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A black, non-troglomorphic amphibian from the karst of Slovenia: *Proteus anguinus parkelj* n. ssp. (Urodela: Proteidae)

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Abstract

A morphologically distinct cavernicolous salamander *Proteus anguinus* from southeastern Slovenia (Bela Krajina) is described as *P. a. parkelj* ssp. n. It differs from *P. a. anguinus* in a dark pigmentation, fully developed eyes, a skull with broader and shorter bones and fewer teeth, a voluminous jaw musculature that gives the head a bulky appearance, a proportionally longer trunk with a higher number of vertebrae, shorter extremities, and a shorter tail. Most of these traits are considered to be plesiomorphic character states. An allozyme analysis over 40 loci has shown the new dark pigmented taxon to be genetically similar to a white and troglomorphic neighbouring population from Stična (D_{Nei} = 0.23). Both populations in turn are genetically dissimilar to a geographically more distant population from Postojna (D_{Nei} = 0.49). The observed level of genetic differentiation suggests that western and southeastern Slovenian populations form separate lineages since the uppermost Miocene but conservatively hitherto only a single species is recognised. The new taxon is only known from a small area and may be rare. *P. a. parkelj*, now under strict legal protection, is threatened by industrial pollution.

Résumé

Un Protée remarquable de Slovénie du sud-est (Bela Krajina) est décrit comme *Proteus anguinus parkelj* ssp. n. Celui-ci se distingue de la sous-espèce nominative par la pigmentation très foncée, les yeux bien développés, le crâne à os plus courts et élargis, un nombre inférieur de dents, la musculature des mandibules plus fortement développée (ce qui donne à la tête un aspect plus massif), le corps relativement plus allongé et à nombre supérieur de vertèbres, ainsi que par les pattes et la queue plus courtes. La plupart de ces caractères sont considérés comme étant plésiomorphes. L'analyse des allozymes à 40 loci montre que ce taxon à pigmentation foncée ressemble au point de vue génétique (D_{Nei} = 0.23) à la population troglomorphe dépigmentée d'une localité située à proximité (Stična). Cependant, ces deux populations diffèrent génétiquement d'une population géographiquement plus distante (Postojna; D_{Nei} = 0.49). Le niveau de différenciation génétique montre que les populations occidentales et sud-orientales représentent des lignées distinctes depuis le Miocène supérieur; on considère tout de même qu'elles appartiennent à une même espèce. Le taxon nouvellement décrit est connu d'un territoire fort restreint, et il est apparemment rare; bien que protégé par la loi, *P. a. parkelj* n. ssp. est menacé par la pollution industrielle.

1 History, habitat, and distribution

The history of the name *Proteus* goes back to Valvasor (1689) who mentioned the species in his treatise "Die Ehre des Herzogtums Crain". With its discovery in the Postojna caves in Central Slovenia by Jeršinovič von Loewengreif in 1797 (Fitzinger, 1850) *Proteus* became the first known specialised cave animal.

The normal habitat of *Proteus* is cave waters, but most places where it was found were in the open when occasionally specimens were washed out of the caves after heavy rainfall. J.N. Laurenti, who formally described the species in a monotypic genus in 1768, obtained his material from such a surface location in Cerknica near the Postojna caves.

The known distribution of *Proteus anguinus* encompasses the Dinaric karst almost entirely, from the lower reaches of the Isonzo-Soča River in Italy in the northwest to the river Trebišnjica in Hercegovina in the southeast. Altogether almost 200 localities are known (Sket, 1983; Sket & Aljančič, in prep.).

Proteus is highly specialised troglomorphic,

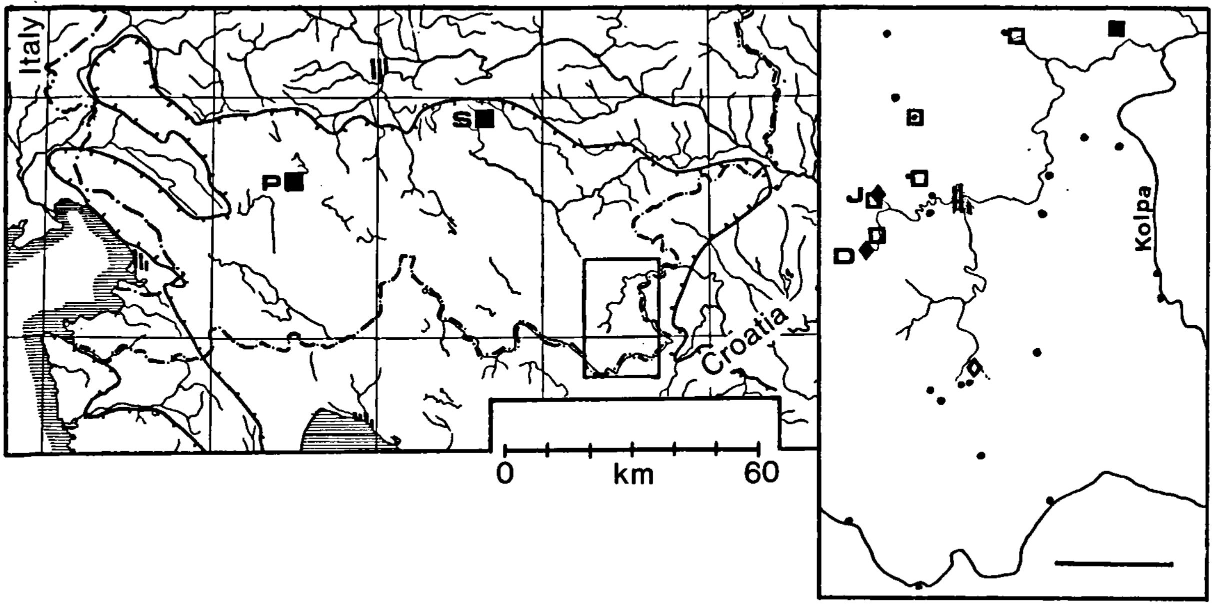

Fig. 1. Southern Slovenia with the area of Dinaric Karst indicated by a denticulated line. Solid squares refer to documented records of white *P. a. anguinus* populations; solid diamonds refer to documented records of *P. a. parkelj*; open symbols refer to reports that require confirmation. The insert highlights the Bela Krajina, with black dots documenting places of access to hypogean waters such as springs and caves. Scale bar is 5 km. D, Dobličica; J, Jelševnik; P, Planina (Postojna and Planinska Jama); S, Stična, with the springs Vir and Rupnica. Three vertical bars indicate (from left to right) the cities Trieste, Ljubljana, and Črnomelj.

strictly neotenic and shows limited morphological variation. Normally *Proteus* is blind and at the most weakly pigmented. However, it is able to synthesise dark pigments and reared in the light it may gradually become dark or even black (Aljančič et al., 1986). During larval development, the initial eye development is retarded; later on in life the eyes degenerate and become covered by skin (Durand, 1973). Attempts to induce metamorphosis failed (Pehani & Seliškar, 1941).

No obvious characters exist that suggest strong intraspecific differentiation. The only serious attempt to elaborate upon the taxonomy of *Proteus* was by Fitzinger (1850) who described seven species under the genus name *Hypochthon* (see also Schreiber, 1912). Of all those taxa only *H. zoisii* has been accepted by some (Mertens & Müller, 1940) at the subspecies level as *Proteus anginus zoisii Proteus anguinus* is the only living representative of the genus. The most likely sister taxon to *Proteus* is the genus *Necturus* from the New World (Duellman & Trueb, 1986).

The first documented finding of a "black" *Proteus* in nature is that by members of the Institute for Karst Research (Slovenian Academy of Sciences and Arts). One black specimen with developed eyes and unusual body proportions was caught in the Dobličica spring, near Črnomelj in southeastern Slovenia on October 18, 1986. As *Proteus* is popular among Slovenes, this finding resulted in a lot of publicity, mostly at the popular level (Aljančič et al., 1986; Istenič & Bulog, 1986; Istenič, 1987; Hodalič, 1993). Another black specimen was sighted and photographed by Miss M. Zlokolica below the Jelševnik springs, some 2.5 km north of Dobličica, on April 21, 1990. In the period up to 1992 a sufficient number of specimens ($n = 15$) was obtained to allow morphometric and other investigations.

For the purpose of the present paper we will refer to densely pigmented *Proteus* as "black" and to unpigmented and slightly pigmented ones as "white".

