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Albumin Evolution and its Phylogenetic and Taxonomic Implications in several Lacertid Lizards

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Abstract. Albumins from several species assigned to *Lacerta* s. l. and *Gallotia* were investigated by means of the Micro-Complement fixation technique. The phylogenetic trees evaluated according to Fitch & Margoliash (1967) and Beverley & Wilson (1982) led us to infer a division, which is in accordance with protein electrophoretic results (Mayer & Tiedemann, 1982) as well as with the subgeneric division used before the studies of Böhme (1971) and Arnold (1973).

Introduction

The clarification of the relationships within the collective genus *Lacerta* is still far from final. Research on these problems has received new stimulus lately, mainly from the works of Böhme (1971) and Arnold (1973). These authors investigated new non-adaptive features, which allow a better understanding of the relationships. Furthermore, Lanza et al. (1977), Lanza & Cei (1977), Engelmann & Schäffner (1981), Mayer & Tiedemann (1982), Guillaume & Lanza (1982) and Lutz & Mayer (1983) contributed completely new interpretations of systematic relationships within the genus *Lacerta* by employing biochemical and immunological protein investigations. Such studies have been found extremely valuable, because the molecular data, contrary to most of the morphological features, do not appear to have important adaptive information. Our immunological data on the albumin evolution of certain species of *Lacerta* s. l. and *G. galloti* serve as confirmation and elaboration of the protein electrophoretic results of Mayer & Tiedemann (1982).

Material and Methods

Table 1 lists the 19 investigated species and their localities. Antisera were prepared to the albumin of the following species: L. (*P.*) *filfolensis*, L. (*P.*) *erhardii*, L. (*P.*) *taurica*, L. *graeca*, L. (*A.*) *bedriagae*, L. (*L.*) *agilis*, L. (*L.*) *trilineata* and L. (*Z.*) *vivipara*.

Table 1. The investigated species with their localities. The abbreviations in brackets refer to table 2 and 3 and figure 2.

L. (P.) erhardii (ER)	Naxos Island	GR
L. (P.) filfolensis (FL)	Malta Island	M
L. (P.) melisellensis (ME)	Neretva Delta	YU
L. (P.) muralis (MU)	Carinthia	A
L. (P.) sicula (SI)	Calabria	I
L. (P.) peloponnesiaca (PE)	Minthi Mountains	GR
L. (P.) taurica (TA)	Chelmos Mountains	GR
L. (P.) tiliguerta (TI)	Corsica	F
L. (P.) wagleriana (WA)	Palermo	I
L. graeca (GR)	Taygetos Mountains	GR
L. (A.) bedriagae (BE)	Corsica	F
L. (A.) horvathi (HO)	Velebit Mountains	YU
L. (A.) oxycephala (OX)	Neretva Delta	YU
L. (Z.) vivipara (VP)	Mariazell	A
L. (L.) agilis (AG)	Orth/Donau	A
L. (L.) trilineata (TL)	Ios Island	GR
L. (L.) viridis (VI)	Mount Olympus	GR
G. galloti (GL)	Teneriffa	E

Purification of the albumins

The lizards were bled to death and to avoid coagulation of the blood, a 3,8% citrate solution was injected into the coelom. The blood was collected and centrifuged to remove red cells. Plasmas from several individuals of one population were pooled. Plasma was dialysed against distilled water at 4°C overnight. After centrifugation of the precipitated fibrin the supernatant was freeze dried. The lyophilisate was dissolved in the smallest volume possible of distilled water and passed over a DEAE Sephadex A-50 column, which binds mainly albumin. Chromatography was carried out according to Curling et al. (1977), except that the pH of the eluting buffer was reduced to 4,5. The fraction containing albumin was dialysed against distilled water, lyophilised and further purified by starch gel electrophoresis (starch: 12% in 1:10 diluted running buffer: 0,17 mol/l Tris, 0.22 mol/l boric acid, 0.005 mol/l EDTA). Albumin was detected with 8-anilino-1-naphtalenesulfonic acid (Mg salt) (0,003% in the buffer described above). This stain makes albumin fluoresce yellow under short wave UV light. The albumin band was cut from the gel and the albumin was eluted by electrophoresis in an apparatus similar to that described by Allington et al. (1978). Each albumin behaved as a single band upon polyacrylamid-electrophoresis at pH 7,8 when stained with amido black.

