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SUGGESTIONS FOR SPECIES GROUPING WITHIN THE FAMILY LIMACIDAE (GASTROPODA, PULMONATA) BY BIOCHEMICAL METHODS

A. V. GROSSU AND C. TESIO

Faculty of Biology, University of Bucharest

In the taxonomy of gastropods, as with other animals, the grouping of species into genera and families is very arbitrary. This is primarily due to differing choices of common characters for making up phylogenetic links and grouping species in higher taxonomic units. It has been demonstrated that in most cases the choice of taxonomic characters has only a relative significance, since different workers may assign a higher value to one or other of these characters, resulting in a great variety of group classification.

There are many suggestions for the grouping of species in Limacomorpha, and particularly in the family Limacidae, each worker in this field being forced to make a choice between the existing ones, or to suggest another classification, thus introducing new complications to an already rather confused situation. It must be noted that the species themselves are always sharply outlined, and only their grouping into higher taxa is questioned. A specific taxonomic value has been given particularly to the following organs or anatomical systems: the crossing between the tentaculum retractor muscle and the penis, the presence or lack of appendages in the penis or intestine, the shell, the radular tooth shape, etc. Though rudimentary, the shell plays an important part, mainly in fossil forms, being the sole available trace from these animals (Zilch, 1960). The various suggestions made for species grouping are due in many instances to the selection as basic taxonomic characteristics of a limited number of such organs.

Following recent population studies it was stated that most of these anatomical features, which were thought to have considerable value, are actually exceedingly variable. On the other hand, many convergences were noted, where anatomically similar organs are found in taxonomically distant species. Such findings resulted in great uncertainty in the characterization and grouping of species, and the classical anatomical criteria were demonstrated not to be unequivocal.

To illustrate the above considerations, we may mention some examples from the taxonomy of Limacidae. This family is divided in two groups of genera: the first comprises species with six intestinal branches (*Limax* L.; *Bielzia* Clessin; *Malacolimax* Malm., etc.) and the second, species having four intestinal branches (*Deroceras*

Rafinesque, *Lytopelte* Boettger, etc.). Other criteria are also used for defining genera, such as the penis form and appendages, the radular tooth structure, etc. Germain (1930) and other workers rely on the radular teeth for making a distinction between the genera *Limax* and *Malacolimax,* but the radular teeth of this latter genus are very similar to those of *Deroceras,* which is a member of another group. The taxonomic value of radular teeth for the identification of the genus *Malacolimax* was questioned by Flasar (1961). This author and other specialists believe this genus to be merely a subgenus of the genus *Limax,* arguing that there is a great deal of evidence that the radula of a very common European species, *M. tenellus* Malm., has a high variability and may therefore result in determinations of doubtful character.

Similarly, the genus *Lehmannia* Heynemann is distinguished from *Limax* by having a well-developed intestinal caecum and some glandular appendages (flagellum) on the penis. It was later found, however, that many *Limax* species have also a well-developed caecum, and that other species of the same genus have glandular penial appendages. By taking some characters as basic or not for certain genera, the distinguished specialist A. Wiktor (1973) has recently suggested that the genus *Lytopelte* is only a subgenus of *Deroceras,* and that the genus *Boettgerilla* Simroth be transferred from the family Parmacellidae to the family Milacidae.

The appreciation of certain organs for taxonomic purposes following subjective criteria, without any hierarchy of the characters used in the grouping of species, results in the lack of a common systematic unity in the work of a number of well-known specialists, such as Hesse (1926), Germain (1930), Pilsbry (1948), Zilch (1960) and others. While for example Hesse and others assign taxonomic value to the structure of the genital system, Germain suggests the radula, and Zilch the limacella, as most important.

On the basis of a combination of characters, a classification of the family Limacidae into twelve genera has been commonly accepted in recent years. Among these genera, those having a limited range, particularly in the Caucasus Mountains, and composed of one or very few species, do not raise any special systematic problem, their distinct characteristics being sufficient for accurate determination and accepted by all authors. The same is true also for the genus *Bielzia,* which has a much wider distribution in Europe but has a genital anatomy completely different from that of the other genera.