2 Material and methods

Samples of *Proteus* used or mentioned in this study originate mainly from the following Slovenian localities: (1) the Na Trati spring at Jelševnik near Črnomelj in the Bela Krajina (in the text usually referred to as "Jelševnik"), (2) the Vir and Rupnica springs, both at Stična, 1.7 km apart, 25 km E.S.E. of Ljubljana (together referred to as "Stična"), (3) the Planinska Jama cave at Planina, near Postojna which forms part of the Postojna-Planina cave system (referred to as "Planina") (Fig. 1).

Only specimens that were damaged or that died in captivity have been preserved, except for specimens that were sacrificed for biochemical taxonomic analysis (see below). As a consequence the quality of most of the material is rather poor. For histological studies the specimens were fixed in a 2% solution of glutaraldehyde and later transferred to 70% ethanol. One skeleton was prepared by maceration of the carcass in a weak solution of KOH and its skeleton then dyed by alizarin. Other specimens or parts thereof have been cleared in KOH and glycerol, stained with alizarin and preserved in glycerol with thymol, following Humason (1967). Eight black *Proteus* are preserved in 70% ethanol and at present six are kept alive at the Department of Biology of the Biotechnical Faculty, University of Ljubljana.

Some osteological details were studied on cleared specimens from Planina and from Luknja near Novo Mesto. In addition, tail tips of some specimens from Vir, Luknja, and Planina were cleared to allow an osteological study. Data on pigmentation were taken from live as well as from preserved specimens. Eyes were measured in some intact specimens and in a skinned head. Data on the structure of the eyes and skin were obtained by electron microscopy (Bulog, 1992). The morphology of the gills was studied in detail but since preliminary observations pointed to a marked phenotypic plasticity, the quantitative aspects were not further taken into consideration. X-ray photographs were taken by standard clinical equipment. For most specimens settings were 31–32 kV at 120 mA on a Siemens "Selenos-4", with a glass plate below serving as a filter. In newly collected specimens listed in Table II, photographs were taken using a Siemens "Polydoros 80" apparatus set at 40–41.5 kV and 1.6–2.5 mA. We did not succeed in obtaining useful pictures of the tiny tail vertebrae, neither by X-ray photography, nor by nuclear magnetic resonance.

A specimen of *Necturus* sp. of unknown provenance and its X-ray image were used for morphological comparison of the eyes and the backbone. Two adult specimens of *Triturus alpestris* (Laurenti, 1768) from the Kamniške Alps in Slovenia and two specimens of neotenic phenotype from Trnovičko Jezero, Maglić Mts., Montenegro were cleared for the same purpose.

A series of 82 individuals, including specimens stored in the collections of the Naturhistorisches Museum, Vienna, and the University of Ljubljana, originating from four localities (Jelševnik, Planina, Rupnica, and Vir) were subjected to the measurement of the following eight variables: total length from the tip of the snout to the tail tip (L); head length from the tip of the snout to the line connecting the bases of the most anterior gills (Lc); head width at the point where head width is largest (Ltc); pre-pedal length from the tip of the snouth to the anterior legs (LaP); trunk length between the hind side of the anterior and the fore side of the posterior leg (LiE); the length of the anterior and the posterior legs from their point of insertion to the tip of the longest toe on the perpendicularly stretched leg (PaL, PpL), and finally tail length from the hind side of a posterior leg to the tip of the tail (Lcd). Measurements were taken to the tenth of a mm using vernier callipers. Presented values are rounded off to their integer values. It should be noted that in soft tissues and in contracted preserved material the precision of the measurements may be diminished.

Two meristic measurements were also taken: (1) In fresh and alcohol-preserved material the number of myomeres (Myo) was counted between both pairs of legs. (2) The number of vertebrae (Ver) from the atlas up to and including the last vertebra before the pelvic girdle (ilium) was determined from X-ray photographs (see Plate I for an example) or counted directly in disarticulated and cleared specimens. Some ambiguity exists in counting the number of vertebrae towards the posterior end of the animal because the cartilaginous pelvic girdle and its attachment to the backbone in particular, is not resolved in X-ray photographs. Not surprisingly, both meristic characters proved highly correlated (cf. Romer & Parsons, 1977), with a product-moment correlation coefficient of 0.70. Because neither data set was complete, for three specimens the vertebrae count was estimated from the regression equation of Ver versus Mio. Teeth counts were not routinely performed, since this would require a partial dissection of precious specimens. Instead we used the data of Dolivo-Dobrovolsky (1926) after comparison with some of our own specimens in order to confirm the reliability of the published data.

At its 18th to 20th year, when *Proteus* matures and starts reproducing, growth has not yet completely ceased (Durand & Delay, 1981). By consequence, absolute morphometric values are of limited use for comparative purposes. L was therefore taken as a reference value for each of the continuous variables in order to reduce the effect of variation in individual size. In addition to a univariate character by character analysis using analysis of variance, the "Wolterstorff Index" ($WI = 100 \times LiE \times Pa^{-1}$ (Wolterstorff, 1923; Arntzen & Wallis, 1993)) was calculated. A Principal Component Analysis (PCA) including *a posteriori* comparisons among localities was performed on ln-transformed data. A further analysis was carried out using Discriminant Analysis on *a priori* grouped specimens with locality as the independent variable. The statistical analyses were carried out using the SYSTAT 5.1 software package (Wilkinson, 1989).

Blood, liver, heart, stomach, and a strip of muscle from the ventrolateral side (in *Proteus*) or from the tail (in *Mertensiella* and *Triturus*, see below) were dissected from freshly sacrificed animals. Erythrocytes were removed from the blood by brief centrifugation. The plasma supernatant was diluted with an aliquot volume of 40% sucrose. Other tissues were ground in homogenising buffer (0.1 M Tris, 10^{-3} M EDTA and 5×10^{-5} M NADP, adjusted to pH 7.0 with HCl) and centrifuged. The aqueous supernatant was decanted and stored at −70 °C for future electrophoresis.

Polyacrylamide slab gel electrophoresis and staining of plasma

Table I. Electrophoretic conditions for 26 protein systems, corresponding to 40 loci, examined in the genera *Proteus*, *Mertensiella*, and *Triturus*. Starch buffers are A: Tris-citrate pH 6.0 (XIII); B: Tris-citrate pH 7.0 (I); C: Tris-citrate pH 8.0 (V); D: Lithiumhydroxide-tris-citrate pH 8.1 (X); E: Tris-malate pH 7.4, electrode buffer is 0.22 M Tris, 0.10 M Maleic acid, 0.01 M EDTA and 0.01 M $MgCl_2$, gel buffer is electrode buffer diluted at 1:10; F: Tris-EDTA-borate pH 8.9 (Ayala et al., 1972); G: Discontinuous tris-citrate-borate pH 8.2–8.7 (Poulik, 1957). Roman numerals refer to the buffer systems of Shaw & Prasad (1970). PAGE refers to acrylamide gels according to Maurer (1971: Table 4.1). Tissues used are: H = heart, L = liver, M = muscle, P = plasma, and S = stomach.

Protein	E.C. No.	Locus	Buffer system	Tissue extract
Acid phosphatase	3.1.3.2	*Acph-2*	A	L
Adenosine deaminase	3.5.4.4	*Ada*	C	L
Albumin	–	*Alb*	PAGE	P
Alcohol dehydrogenase	1.1.1.1	*Adh-1, Adh-2*	C	L
Catalase	1.11.1.6	*Cat*	D	L
Esterase	3.1.1.1	*Est-1, Est-2*	D	P,L
General Protein	–	*GP-2*	PAGE	P
Glucose 6 phosphate dehydrogenase	1.1.1.49	*G6pd-1, G6pd-2*	C	L
Glucose dehydrogenase	1.1.1.47	*Gdh*	C	L
Glucose phosphate isomerase	5.3.1.9	*Gpi*	A	L
Glutamate oxaloacetate transaminase	2.6.1.1	*Got-1*	G	L
α-Glycerophosphate dehydrogenase	1.1.1.8	*Gly-1, Gly-2*	C	L
Isocitrate dehydrogenase	1.1.1.42	*Icd-1, Icd-2*	E	L
Lactate dehydrogenase	1.1.1.27	*Ldh-1, Ldh-2*	A,B	H,M
Leucine aminopeptidase	3.4.11	*Lap*	F	L
Malate dehydrogenase	1.1.1.37	*Mdh-1, Mdh-2*	A	L
Malic enzyme	1.1.1.40	*Me*	C	L
Mannose phosphate isomerase	5.3.1.8	*Mpi-1, Mpi-2*	C	L
NADH dehydrogenase	1.6.99.2	*Nadhdh-1, Nadhdh-2*	G	L
Peptidase	3.4.11–13	*Pep-1, Pep-2, Pep-3*	D	S
Phosphoglucomutase	2.7.5.1	*Pgm-1, Pgm-2*	A	L
Phosphogluconate dehydrogenase	1.1.1.44	*Pgd*	A	L
Sorbitol dehydrogenase	1.1.1.14	*Sdh*	A	L
Superoxide dismutase	1.15.1.1	*Sod-1, Sod-2*	E	L,M
Transferrin	–	*Trf*	PAGE	P
Xanthine dehydrogenase	1.1.1.204	*Xdh-1, Xdh-2*	E	L