Antiserum production

Antisera to purified albumin were raised in Yellow-Silver rabbits. Three rabbits were used for one antiserum. Each rabbit received at first several intradermal and intra-

muscular injections of 1 ml of Freund's complete adjuvent mixed thoroughly with 1 ml of an albumin solution prepared in the following way: 500 µg albumin were diluted in 0,5 ml 0,9% NaCl solution and 0,5 ml 1% $Al_2(SO_4)_3$-solution. Then 0,1 mol/l NaOH was added dropwise until $Al(OH)_3$ was precipitated.

Three weeks later each rabbit was given a booster with 250 µg albumin and Freund's incomplete adjuvent. This was repeated three times. Thus each rabbit was immunized with 1,5 mg albumin.

The rabbits were bled to death from the heart, the individual antisera were tested for purity by means of the double diffusion according to Ouchterlony and immunoelectrophoresis.

Micro-Complement fixation tests

Micro-Complement fixation experiments were performed according to the methods described by Champion et al. (1972) and Zwilling (1974). The individual antisera were pooled in inverse proportions to their MC'F titers. The titers of the pools reached from 1/2100 to 1/9500 (the incubation time was 18 hours at 4°C).

Construction of the phylogenetic tree

To adjust the variation of the individual antisera, the distances of the reciprocal matrix were scaled according to Uzzell (1982). The distances, which were determined by the antiserum to *L. trilineata*, were used as constants. After multiplying the immunological distances with their corresponding factor, the resultant scaled values were averaged. (This was possible for bidirectional distances only).

Dendrograms were calculated according to Fitch & Margoliash (1967). For the construction of such trees a complete matrix is required. Therefore we used only those distances, which were obtained with the eigth taxa utilized for the preparation of antisera and *G. galloti*.

As the experimental error of the MC'F is +/- 2 units, branches shorter than 1 unit were avoided by the following procedure. Such taxa were allied to one last common ancestor. The calculation of these branches is shown in figure 1.

To join taxa with undirectional distances to the tree, we used the method described by Beverley & Wilson (1982).

For lack of appropriate fossils within *Lacerta*, it is very difficult to evaluate the times of the last common ancestors. Among the species groups we investigated, the rate of albumin evolution is scattered very much. Therefore we cannot use a linear timescale for our data. For the estimation of isolation times usually the equation: 100 units = 60 m.y. (Maxson et al. 1975) is applied. Nevertheless we have to use this equation, because there are no other possibilities to calculate isolation times, which therefore are only coarse estimates.

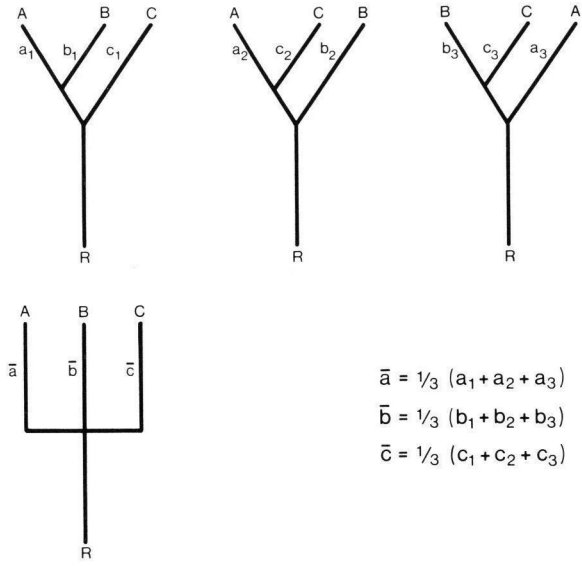

Fig. 1. The calculation to ally three taxa to one common ancestor.