The most difficult problems arise mainly in relation to the genera *Limax, Lehmannia, Malacolimax, Deroceras* and *Lytopelte,* comprising a great number of species with a wide distribution in Europe. The species belonging to these genera are frequently grouped together into subgenera, which are considered by some workers to be in fact true genera: in other instances, the genera are assumed to be only subgenera. Thus we may find in the literature taxa which belong sometimes to one genus and sometimes to another, according to the priority given by a worker to one character or another. This is the case, for example, with the species *Limax flavus* L. which is put into the same genus as *Lehmannia,* while many other authors suggest that *Lehmannia* is only a subgenus of *Limax* (Walden, 1961; Altena, 1966; Pilsbry, 1948; etc.).

These controversial questions, which have also given rise to some polemics (Walden, 1961; Lupu, 1971), prompted us to direct our attention to them, in order to check a number of the authors' proposals. To achieve this aim we made use of a rich collection of preserved and living material, which was studied according to the classical criteria as well as by the new taxonomical methods, the biochemical methods.

In the present study we shall not discuss the genera *Lytopelte* and *Malacolimax,* since the lack of living material prevented us from making comparative biochemical studies. We directed, therefore, our attention to the taxonomic position of *Lehmannia,* since it is a subject of controversy, and since rich material was available for anatomical and biochemical studies and for comparison with related species of the genus *Limax.*

Bibliographical information as well as our anatomical researches demonstrate that species referred to the genera *Limax* and *Lehmannia* have actually many common characters such as large or medium size, a digestive tract with six branches, a retractor and an ommatophore crossing the penis, and also some genital characters. However, study of a number of species belonging to the genus *Limax* (Fig. 1) showed a high variation in the terminal part of the genital system and particularly in the structure and dimensions of the penis, which form the anatomical basis for the separation of species. In all species the ductus deferens is short, being generally inserted at the end of the penis, irrespective of its length.

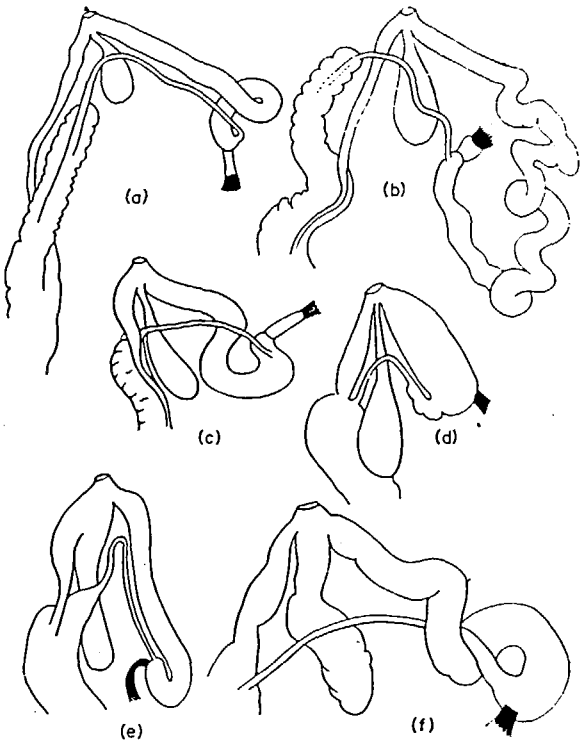

FIG. 1. Terminal part of the genital system of: (a) *Limax maximus* L.; (b) *L. cinereoniger* Wolf; (c) *L. dobrogicus* Grossu-Lupu; (d) *L. tenellus* Nilson; (e) *L. flavus* L.; (f) *L. nyctelius* Bourg.

By observing the same portion of the genital apparatus in *Lehmannia* spp. (Fig. 2), we found the same variation in penis shape and size, but always with a short ductus deferens. The difference between these two species groups is the presence of a flagellum or an appendix attached at the penis, which is more or less well developed in *Lehmannia*, but is lacking in the species putatively belonging to *Limax*. Some workers feel that the presence or the absence of this appendix is a sufficient reason for the classification of the respective species into two distinct genera.