proteins were performed according to Maurer (1971). Enzyme electrophoresis was performed using Connaught starch in horizontal gels. The enzymes were visualised by standard histochemical techniques (Shaw & Prasad, 1970; Harris & Hopkinson, 1976) with few modifications. Proteins assayed and buffer systems used are presented in Table I. Presumptive loci and alleles (electromorphs) were assigned numbers and letters, respectively, when more than one zone of activity was observed in sequence starting from the most anodally migrating forms. For two enzyme systems, ACPH and GOT, two zones of activity were observed, but only one could be consistently scored. These loci were designated *Acph-2* and *Got-1*, respectively.

Three different enzymes with protein digesting properties were resolved. PEP-3, anodally migrating in the *Proteus* samples, showed substrate specificity for the tripeptide Leucyl-Glycyl-Glycine, while the cathodally migrating PEP-1 and PEP-2 were most clearly resolved when the dipeptide Leucyl-Tyrosine was supplied as a substrate. Three plasma proteins were scored, although in one unidentified protein (GP-2) the corresponding electromorph(s) could not be identified for one taxon. The faster anodally migrating fractions were identified as Albumin and Transferrin, based on their phenotypic characteristics and their representation in the most concentrated fraction in the plasma (cf. Rafinski & Arntzen, 1987).

To assess the degree of genetic variability within the *Proteus* individuals, populations, and the outgroup taxa, we counted the number of polymorphic loci and calculated the unbiased estimate for mean heterozygosity based on Hardy-Weinberg expectations (H_e) and the accompanying standard error (Nei, 1987). In order to assess the degree of genetic differentiation across populations we calculated the genetic distance measure of Nei and its standard error (D_{Nei}; Nei, 1972), which may, for low and intermediate values, be linearly correlated to time (Nei, 1987).

The matrix of D_{Nei} was converted into a phenogram using the UPGMA method (Sneath & Sokal, 1973). A main objection against this method is that the phenogram can be interpreted in a phylogenetic sense only when rates of evolutionary change are homogeneous across the phyletic lines, a condition almost never satisfied in reality. This strong and unreal assumption of uniformity of evolutionary rates is relaxed in the distance-Wagner procedure (Farris, 1972). This procedure requires a distance measure which is metric. Since Nei's distance is not metric, we applied the widely used Rogers' genetic distance D_R (Rogers, 1972). From the resulting undirected trees the most parsimonious one, in terms of tree length, was selected. For an evolutionary interpretation the undirected trees have to be rooted. As appropriately preserved specimens of the genus *Necturus*, the assumed sister group of *Proteus*, were not available for comparison, instead seven *Triturus cristatus* (Laurenti, 1768) from Canterbury and Peterborough, United Kingdom and two *Mertensiella caucasica* (Waga, 1876) from near Rize in Turkey were used as outgroups.

To distinguish on the generated tree the branches for which the data show strong support from the areas in which the branching order is more uncertain, we applied the jack-knife test as advocated by Lanyon (1985) for distance data. The computer programme employed for analysis was the micro-computer version of BIOSYS-1 (Swofford & Selander, 1981).

The locality Jelševnik was visited after heavy rainfalls, when local inhabitants notified us about the welling up of the so-called "boiling-holes". To obtain an impression of the composition of the cave fauna that may serve as prey to *Proteus*, drift nets of various size and mesh width were placed immediately below the holes as long as the discharge continued (generally from one to three days). The catches were generally very small and no animals at all were obtained from the main Jelševnik spring. Since only empty gastropod shells could be collected from sediments of the outflow, it is not possible to establish their origin precisely.

3 Results

3.1 *Description of the black* Proteus

Anticipating the discussion we assign taxonomic status to the black *Proteus* that will be named *Proteus anguinus parkelj* Sket & Arntzen.

Material. – Holotype: coll. nr. J8 is a female from Na Trati, Jelševnik near Črnomelj, Slovenia (plate I), preserved in ethanol. Paratypes: same locality, six specimens (J1–J3, J6–J7, J9) and one embryo/larva of 21 mm (J10), preserved in ethanol. One specimen cleared and stained (J5) and one disarticulated skeleton (J4) are preserved in glycerol. Others: one specimen (D1) from Dobličica spring, Dobliče near Črnomelj, is ethanol preserved. All material is kept in the collection of the Department of Biology of the University of Ljubljana, Slovenia, except for J9, which is deposited in the Zoological Museum, Amsterdam, the Netherlands and stored under nr. ZMA Herp. 9239.

3.1.1 Etymology. – "Parkelj" is one of the Devil's names in Slovene Christian mythology. In Ljubljana, around St. Nicholas day, small "parkeljni" (plural of "parkelj") with a black body and a red tongue are sold. These allegedly hypogean creatures resemble the black hypogean amphibian with its red gills. Note that the ending "*j*" of the word parkelj is not to be pronounced.

3.1.2 Diagnosis. – A dark pigmented and short-snouted subspecies of *P. anguinus* with externally visible eyes, a head with angular and convex lateral sides, a long trunk, short legs, and a short tail.

3.1.3 Description of the type series. – Holotype: morphometric characters, variability indices, and corresponding data for white *Proteus a. anguinus* can be taken from Table II. The dorsal and lateral parts of the body are almost completely black with a violet or brownish hue. In some specimens the colour is only very dark brown. The snout is broadly bordered black as soot, in some specimens with a pale or whitish triangle in the preocular region. Limbs pale with a dark patterning. Ventral side of the body pale with a bluish or pinky shade. Skin surface finely granulated in preserved specimens. An alcohol preserved specimen (D1) has retained a dark brownish black coloration for seven years. Gills red in live specimens, with dark brown principal branches.

The skin contains large quantities of pigment, mainly in the upper part of the dermis. The upper dermis also contains more numerous multicellular glands than is found in white specimens and Leydig cells are present in the epidermis (Bulog, 1991, 1992).

The eyes are small and externally visible as black dots that are surrounded by a white circle. The eye is covered by a distinct transparent conjunctiva but without lids or similar structures. The diameter of the conjunctiva is ca. 5% of the head length. The general structure of its bulb is similar to that in epigean amphibians with a well-developed retina. In a 190 mm long specimen from Dobličica, the bulb diameter was measured to 1.3 mm (7% of Lc) and the diameter of its lens 0.2 mm (glutaraldehyde preparations for electron microscopy were made by

Table II. Morphometric data (mm) for *Proteus anguinus parkelj* spp. n. from Jelševnik and *P. a. anguinus* from Planina, Rupnica, and Vir. L = total length; Lc = head length; Ltc = head width; LaP = pre-pedal length; LiE = trunk length; PaL = anterior leg length; PpL = posterior leg length; Lcd = tail length; Myo = number of trunk myomeres; Ver = number of neck and trunk vertebrae; WI = Wolterstorff Index. Values in parentheses are estimated from the regression of Lcd versus L and Ver versus Myo. Data are presented individually for all biochemically studied specimens and for the type series from Jelševnik. Statistical data include the specimens measured by Kranjec (1981).