Results and Discussion

The 8 x 8 matrix of reciprocal distances is given in table 2. The percent standard deviation (Champion et al., 1975) is 12,9%. After the data were scaled (table 3) the percent standard deviation was reduced to 6.7%.

Figure 2 shows the dendrogram with the smallest error (f = 4,7%), calculated according to Prager & Wilson (1976). The dotted lines indicate the taxa which were fitted in the tree later. Neglecting the surprising position of *L. graeca*, several species groups have to be distinguished. This grouping is in good accordance with the division into the categories *Podarcis, Archaeolacerta, Lacerta* s. str., *Zootoca* and *Gallotia* valid before Böhme (1971) and Arnold (1973).

1. *Podarcis*:

Six species (*L.* (*.P.*) *erhardii, L.* (*P.*) *filfolensis, L.* (*P.*) *peloponnesiaca, L.* (*P.*) *tiliguerta, L.* (*P.*) *taurica, L.* (*P.*) *wagerliana*) form an extremely closely related species group. As the experimental error of the MC'F is +/- 2 units, the radiation of these species, which results in the dendrogram of figure 2, is very doubtful. Therefore we have to leave the relationships within this group uncertain. We estimate, that their radiation began 4-6 m.y. ago at the time of the desiccation of the Mediterranean Sea (Cita, 1980; Hsü, 1977).

There exist also some incomplete distances to *L.* (*P.*) *milensis*, which—in good accordance with the electrophoretic results (Mayer & Tiedemann, 1980)—allow to assume a

Table 2. Immunological distances between the albumins of the investigated lizards. AG ... antigen, AS ... antiserum, other abbreviations see table 1.

	AS	FL	TA	ER	GR	BE	VP	AG	TL
AG									
filfolensis		0	12	9	40	32	45	45	42
taurica		6	0	8	40	33	43	47	41
erhardii		8	10	0	40	31	44	47	41
graeca		41	48	41	0	33	53	56	49
bedriagae		32	42	34	36	0	35	39	40
vivipara		47	55	49	54	39	0	45	38
agilis		39	45	40	35	22	30	0	7
trilineata		40	49	40	39	25	29	9	0
wagleriana		6	11	12	/	/	/	/	/
tiliguerta		8	12	10	/	/	/	/	/
peloponnesiaca		8	8	0	/	/	/	/	/
melisellensis		14	21	13	/	/	/	/	/
sicula		17	31	22	/	/	/	/	/
muralis		22	36	16	/	/	/	/	/
horvathi		30	42	30	35	8	30	38	44
oxycephala		33	36	33	31	18	34	43	45
viridis		41	49	41	44	24	29	5	6
galloti		64	64	54	59	56	56	58	57

Table 3. The upper right-hand section of the matrix shows the immunological distances reconstructed from the phylogenetic tree shown in figure 2. The lower left-hand section of the matrix shows the scaled immunological distances evaluated according to Uzzell (1982). Abbreviations see table 1. AG ... antigen, AS ... antiserum.

	AS	FL	TA	ER	GR	BE	VP	AG	TL	GL
AG										
filfolensis		0	10	10	49	40	56	41	44	68
taurica		9	0	8	47	37	54	38	41	65
erhardii		10	9	0	47	37	54	39	42	66
graeca		48	46	47	0	44	61	45	48	72
bedriagae		39	40	38	43	0	51	36	38	63
vivipara		55	52	54	66	47	0	36	39	68
agilis		42	41	42	45	31	38	0	7	48
trilineata		44	43	42	48	36	37	7	0	55
galloti		74	59	59	71	71	70	49	57	0
wagleriana		7	10	13	/	/	/	/	/	/
tiliguerta		9	11	11	/	/	/	/	/	/
peloponnesiaca		9	7	0	/	/	/	/	/	/
melisellensis		16	19	14	/	/	/	/	/	/
sicula		19	28	24	/	/	/	/	/	/
muralis		25	33	17	/	/	/	/	/	/
horvathi		34	38	33	42	10	38	32	44	/
oxycephala		38	33	36	37	23	43	36	45	/
viridis		47	44	45	52	31	36	4	6	/

close relation of this species to the group mentioned above. *L.* (*P.*) *erhardii* and *L.* (*P.*) *peloponnesiaca* cannot be distinguished from one another according to our results. Therefore we had to put them on the same branch of the dendrogram. This result agrees very well with the enormous morphological and electrophoretic uniformity, which makes a separation into two species doubtful.