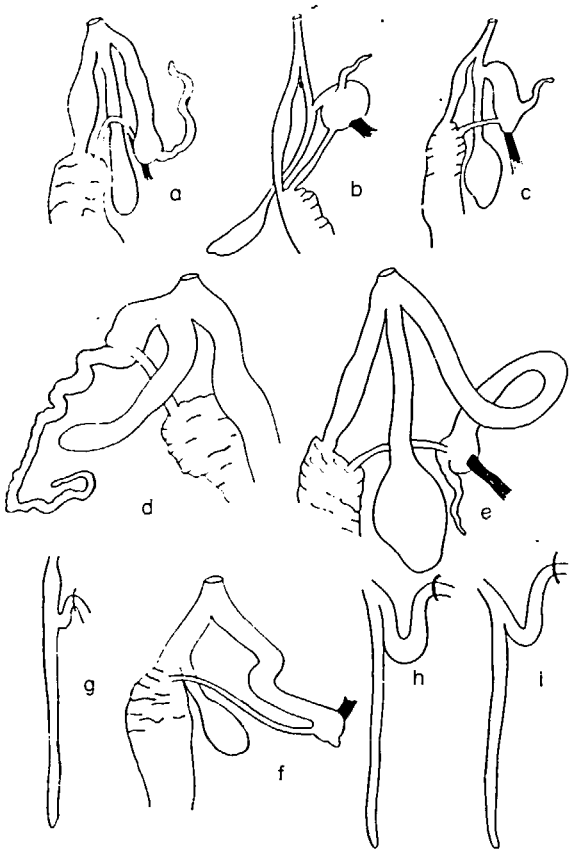

FIG. 2. Terminal part of the genital system of: (a) *Limax* (*Lehmannia*) *marginatus* Müller; (b) *L. marginatus* Müller (juv.); (c) *L. marginatus* Müller (juv.); (d) *L. macroflagelatus* Grossu-Lupu; (e) *L. jaroslaviae* Grossu; (f) *L. jaroslaviae* Grossu (juv.); (g) caecum (intestinal appendix) of *L. flavus* L.; (h) caecum of *L. nyctelius* Bourg.; (j) caecum of *L. sarmizegetusae* Grossu.

In fact this character, to which so high a taxonomic value is given by several authors, is far from constant, having many different shapes and sizes, and sometimes even lacking, as in some species referred to *Lehmannia* (Grossu-Lupu, 1964); conversely, it may be present but in an atrophied state in the *Limax* group. *Limax carbonarius* Böttger and *L. voronovi* Simroth, for example, have a short appendix to the penis, while in some specimens of *Lehmannia marginata* Müller and *L. valentiniana*

Férussac it is atrophied or even lacking. The siting of the penis in relation to the receptaculum seminis is also very variable (Fig. 2b, c). The presence and variation of the flagellum may support a species separation, but if we take into account other characteristics too, we may find that there is no sharp delimitation between the two groups.

The presence of an intestinal appendix (caecum) is also considered to be a basic character of *Lehmannia*; our anatomical studies, however, have revealed that a well-developed appendix also occurs in some species of the *Limax* group (Fig. 2g, h, i). Certain authors, applying their principles consistently, put these species into the *Lehmannia* group (*L. flavus* L., *L. nyctelius* Bourg.), although the penis has no flagellum—a character indissolubly related to *Lehmannia* (Altena, 1966; Lupu, 1972).

Thus, by taking these anatomical characters into account, we may classify these species with some approximation in smaller groups at the subgeneric level. We may obtain in this way a group belonging to the subgenus *Limax* s.str. (large size, penis and intestine without appendages, such as *Limax maximus* L., *L. cinereoniger* Wolf, *L. zilchi* Grossu-Lupu, etc.); a second group may comprise the subgenus *Limacus* Lehmann (large and medium size, penis without a flagellum, very well-developed intestinal caecum: *L. flavus, L. nyctelius*); and a third group, the subgenus *Lehmannia* (medium size, penis and intestine provided with an appendage: *L. marginata* Müller *L. horeziae* Grossu-Lupu, *L. jaroslaviae* Grossu, *L. getica* Grossu etc.). Some more groups may be added on a basis of anatomic criteria, such as the subgenera *Malacolimax* Malm, *Vitrinoides* Simroth, and *Caspilimax* Hesse (the latter two occurring only in the Caucasus), belonging also to the genus *Limax*.

All these contradictions, and particularly the species classification in one or other systematic group, in distinct genera or subgenera, as well as the selection of some characters without taking into account their validity, prompted us to consider these classical, mainly anatomical, criteria as inadequate to support the different taxonomic proposals. The grouping of species into a higher taxonomic unit must be based upon a constant and common complex of characters with low variability. Our taxonomic researches in other gastropod groups have also demonstrated a high characteristic variability at the generic or specific level, where many contradictions exist between the taxonomic proposals made according to classical criteria. Such studies were made in the genera *Campylaea* Beck, *Helicigona* Risso, *Cochlodina* Férussac and especially in the genus *Alopia* H. and A. Adams, where the anatomical characteristics alone could not elucidate certain difficulties in grouping the species into genera. In order to overcome them, we resorted to biochemical methods, making use of the non-specific esterase electrophoresis in starch gel or in polyacrilamide originating both from the whole animal and from its foot muscle. Thus, we tried to solve the existing contradiction by means of a new taxonomic method, testing structure at the protein level; we obtained by this method positive results, with a safe documentation in support of our views (Grossu and Tesio, 1971, 1972a, b, 1973).