Locality	Specimen number	L	Lc	Ltc	LaP	LiE	PaL	PpL	Lcd	Myo	Ver	WI
Jelševnik												
	J1	227	27	16	31	130	14	13	64	31	(33)	10.8
	J2	247	26	16	32	138	14	12	(69)	–	–	10.1
	J3	240	25	17	34	140	16	13	67	32	35	11.4
	J5	205	23	14	31	120	13	11	55	–	34	10.8
	J7	199	20	12	27	113	13	10	57	30	(33)	11.5
	J8*	276	28	17	37	158	16	13	78	30	34	10.1
	J9	232	24	16	30	135	14	13	65	33	35	10.4
	sample size	7	7	7	7	7	7	7	6	5	4	7
	mean	232.3	24.7	15.4	31.7	133.4	14.3	12.1	64.3	31.2	34.5	10.7
	SD	26.0	2.69	1.81	3.15	14.6	1.25	1.22	8.19	1.30	0.58	0.57
	minimum	199	20	12	27	113	13	10	55	30	34	10.1
	maximum	276	28	17	37	158	16	13	78	33	35	11.5
Planina												
	P5	235	26	17	35	118	19	16	75	27	31	16.1
	P6	255	30	17	37	129	19	20	82	28	31	14.7
	sample size	54	54	54	54	54	54	54	54	54	53	54
	mean	223.2	29.0	15.3	36.0	109.9	18.7	17.5	72.6	25.5	30.8	17.1
	SD	26.6	3.47	2.40	4.02	14.1	2.07	2.15	9.29	0.79	0.51	1.20
	minimum	147	19	9	24	72	13	12	48	24	30	13.8
	maximum	299	37	23	46	152	24	23	100	28	32	19.6
Rupnica												
	R1	270	31	19	42	133	25	21	90	25	29	18.8
	R2	225	24	16	31	111	18	15	77	26	31	16.2
	sample size	7	7	7	7	7	7	7	7	7	7	7
	mean	245.0	27.7	17.5	36.6	118.4	20.4	17.2	83.9	25.4	29.9	17.3
	SD	40.6	4.82	3.37	6.45	21.1	3.40	2.53	11.7	0.54	0.69	1.64
	minimum	188	23	14	29	88	16	15	67	25	29	15.7
	maximum	294	34	22	45	144	25	21	102	26	31	19.7
Vir												
	V7	219	24	15	32	108	19	17	76	27	31	11.1
	sample size	14	14	14	14	14	14	14	14	14	14	14
	mean	228.4	27.8	16.0	34.6	111.4	19.1	16.7	76.5	26.1	30.6	16.7
	SD	34.7	3.90	2.61	4.55	16.9	3.08	2.00	12.8	0.66	0.65	1.95
	minimum	154	21	11	26	76	14	14	49	25	29	11.1
	maximum	285	34	20	41	138	23	20	100	27	31	18.7

* Holotype.

Bulog (1991, 1992)). In a skinned specimen of 205 mm, at 0.9 mm the eye bulb's diameter was 3.9% of Lc (ethanol preparation).

The head is slightly broader than the trunk and approximately 11% of the length of the body (L-Lcd). In its postocular part the head is parallel-sided, the short snout shaped as a trapezoid with rounded corners. The snout comprises on average 27% of Lc. Three pairs of subdermal muscular cushions (that we tentatively identify as the *leva-*

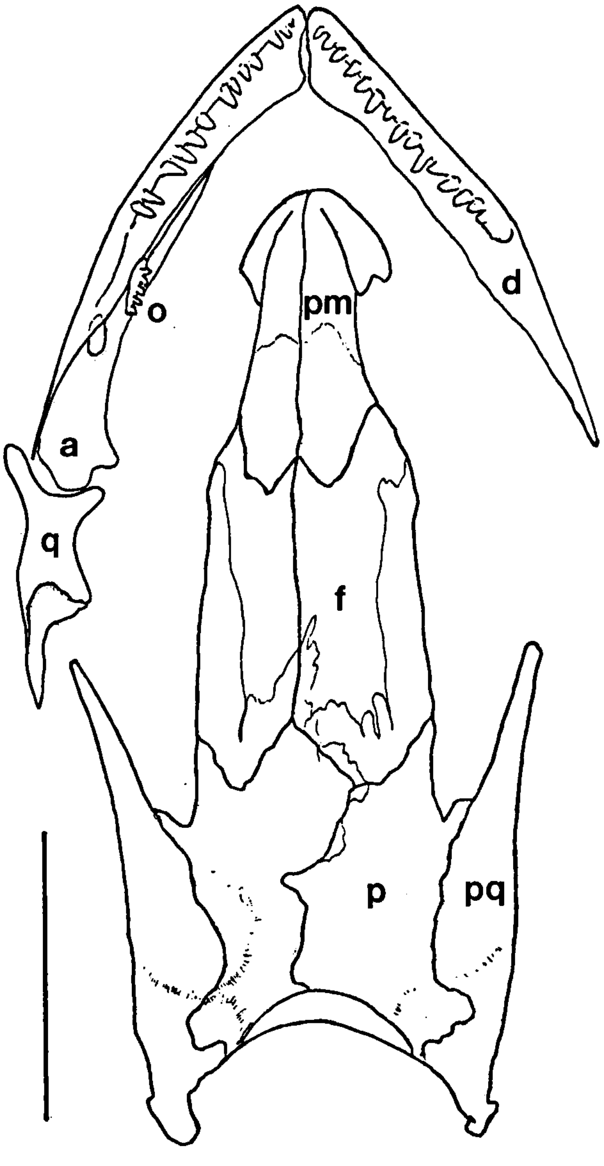

Fig. 2. Cranial and mandibular bones in *Proteus anguinus parkelj* from Jelševnik (specimen no. J4): a, angulare; d, dentale; f, frontale; o, operculare; p, parietale; pm, praemaxillare; pq, paraquadratum; q, quadratum. Scale bar 5 mm.

tores mandibulae anteriores, *levatores mandibulae externi*, and *depressores mandibulae posteriores*) give the head a bulky appearance. Three pairs of short, bushy gills are well developed with a length of approximately 20% of Lc.

The trunk is cylindrical and anguilliform, on average 58% of the length of the body. The flanks of the body are running parallel with shallow furrows between the myomeres. The tail is short, comprising 28% of the entire body length and flattened laterally with the ventral and dorsal sides running almost parallel. The tail is as high or slightly higher than the trunk. The tail tip is broadly rounded or triangular with a rounded tip. The legs are short and slender. The anterior legs form on average 6% of the body length and have three toes. The posterior legs are slightly shorter and have two toes.

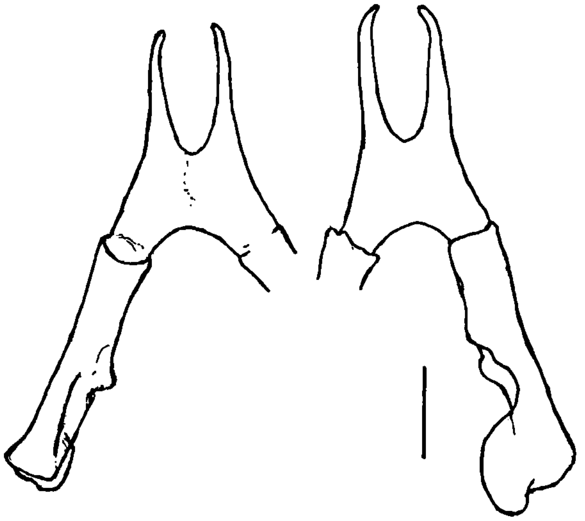

Fig. 3. Rostral part of the *trabecula cranii* with the anterior ossification and ethmoidal plaque in *Proteus anguinus parkelj* from Jelševnik (specimen no. J4). Scale bar 1 mm.

The skull is similarly built to that of white specimens (cf. Dolivo-Dobrovolsky, 1926) but proportioned differently. The neurocranium is wider, with a rostro-occipital length that is slightly less than the length of four pectoral vertebrae. The dentalia are placed in a wide arch, the largest width equals the lengths of 2.3 pectoral vertebrae. The ratio rostro-occipital length versus largest mandibular width averages 1.4 to 1. The praemaxillaria are less than twice as long as wide (Fig. 2). The ethmoidal plaque in the only macerated skull that we possess has a rather peculiar shape with its rostral tines curved and with convergent tips (Fig. 3). In the frontalia the width versus length ratio averages at 0.6 to 1. The frontalia slightly protrude along the praemaxillaria. The other bones of the neurocranium are

Table III. Number of teeth observed in *P. a. anguinus* (four specimens from Luče and Stična, according to Dolivo-Dobrovolsky, 1926) and in two specimens of *P. anguinus parkelj* ssp. n. from Jelševnik. Figures in parentheses denote the accessory smaller teeth standing outside the main row. Maximal numbers for white specimens are given separately for different combinations of principal (large) teeth and accessory teeth. In both taxa the opercular teeth may be arranged either in one or in two to three rows.