L. (*P.*) *melisellensis* does not belong to this group, this species seems to be isolated for approximately 10 m.y. It has to be noticed, that this result is contrary to electrophoretic data (Mayer, unpublished). Considering these data one would have expected a much closer relationship to *L.* (*P.*) *filfolensis* and especially to *L.* (*P.*) *taurica*.

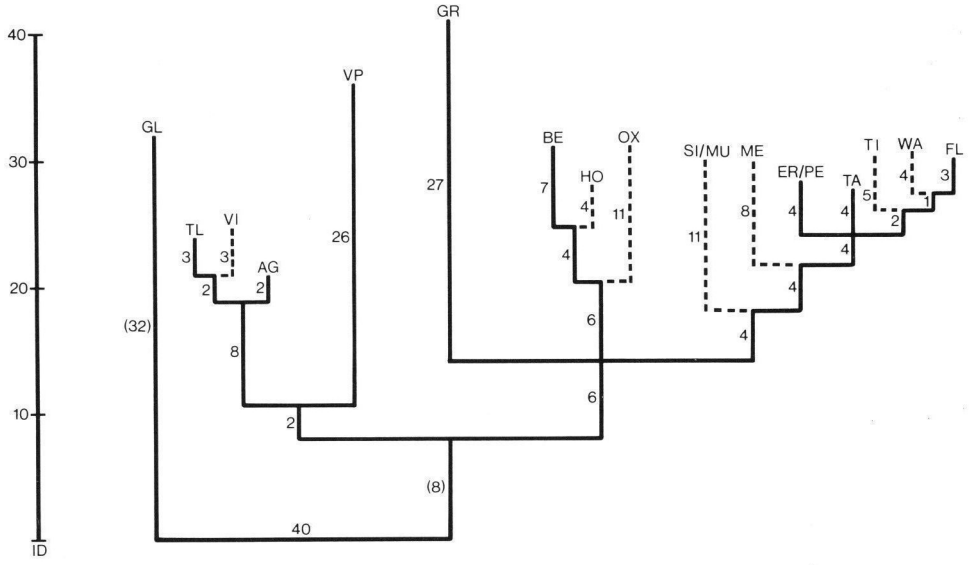

Fig. 2. This tree was constructed by the methods of Fitch & Margoliash (1967) and Beverley & Wilson (1982). ID ... Immunological distances, other abbreviations see table 1.

L. (*P.*) *muralis* and *L.* (*P.*) *sicula* are obviously isolated already very long from the other *Podarcis* species. The immunological distances are so scattered, that it is impossible to make a statement concerning their relationships between them; a decision would have been possible only, if we had a specific antiserum to either of these two species. From our results we believe that these two species were separated from the stock of the other *Podarcis* as long ago as 15 m.y.

1. *Archaeolacerta*:

The species *L.* (*A.*) *bedriagae, L.* (*A.*) *horvathi* and *L.* (*A.*) *oxycephala* form a homogenous and closely related species group, whereas *L. graeca* does not seem to belong to it. This result is confirmed by Böhme (1971), who found a very divergent hemipenis epithel.

The protein electrophoretic results (Mayer & Tiedemann, 1983), however, lead us to assume a completely different relationship. Considering these data *L. graeca* shows an enormous similarity to *L.* (*A.*) *oxycephala*. Moreover *L.* (*A.*) *bedriagae* should be separated from the rest. Thus the position of *L. graeca* seems still unclear. We prefer not to assign *L. graeca* to any subgenus for the present, especially an assignment to *Archaeolacerta* seems extremely doubtful.