For using this method in the family Limacidae also, we needed living animals. These were difficult to obtain from other countries, and we therefore limited our researches to species occurring in Rumania, which are numerous and according to the

classical classification represent many genera and subgenera. Our main purpose was to analyse certain constant biochemical characteristics which might support the actual unity of some species with high anatomical variability if they had constant biochemical characteristics showing a number of common features which could justify putting them together in supraspecific taxonomic units.

Great attention was also directed to the *Lehmannia* group, to see whether certain anatomical characters are consistent with the biochemical results; in other words if the biochemical tests support one or other among the taxonomic proposals based upon anatomical criteria. The experiments were made with an extract from the foot muscle. For the unspecific esterase determinations by means of the starch gel electrophoresis, according to previously reported procedures (Grossu-Tesio, 1971), we used a 10% concentration of Canadian hydrolysed starch (Connaught Medical Research Laboratories, Lot 281–1).

During our frequently repeated determinations we always obtained identical patterns for each species, regardless of whether the individuals composing the populations studied had a high variability in colour or size, or of whether their basic anatomical characteristics showed a tolerable variability, particularly in genital apparatus structure. Analysis of these electrophoretic patterns results in the confirmation as independent species of certain taxonomic units previously described according to anatomical criteria only (*L. dobrogicus, L. macroflagelata, L. zilchi,* etc.).

The electrophoretic patterns of the species of both *Limax* and *Lehmannia* demonstrate a number of common characters. For both species categories, there is a high similitude in the disposition of bands as well as in their number and intensity. Each of the species studied (Fig. 3a, b) has a number of bands with a low or median migration speed, usually three, one of which has a higher intensity. The *Limax* species have patterns with 9 to 10 bands, while those of *Lehmannia* species have only 7 to 8 bands. We have, however, found that the patterns of some species which are supposed to belong to the genus *Limax* are very close to those of some species of *Lehmannia*. From this standpoint the position of *L. nyctelius* seems to be somewhat questionable among the *Limax* species. Similarly, *L. horeziae* from the genus *Lehmannia* has an electrophoretic pattern different from those of the other species (only 5 to 6 bands with dissimilar intensities).

From a comparison of these patterns some rather obvious conclusions may be drawn. Although each species studied exhibits characteristic biochemical patterns, some constancy may be observed in both categories, a common characteristic consisting of a 3-band group, one band being more intense. No significant biochemical differentiation may be noted between the groups of *Limax* and *Lehmannia*; therefore both groups belong to the same genus, which by reason of priority must be the genus *Limax*. The differences occurring in some species of these groups could not justify their separation in two higher taxonomic units. In other words, the results obtained by anatomical analysis in favour of the occurrence of two distinct genera and several subgenera are not confirmed by the biochemical method. Taxonomic units sharply outlined biochemically comprise only species and genera. This is, however, the case