		Number of teeth on				
		Praemaxillare	Vomer	Pterygo-palatinum	Dentale	Operculare
*P. a. anguinus** (minimum)	left	7	22	5	22	6
	right	7	22	6	21	5
*P. a. anguinus** (maximum)	left	10 or 8 (2)	24 (8)	6	33 or 23 (11)	12
	right	9 (2) or 8 (4)	25 (8)	6	31 (2) or 23 (8)	12
P. a. parkelj	J4–left	5	19	4	19	7
	J4–right	5	18	4	16	?
P. a. parkelj	J5–left	7	20	5	17 (6)	8
	J5–right	8	22	5	17 (4)	6

* According to Dolivo-Dobrovolsky (1926).

short and wide. There are fewer teeth than in the white morph, particularly so on the dentale. On the operculare, teeth are arranged in two or three rows. The dentale bears 16 to 19 teeth with another 4 to 6 small auxiliary teeth that are placed outside the main row (Table III).

The number of vertebrae between the skull and the posterior legs is 34 to 35. The neck vertebrae are of the same length as the pectoral ones. The caudal vertebrae get gradually smaller till number XXIII or XXIV and are then followed by 3 to 5 rudimentary vertebrae that have no processes. Up to number XVII, the neural spine of the vertebrae is in the shape of a sharp and caudally extended triangle. The posterior spines form irregular trapezoidal crests and owing to the fact that they are remarkably short, the vertebrae appear to be high.

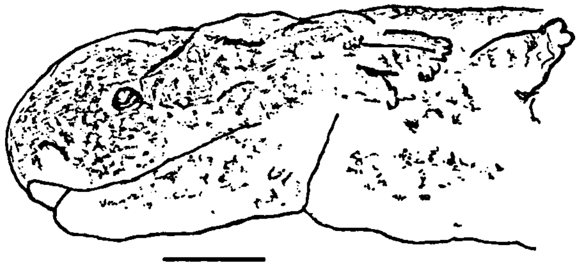

Fig. 4. Anterior part of embryonic larva of *Proteus anguinus parkelj* from Jelševnik (specimen no. J10) at Briegleb stage 21. Scale bar 1 mm.

A drifted larva of 21 mm L (specimen no. J10) was in Briegleb's developmental stage 21 (Fig. 4). It did not have the appearance of an active feeder. Its anterior legs are short and three-toed, the posterior ones are only short stumps without toes. The eye diameter is 8% of Lc. Ventrally the specimen is pale white. Dorsally it is densely speckled grey and less so on the sides.

As a result of the allozyme study, four alleles, Cat^{b}, Cat^{d}, $Nadhdh\text{-}1^{a}$, and Pgd^{a}, were uniquely observed in black specimens.

3.2 *Comparison with related taxa*

3.2.1 General appearance, pigmentation, eyes, and gills. – Differently from the new subspecies, all *Proteus* from other populations than Jelševnik and Dobličica exhibit a nearly white or pinkish skin, sometimes with pale yellow or pale grey patches. They only become dark after a long exposure to light.

In the stages immediately before or after the larval hatching (corresponding to an advanced stage 21 of Briegleb) the eye, with a diameter of 6 to 8% of Lc, is not evidently larger in *P. a. parkelj* than in *P. a. anguinus* (corresponding value is 8%; mea-

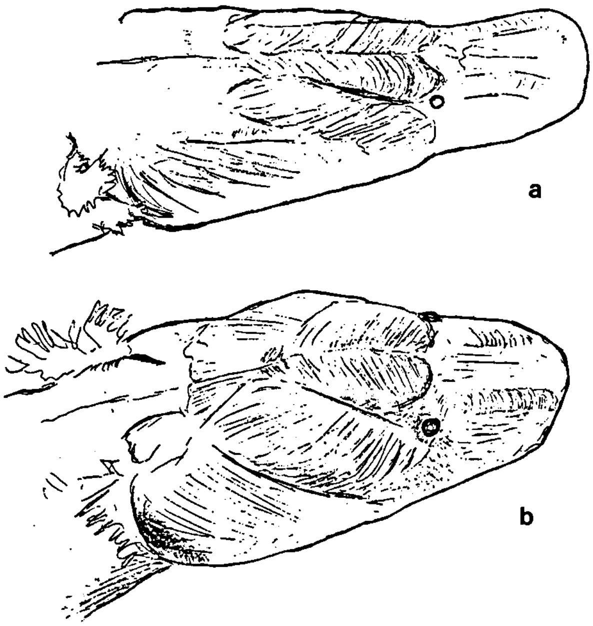

Fig. 5. Skinned heads of *Proteus anguinus anguinus* from Planina (a) and *P. a. parkelj* from Jelševnik (b).

surements taken from a drawing in Briegleb (1962) and from photographs by Vandel & Bouillon (1959) and Vandel et al. (1966)). The dark pigmentation in the embryo of *P. a. parkelj* seems to be only a little denser than in *P. a. anguinus*. The intense pigmentation of adult *P. a. parkelj* differs from most surface amphibians by the absence of a more vivid pattern of coloration. Also in the genus *Necturus*, sister group of *Proteus*, most species are without any bright coloration (cf. Conant, 1975; Behler & Wayne King, 1979).

The eye in the black morph is small and without lids and superficially similar to that in *Necturus*. In the white morph the degenerated eyes are always covered by skin, although in some cases the eye might be shining through as a blackish dot. In a skinned specimen of white *Proteus*, the eye bulb's diameter at 2% of Lc was half that of the black specimen. As in other species with rudimentary eyes (Peters et al., 1973; Sket, 1985) the diameter of the rudiment is expected to be highly variable.

The length and ramification of the gills is variable within white populations. It may be that the varying oxygen concentration in the water affects increase or decrease of gill size. A similar phenomenon has been supposed for *Necturus* (Conant, 1975). Compared to other *Proteus* populations, the gills in the black morph are of medium size.

3.2.2 Univariate morphometric and osteological comparisons. – The total length of the black *Proteus* ranged from 199 mm to 276 mm, which falls within the range observed in our sample of white *Proteus* (range 147–299 mm).

For most measurements relative to L (Lc, LaP, LiE, Lpa, Lcd) differences are found between populations. Relative Lc is smaller in the black *Proteus* than in the whites, while relative Ltc of both forms is nearly equal. The head differs strikingly in shape, more strongly than is brought about by the morphometric data. In the black morph the head is parallel-sided behind the snout. In the whites, due to a weaker musculature behind the eyes, the whole head has the shape of a long, truncated triangle (Fig. 5). It even can be pear-shaped with concave flanks.

The relative length of the posterior legs, strongly correlated with relative head length (product-moment correlation coefficient = 0.94), is smaller in the black morph than in the white morph. Making up on average 58% of L, relative trunk length is larger in the black morph than in the white morph in which the corresponding value is 49%. Correspondingly, in the black morph the absolute number of trunk vertebrae and myomeres is 4; 5 or 6 higher than in the white morph.

Both pairs of legs are remarkably shorter in the black specimens than in the white ones. In both morphs the legs are similarly built with only three and two toes at the fore and hind limb, respectively, and with a simplified skeleton (cf. Aljančič & Sket, 1964).

