – Gene flow across the Pillars of Hercules: the role of North Africa in the postglacial recolonization of Europe” PTDC/BIA-BDE/74349/2006 (RC) and “Programa Ciência 2007” (AL). T. Backeljau was supported by BELSPO project MO/36/017 and FWO research network W0.009.11N.

LITERATURE CITED

BACKELJAU, T. & L. DE BRUYN, 1990, On the infrageneric systematics of the genus *Arion* Férussac, 1819 (Mollusca, Pulmonata). *Bulletin de l’Institut Royal des Sciences Naturelles de Belgique, Biologie*, 60: 35–68.

BACKELJAU, T., B. WINNEPENNINCKX, K. JORDAENS, H. DE WOLF, K. BREUGELMANS, C. PAREJO & T. RODRIGUEZ, 1996, Protein electrophoresis in arionid taxonomy. *BCPC Symposium Proceedings no. 66, Slug and Snail Pests in Agriculture*: 21–28.

BARKER, G. M., 2001, Gastropods on land: phylogeny, diversity and adaptive morphology. Pp. 1–146, in: G. M. Barker, ed., *The biology of terrestrial molluscs.* CABI Pub., New York, 558 pp.

BEESON, G. E., 1960, Chromosome numbers of slugs. *Nature*, 186: 257–258.

BURCH, J. B., 1965, Chromosome numbers and systematics in euthyneuran snails. *Proceedings of the 1st European Malacological Congress*: 215–241.

BURCH, J. B. & C. M. PATTERSON, 1971, The chromosome number of the athoracophorid slug, *Triboniophorus graeffei*, from Australia. *Malacological Review*, 4: 25–26.

CASTILLEJO, J., 1998, *Guía de las babosas Ibéricas*. Real Academia Galega de Ciencias, Santiago, 154 pp.

CASTILLEJO, J. & T. RODRIGUEZ, 1991, *Babosas de la Peninsula Iberica y Baleares: Inventario critico, citas y mapas de distribución:* (*Gastropoda, Pulmonata, Terrestria nuda*). Universidade de Santiago de Compostela Servicio de Publicacions e Intercambio Cientifico, Santiago de Compustela, 162 pp.

CASTILLEJO, J., C. GARRIDO & J. IGLESIAS, 1994, The slugs of the genus *Geomalacus* Allman, 1843, from the Iberian Peninsula (Gastropoda: Pulmonata: Arionidae). *Basteria*, 58: 15–26.

COLOMBA, M., R. VITTURI, M. RAMPIN, A. LANNINO, A. TARAVELLA & A. LIBERTINI, 2009, Comparative cytogenetic analysis of three stylommatophoran slugs (Mollusca, Pulmonata). *Malacologia*, 51: 173–179.

HESSE, P., 1926, Die Nacktschnecken der Palaearktischen Region. *Abhandlungen des Archiv für Molluskenkunde*, 2: 1–152.

KONGIM, B., C. SUTCHARIT, P. TONGKERD & S. PANHA, 2009, Karyotype differentiation within the elephant pupinid snail, *Pollicaria mouhoti* (Pfeiffer, 1862) (Caenogastropoda: Pupinidae). *The Natural History Journal of Chulalongkorn University*, 9: 201–208.

KONGIM, B., C. SUTCHARIT, P. TONGKERD, S. H. A. TAN, N. X. QUYNH, F. NAGGS & S. PANHA, 2010, Karyotype variations in the genus *Pollicaria* (Caenogastropoda: Pupinidae). *Zoological Studies*, 49: 125–131.

LEITÃO, A., P. VASCONCELOS, R. BEN-HAMADOU, M. B. GASPAR, C. M. BARROSO & F. RUANO, 2009, Cytogenetics of *Bolinus brandaris* and phylogenetic inferences within the Muricidae (Mollusca: Gastropoda). *Biological Journal of the Linnean Society*, 96: 185–193.

LEVAN, A., K. FREDGA & A. A. SANDBERG, 1964, Nomenclature for centromeric position on chromosomes. *Hereditas–Genetiskt Arkiv*, 52: 201–220.

PARK, G. M. & J. J. KIM, 1997, Chromosome study on the *Oxyloma hirasei* (Gastropoda: Succineidae) in Korea. *Korean Journal of Malacology*, 13: 125–130.

PATTERSON, C. M., 1969, Chromosomes of mollusks. *Proceeding of the Symposium on Mollusca*, *Part II. Marine Biology Association India*: 635–686.

POLLONERA, C., 1890, Recensement des Arionidae de la Région Paléarctique. *Bollettino dei Musei di Zoologia ed Anatomia Comparata della Reale Università di Torino*, 5: 1–42.

RODRIGUEZ, T., P. ONDINA, A. OUTEIRO & J. CASTILLEJO, 1993, Slugs of Portugal. 3.Revision of the genus *Geomalacus* Allman, 1843 (Gastropoda, Pulmonata, Arionidae). *The Veliger*, 36: 145–159.

THIRIOT-QUIÉVREUX, C., 2003, Advances in chromosomal studies of gastropod molluscs. *Journal of Molluscan Studies*, 69: 187–202.

THIRIOT-QUIÉVREUX, C. & N. AYRAUD, 1982, Les caryotypes de quelques espèces de bivalves et de gastéropodes marins. *Marine Biology*, 70: 165–172.

VITTURI, R., A. LIBERTINI, L. SINEO, I. SPARACIO, A. LANNINO, A. GREGORINI & M. COLOMBA, 2005, Cytogenetics of the land snails *Cantareus aspersus* and *C. mazzullii* (Mollusca : Gastropoda : Pulmonata). *Micron*, 36: 351–357.

WHITE, M. J. D., 1978, *Modes of speciation*. W. H. Freeman, San Francisco, California, 455 pp.

WIKTOR, A., 1983, Some data on slugs of Morocco and Algeria with description of a new *Deroceras* species (Gastropoda, Pulmonata). *Malakologische Abhandlungen*, 8: 155–165.

WIKTOR, A. & A. NORRIS, 1991, *Geomalacus malagensis* sp. n. and some remarks concerning generic level systematics of Arionidae (Gastropoda, Pulmonata). *Journal of Conchology*, 34: 19–29.

Revised ms. accepted June 3, 2013