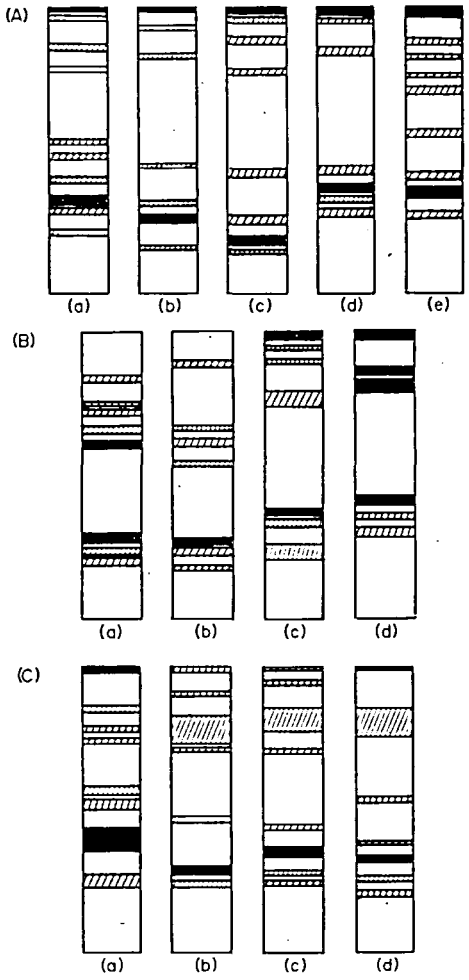

FIG. 3. (A) Electrophoretic patterns (muscle esterases) of: (a) *Limax maximus altenae* Grossu-Lupu; (b) *L. cinereoniger* Wolf; (c) *L. dobrogicus* Grossu-Lupu; (d) *L. nyctelius* Bourg.; (e) *L. flavus* L. (B) Electrophoretic patterns (muscle esterases) of: (a) *Limax* (*Lehmannia*) *marginata* Müller; (b) *L. macroflagelatus* Grossu-Lupu; (c) *L. geticus* Grossu; (d) *L. horeziae* Grossu. (C) Electrophoretic patterns (muscle esterases) of: (a) *Bielzia coerulans* M. Bielz; (b) *Deroceras waldeni* Grossu; (c) *D. reticulatum* Müller; (d) *D. sturany* Simroth.

only for the genus *Limax* and its species, and must not be generalized. Within the genus *Limax* a division into subgenera is possible only by using anatomical criteria.

In order to check our findings about the biochemical unity of the genus *Limax*, we studied electrophoretic patterns in species of other genera having a wide geographical distribution. These are species well outlined by their anatomical characteristics and closely related phylogenetically within the family Limacidae. We also obtained patterns of *Bielzia coerulans* and of several species of the genus *Deroceras*, by studying

with the same methods the characteristics of the muscle extract esterases (Fig. 3,C). The genus *Bielzia*—which was represented by only one species—exhibits an electrophoretic pattern (Fig. 3,C a) very different from the other two genera in the disposition, number and width of its bands. Within the genus *Deroceras*—represented by numerous species among which we selected only three (Fig. 3,C b, c, d)—the patterns are distinctive of each species, but all of them have in common a wide band with high migration speed, and two or three bands with slow migration speed, one of them very intense. These bands are characteristic for the genus. The similarity of pattern demonstrates the unity of the genus *Deroceras,* but shows at the same time a high dissimilarity as compared to *Limax.*

Hence, by comparing the three kinds of patterns we may demonstrate a sharp difference amongst them, obvious evidence that these are three different genera. The slight differences seen in the patterns of the *Limax* species do not justify the view that they belong to different genera, the more so as all the patterns have many common characters.

The biochemical tests support the proposals of the well-known specialist H. Walden (1961) and workers of experience such as Pilsbry (1948), Altena (1966), Flasar (1964) and others, who suggested on classical criteria that *Lehmannia* represents a subgenus within the genus *Limax.*

From our researches on problems concerning the systematics of the family Limacidae and particularly of the genus *Limax,* and from the results obtained by using both biochemical and classical criteria, the following conclusions may be drawn.

Electrophoretic separation in starch gel of the nonspecific esterases of the muscle extract, results in constant patterns which are species characteristic, thus supporting the species description made by means of classical criteria.

Concerning the questionable taxonomic position of *Lehmannia,* the electrophoretic patterns show that it belongs to the same genus as *Limax,* being eventually considered as a group at the subgenus level, on anatomical criteria.

The patterns of *Limax* species have a similar appearance and are well differentiated from those of the genera *Bielzia* and *Deroceras* of the same family.

Our biochemical taxonomic studies in the family Limacidae demonstrate that electrophoretic separation of the muscle extracted nonspecific esterases may be used for differentiation of species and grouping them into genera.

SUMMARY

The authors' aim is to point out the genus grouping of some species belonging to the family Limacidae by means of biochemical methods, since classification based on classical methods is controversial. By electrophoretic separation in starch gel of the unspecific esterases from muscle extract, constant characteristic patterns of every species were obtained. Moreover, comparison of these patterns with one another demonstrates the occurrence of bands with the same position and intensity, thus forming a basis for grouping the species in genera. By using these results, the authors include the species of the genera *Limax* and *Lehmannia* in a single genus, *Limax,*

since the electrophoretic findings invalidate the position of *Lehmannia* as an independent genus.

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