The tail is longer in white *Proteus* than in black *Proteus*. The tail shape shows a great interpopulational variability but some contour characters are quite constant within populations. In specimens from Planina, the tail with its crest is of the same height as the body, gradually lowering towards the tail tip, which is usually bluntly pointed. In specimens from Rupnica and Vir the tail is usually higher than the body. It does not narrow caudally and ends broadly rounded. In the black morph the tail tip is

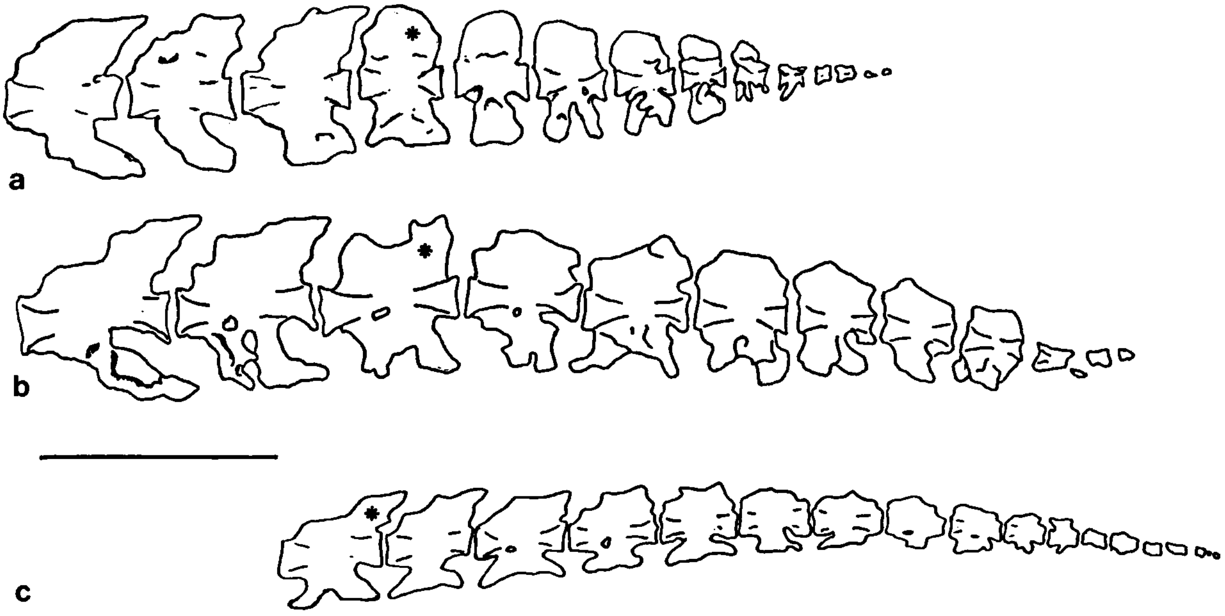

Fig. 6. Last caudal vertebrae in *Proteus anguinus parkelj* from Jelševnik (a) and *P. a. anguinus* from Vir (b) and Planina (c). The XVIIIth caudal vertebra is indicated by an asterisk. Scale bar is 5 mm. The vertebrae are seen here from the same perspective as the heads in Fig. 5.

of a shape somewhere in between the recorded extremes.

In the skull of the black morph, the flat bones such as the parietalia, frontalia, and praemaxillaria are shorter or wider than in the white morph and the dorsally visible intercalations are shorter. Also some other bones (dentalia, paraquadrata) are differently shaped. The mandibular arch such as formed by the dentalia and angularia is wider than in the white morph. The number of teeth (Table III) is higher in the white morph than in the black morph, the difference being most pronounced in the dentale. The fossil relative *Mioproteus caucasicus* Estes & Darevski with 10–12 teeth on its vomer (Estes & Darevski, 1977) was markedly less toothed.

Some significant morphometric differences are also found between white *Proteus* populations from Planina and Stična. The two Stična populations (Rupnica and Vir), however, are mostly indistinguishable.

In some white specimens the neck vertebrae are elongated, a feature that has not been observed in the black morph. Otherwise the trunk vertebrae are similarly shaped, and even in the black morph the vertebrae are not as stout as in *Mioproteus caucasicus* (cf. Estes & Darevski, 1977; Estes & Schleich, 1993). In *Necturus* as well as in all *Proteus* the centra and particularly the processes of the tail vertebrae are less well developed than in *Paleoproteus* (cf. Herre, 1935) or in neotenic and regular *Triturus alpestris*. In *Proteus* the centre of the individual vertebra is thinner, the dorsal spine and ventral process are narrower and the interspaces between subsequent vertebrae are substantially larger. Altogether their shape is variable. In black specimens, the caudal vertebrae get gradually smaller till the XXIIIrd or XXIVth and are followed by three to five rudimentary ones which are without processes (Fig. 6). In the white specimens from Vir the tail vertebrae are similar to the inspected black specimens, although markedly shorter. In the white specimens from Planina the vertebrae are lower and gradually diminishing in size towards the tip of the tail. One specimen has a chain of seven small, spineless vertebrae after the XXVIIIth that still bears spines (Fig. 6), while there are only three of such in another specimen.

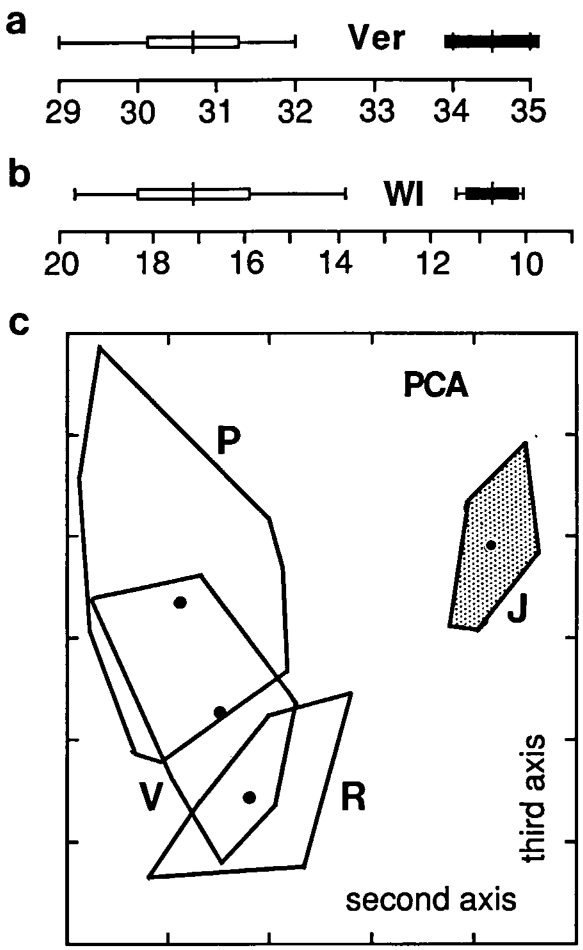

Fig. 7. Morphometrics of *Proteus anguinus anguinus* (outline symbols) and *P. a. parkelj* (solid symbols). Upper part (a): univariate – number of trunk vertebrae (Ver); to the middle (b): bivariate – Wolterstorff Index (WI, details see text); lower part (c): second and third axes of a Principal Component Analysis (PCA) with centroids and convex outline polygons for P = Planina, R = Rupnica, and V = Vir (*P. a. anguinus*) and J = Jelševnik (*P. a. parkelj*).

3.2.3 Bivariate and multivariate morphometric analysis. – The Wolterstorff Index is significantly different in the black and white morph of *Proteus* ($P<0.001$; Table II). Similar to the situation in *Triturus cristatus* for which the WI was originally devised (Wolterstorff, 1923), the number of trunk vertebrae (Ver) is an equally good or better discriminatory feature than WI itself (Arntzen & Wallis, 1993) (Fig. 7ab).

In the multivariate PCA all eight variables have high component loadings on the first axis, which is interpreted as representing general size. In line with the univariate analysis, size is not population dependent and not discriminatory for black versus white populations. The second axis is dominated by LiE, PaL, and PpL. Component loadings have contrasting signs, which means that animals have either a short trunk and long extremities, or a long trunk and short extremities. Hence the second PCA axis is essentially the same as the WI (Fig. 7bc).

On the third axis Lc and PpL dominate together with Lcd, with contrasting signs. This axis helps separating all populations from one another, especially those from Planina and Jelševnik from Rupnica and Vir. Plotting the second versus the third axis provides excellent overall separation of black and white specimens (Fig. 7c).

Discriminant analysis reveals that the morphometric differentiation for Planina versus Rupnica and Vir is not statistically significant, with 8 out of 54 specimens (15%) wrongly classified. The populations of Rupnica and Vir are morphometrically virtually indistinguishable, with 7 out of 21 specimens (33%) wrongly classified.

3.2.4 Protein analysis. – In 23 different enzyme systems and three plasma proteins the variability could be consistently scored, with the exception of some loci in the outgroup taxa (two loci and four loci in *Mertensiella* and *Triturus*, respectively). Data on allelic composition for altogether 40 presumptive loci and the associated estimates of genetic variability of populations are presented in Table IV. The *Mpi-1* locus showed no variability over any of the assayed populations, while an additional 11 loci showed no variation across *Proteus* samples. In 12 loci, inter- but no intra-populational variation was observed for *Proteus* populations. The remaining 16 loci showed variability in at least one of the *Proteus* samples. The mean heterozygosity (H_e) for the combined *Proteus* populations is 12.0 ± 4.0%, which is similar to the value found for *M. caucasica* (11.2 ± 3.3%) and substantially higher than $H_e = 1.3 \pm 1.0\%$ found for *T. cristatus*. Among *Proteus* populations H_e ranges from 7.5% for the two studied specimens from Planina to 22.5 ± 6.7% for the single individual from Vir.