L. (*A.*) *bedriagae* and *L.* (*A.*) *horvathi* possibly are isolated since 5 m.y. and *L.* (*A.*) *oxycephala* has been isolated from both species for about 10 m.y. If we may trust in the isolation times so calculated, we cannot accept *L.* (*A.*) *bedriagae* as a praemiocene element of Corso-Sardinia (Guillaume & Lanza, 1982), the introgression of their ancestor, however, must have taken place during the desiccation of the Mediterranean Sea.

3. *Lacerta* s. str.:

The sibling species *L.* (*L.*) *trilineata* and *L.* (*L.*) *viridis* show an extremely close relationship in their albumins, which is in good agreement with their strong morphological and electrophoretic similarity (Mayer & Tiedemann, 1985). Their immunological distance of 6 units is equal to approximately an isolation time of 3 to 4 m.y.

The calculated isolation time of *L.* (*L.*) *agilis* is approximately 4-6 m.y., which is probably an underestimation. *L.* (*L.*) *agilis* shows a greater morphological and electrophoretic difference to *L.* (*L.*) *trilineata* and *L.* (*L.*) *viridis* than the immunological distances would indicate.

12. *Zootoca*:

L. (*Z.*) *vivipara* seems to be related most nearly to the subgenus *Lacerta*. Our results speak clearely against a relationship to the species of the subgenus *Archaeolacerta* (Arnold, 1973; Böhme, 1971).

5. *Gallotia*:

The distances to *G. galloti* are always the highest, which were measured with one antiserum. This means that this species is a representative of a totally distinct group.

Based on our immunological data we assume the following order of divergence: *Gallotia* diverged about 40 m.y. ago, *L.* (*Z.*) *vivipara* and *Lacerta* s. str. have been isolated from *Podarcis* and *Archaeolacerta* for about 30 m.y. The isolation of the latter two took place about 25 m.y. ago. We have to put also the isolation of the species group around *L. lepida* on the same time level (Lutz & Mayer, 1983). Neglecting *L. graeca*, which is difficult to interpret, the immunological results on the whole confirm the data which were obtained with protein electrophoresis (Mayer & Tiedemann, 1982). In particular a special position of *Podarcis* in opposite to all other species as well as a closer

relationship of *Zootaca* to *Archaeolacerta* which were investigated here has to be excluded (Arnold, 1973).

Genera or Subgenera?

Guillaume & Lanza (1982) considered *Lacerta* s. str., *Archaeolacerta, Zootoca* and *Podarcis* as independent genera, which is an interpretation in opposite to Mayer & Tiedemann (1982). Guillaume & Lanza (1982) support their conclusion with "the well known data of hybridisation, genetic distances and ecology". The "rare" (?) species of which the relationship is still unknown, should according to their point of view be left in a separate category (subgenus?) within the genus *Lacerta*.

A critical view on this hypothesis leads us to the following argumentation:

1. Hybrids within *Lacerta* s. l. occur only exceptionally and between very closely related taxa (even *L. mixta* is, as Uzzell & Darevsky (1973) have shown, no hybrid but a proper well isolated species). The existance of hybrids is thus a good argument to unite the parent species into a genus, however their absence cannot justify a separation of species or species groups as individual genera.

2. Genetic and immunological distances are, according to our opinion, an excellent quantitative measure for the degree of phylogenetic relationship, though we see no possibility of associating such values with distinct taxonomic categories in a objective way.

3. No ecological gaps exist between closely related groups in *Lacerta*. The slight ecological preference of some groups cannot serve as an argument to establish genera in the sense of evolutive units (Dubois, 1981).

4. If we establish a genus *Lacerta* with the subcategory *Lacerta* s. str. and an "autre renfernement" as a waiting room for such species with an unclear relationship, we form an extraordinary heterogenous group, which is at variance with the very principle of a phylogenetic unit.

As a result, the only obstacles accepting the generic division of the collective genus *Lacerta* at the moment, are the "difficult" species, because the arguments listed under point 1 to 3 are not against such a division; they only show that there are no forcing arguments for this division.

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