Table IV. Allelic composition for 40 gene loci (upper part) and measures of genetic variability (lower part) as observed in the genera *Proteus*, *Mertensiella*, and *Triturus*. Individuals from two populations of the latter taxon are pooled. Allelic composition in parentheses refers to single individuals. Missing data are indicated by a hyphen. *P* is the number of polymorphic loci, H_e is the average heterozygosity calculated on the basis of Hardy-Weinberg expectation and SE is the corresponding standard error (in percent values).

Species	*Proteus anguinus*							*Mertensiella caucasica*		*Triturus cristatus*
Locality	Jelševnik, Slovenia		Planina, Slovenia		Rupnica, Slovenia		Vir, Slovenia	Azaklihoca Köyü, Rize, Turkey		Canterbury and Peterborough, U.K.
Reference no.	J1	J2	P5	P6	R1	R2	V7	ZMA Herp. 7277 ($n=2$)		ZMA Herp. 9199 and 9200 ($n=7$)
Locus										
Acph-2	CC	CC	DD	DD	CC	CC	CC	BB	BC	AA
Ada	BB	BB	BB	BB	BB	BB	BB	AA	AA	CC
Alb	DD	DD	CC	CC	DD	DD	CD	AA	AA	BB
Adh-1	BB	BB	AA	AA	BB	BB	BB	–	–	–
Adh-2	DD	DD	AA	AA	AA	AD	AD	CC	CC	BB
Cat	AB	AD	AA	AA	AA	AA	AA	–	–	CC
Est-1	CC	CC	CC	CC	CC	CC	CC	BB	BB	AA
Est-2	DD	DD	DD	DD	DD	DE	CE	BB	BB	AA
GP-2	BB	BB	BB	BB	CC	CC	CC	AA	AA	–
G6pd-1	AA	AA	BB	BB	AA	AA	AA	DD	DD	CC
G6pd-2	BB	BB	CC	CC	BB	BB	BB	AA	AA	DD
Gdh	AA	AA	AB	BB	AB	BB	BC	DE	DD	–
Gpi	AA	AA	AA	AB	AA	AA	AA	DE	EE	CC (CD, DD)
Got-1	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA
Gly-1	BC	CC	CC	CC	CC	CC	BC	CC	CC	AA
Gly-2	CC	CC	CC	CC	CC	CC	CC	BB	BB	AA
Icd-1	BB	BB	BB	BB	BB	BB	BB	CC	CC	AA
Icd-2	DD	BD	DD	DD	BB	BB	BB	CC	CC	AA
Ldh-1	BB	BB	BB	BB	AA	AA	BB	CC	CC	CC
Ldh-2	AB	AB	BB	BB	BB	BB	AA	CC	CC	DD
Lap	CC	CC	CC	CC	CC	CC	CC	AA	AA	BB
Mdh-1	BB	BB	BB	BB	BB	BB	BB	CC	CC	AA
Mdh-2	DD	DD	CC	CC	DD	DD	DD	AA	AA	BB
Me	BB	BB	BB	BB	AA	AA	AA	DD	CD	EE
Mpi-1	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
Mpi-2	BB	BB	AB	AB	AB	AA	AB	DD	DD	CC
Nadhdh-1	BB	AB	BB	BB	BC	BC	BB	CC	CC	DD
Nadhdh-2	AA	AA	AA	AA	AA	AA	AA	AA	AB	CC (CD)
Pep-1	EE	DD	DD	DD	DD	DD	DE	CC	BC	AA
Pep-2	BB	BB	CC	CC	BB	BB	BB	AA	AA	–
Pep-3	DD	DD	CC	CC	DD	DD	DD	AA	AB	AA
Pgm-1	CC	CC	AC	AC	AC	AC	BC	CC	CC	DD
Pgm-2	BB	BB	BB	BB	BB	BB	BB	DD	CD	AA
Pgd	AA	BB	BB	BB	BB	BB	BB	DE	DD	CC
Sdh	BC	BC	BC	BC	BB	BC	CC	DD	DD	AA
Sod-1	CC	CC	CC	CC	CC	CC	CC	AA	AA	BB
Sod-2	CC	CC	DD	DD	CC	CC	CC	AA	AA	BB
Trf	BB	BB	BB	BB	CC	CC	CC	AA	AA	AA
Xdh-1	AA	AA	AA	AA	AA	AA	AA	BB	BB	BB
Xdh-2	BB	AB	BB	BB	BB	BB	AB	CC	CC	DD
P (individual)	4	6	4	4	4	5	9	3	6	0–1
P (population)	9		5		7		9	9		2
$H_e \pm$ SE (population)	13.8 ± 4.2		7.5 ± 3.2		9.6 ± 3.4		22.5 ± 6.7	11.2 ± 3.3		1.3 ± 1.0
$H_e \pm$ SE (species)	12.0 ± 4.0							11.2 ± 3.3		1.3 ± 1.0

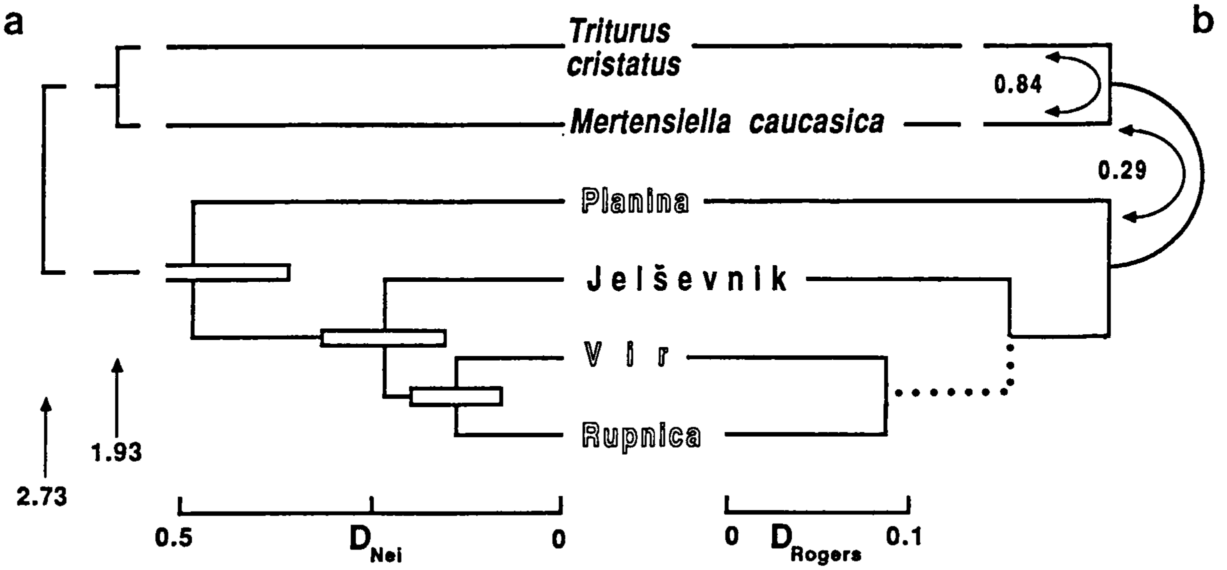

Fig. 8. To the left (a): UPGMA-dendrogram for four populations of *Proteus* (white morph with "outline" printed population name) based upon Nei's genetic distance as measured over 40 gene loci. Boxes indicate estimated standard error (Nei, 1987). The cophenetic correlation coefficient is 0.96. To the right (b): minimum length distance-Wagner tree on the basis of Rogers' genetic distance, rooted by the outgroup. Length of the tree is 0.62 (1.75 with outgroup taxa included); the cophenetic correlation coefficient is 0.99. The interrupted line refers to a branch that is not robust under the jack-knife test (see text). Outgroup taxa *Mertensiella* and *Triturus* are included for comparison.

High values of D_{Nei} (> 1.8; Fig. 8a) were obtained for all comparisons among *Proteus* and the outgroup taxa. Among *Proteus* populations relatively high values, with D_{Nei} ranging from 0.41–0.56, were obtained for comparisons involving the Planina population versus the others. Substantially lower values (D_{Nei} = 0.23 and 0.24) were recorded for comparisons between the black population from Jelševnik and the white populations from Rupnica and Vir, respectively. In all cases the standard error of these estimates, averaging at 32% of D_{Nei}, is substantial and taking the standard error into account, the latter populations are not clearly separated (Fig. 8a). Using the UPGMA-method with the matrix of D_{Nei} results in a tree in which, seen from the root, the population from Planina branches off first, followed by Jelševnik and the two remaining clustering populations of Rupnica and Vir (Fig. 8a). Each of the jack-knife replicates supports this branching topology.

In the distance-Wagner tree that displays maximum parsimony the results are essentially the same. Inspecting the distribution of alleles, seven alleles (*Adh-2*^{d}, *Alb*^{b}, *G6pd-1*^{a}, *G6pd-2*^{b}, *Icd-2*^{b}, *Mdh-2*^{d}, and *Sod-2*^{c}) are potentially synapomorphic character states defining the grouping together – separate from Planina – of the Jelševnik and both Stična populations. Conflicting results and hence no robust phylogenetic resolution is found within the latter group (Fig. 8b).

Four alleles, *Cat*^{b}, *Cat*^{d}, *Nadhdh-1*^{a}, and *Pgd*^{a}, were observed uniquely in the black *Proteus* from Jelševnik. Larger reference samples are required to find out whether or not these alleles are strictly autapomorphic. No loci with fixed alleles diagnostic for the black phenotype were observed. The populations from Rupnica and Vir (D_{Nei} = 0.14) differ from one another most markedly on both *Ldh* loci.

3.3 *Ecology and reproduction of the black* Proteus

3.3.1 Habitat: abiotic parameters. – Up to the present, *P. a. parkelj* has only been documented for two localities near the town of Črnomelj in the Bela Krajina, which is in the southeasternmost part of Slovenia. The Dobličica and Jelševnik springs both discharge into the river Dobličica. They are situated in close proximity to one another (about 2.5 km apart), below the higher conical karst of the Poljanska Gora mountain ridge, with deeply set water channels. At an altitude of 150 m above sea level, the conical karst runs out in karst plains (Habič, 1991). On the plain, some small rivers flow on the surface while others are subterranean at a depth of several meters.

Dobličica is a permanent spring, rising from a deep pool of ca. 25 m diameter. There are some 5–11 m deep pits in the pool and its outflow. One black *Proteus* was caught in the outflow (not in the main spring) after a pumping experiment, which lowered the water level by two meters (Aljančič et al., 1986). Soon after, one of us (BS) inspected the spring by diving, but no amphibians were seen. At Jelševnik, there is a permanent limnocrene spring (called Jezero), similar to the Dobličica spring, and two associated groups of temporarily active "boiling holes", all within a reach of 150 m. One black specimen was photographed below the southern group of boiling holes (Jamnice), while the typical series was caught at the northern group of holes of 1 to 100 cm in diameter (Na Trati). The boiling holes eject water only a few times a year after heavy rains. When they are active or soon afterwards, black specimens have occasionally been found crawling around on the wet meadow or they were collected straight from a narrow hole in the meadow covering the karstified rock. The boiling holes at Jelševnik, as well as some accessory springs in the outflow of Dobličica, are probably fed by shallow local aquifers from the mountain or from the low karst zone. When active, they discharge large amounts of soil and some terrestrial animals. The presence of some epigean aquatic organisms in the drift of Na Trati suggests its partial origin from a surface stream in the background.

Physical and chemical parameters of the permanent springs were measured by Habič et al. (1990) on 17 occasions in spring and autumn of 1986–1990. In the permanent springs of Dobličica and Jelševnik, water temperature fluctuated between 10.0 and 11.3 °C and temperatures in winter and summer are expected to deviate only marginally from this range (the extreme value of 14.8 °C as mentioned for Dobličica is almost certainly erroneous). The total hardness of the water ranged from 200 to 250 mg $CaCO_3 \times l^{-1}$, with Ca × mg^{-1} ratios fluctuating. Oxygen content in Dobličica in the autumn of 1986 was near saturation. In May 1987, O-phosphates in Dobličica and Jelševnik springs corresponded to 0.01 mg PO × l^{-1}. The dissimilar response of the springs to large scale water extraction and their differing chemical properties suggest complex and perhaps remote connections between these springs and the areas from where the water originates (Habič et al., 1990).

The hydrological complexity of the region can be illustrated by the situation at Jelševnik on November 1, 1992. While the temperature of the Jezero permanent spring and some of the boiling holes was in the range of 10.1 to 10.3 °C, most holes discharged water of 11.2 °C, which was also the temperature of a nearby epigean stream. Close monitoring of the system, especially during upwelling periods, will be required to gain a proper understanding of the situation.

3.3.2 Associated biocoenoses and food. – Some stygobiont animals have been ejected from the permanent Dobličica and Jelševnik springs (Table V). Since Na Trati has no steady outflow and associated surface fauna, the surface animals in its drift either originate from an unidentified stream, or they are sucked in hydrodynamically from the main spring.

Upon capture, some of the *Proteus* gave up their stomach contents. Surprisingly, food items predominantly consisted of surface-dwelling prey species. Of these, the gastropod *Vitrea* sp., earthworms (Oligochaeta: Lumbricidae), and the chilopod *Lithobius* sp. may have actively penetrated subterraneously, but the aquatic gastropod *Sadleriana fluminensis* Kuester has been shown not to enter cave waters (Sket, 1970). These observations are in favour of suggestions that *Proteus* forages at night out in the open. In captivity, black *Proteus* are seen to be active and aggressive feeders that may, at least in an illuminated aquarium, attack and bite their white counterparts. Indeed, a violent attack on a white specimen during feeding has been registered on videotape.

Table V. Fauna drifted from the habitat of *Proteus anguinus parkelj*: J, Jelševnik Jezero; Ja, Jamnice; N1, Na Trati (big hole); N2, Na Trati (small holes). Gastropods were collected from sediments. Taxa of which the origin is difficult to determine, such as Nematoda, Oligochaeta, and Cyclopoida, are excluded (for details see text).

	Dobličica	Jelševnik			
		J	Ja	N1	N2
Stygobiont and stygophilic species					
Gastropoda					
Belgrandiella fontinalis (Schmidt, 1847)	x	x	x	x	
Belgrandiella sp.	x	x	x	x	
Hauffenia cf. *michleri* Kuščer, 1932			x		x
Hauffenia sp.	x	x	x	x	x
Iglica cf. *hauffeni* (Brusina, 1885)		x			
Iglica sp.	x				
Sadleriana cavernosa Radoman, 1978	x	x	x		
Sadleriana sp.		x		x	x
Crustacea					
Microcharon sp.					x
Monolistra racovitzai spp. Strouhal, 1928				x	x
Monolistra velkovrhi Sket, 1960			x		x
Niphargus sp.				x	x
Niphargus sp.					x
Niphargus sp.					x
Niphargus subtypicus Sket, 1960					x
Proasellus parvulus (Sket, 1960)					x
Troglocaris sp. (larva)				x	x
Troglodiaptomus sketi Petkovski, 1978		x			
Surface aquatic species					
Cyclopoida			x	x	x
Cyclostomata (larva)				x	
Gammarus fossarum Koch, 1836				x	
Hydra sp.					x
Pisces (larvae)				x	
Terrestrial species					
Chilopoda			x	x	
Diplopoda			x	x	
Diplura				x	

3.3.3 Reproduction. – The finding at Jelševnik of an embryonic larva of stage 21 (still swollen by vitellus and probably not able to move around in an organised way) speaks in favour of oviparity in *P. a. parkelj*, as is likely to be the case in *P. a. anguinus* (Briegleb, 1962; Sket & Velkovrh, 1978).

4 Discussion

4.1 *Taxonomic status of the black* Proteus

The black morph of *Proteus* can be distinguished from the white morph by: (1) synthesis of pigment in the absence of light, (2) fully developed eyes, (3) the shape of some cranial bones and the number of teeth, (4) a different head shape stemming from a well-developed head musculature, (5) a high number of trunk vertebrae and its associated long trunk, (6) short relative length of the tail, and (7) short relative length of the legs.

On the basis of these evidently constant differences the black and white morph deserve attribution to separate taxa. On many occasions differences of similar magnitude have led to the recognition of separate species or even genera. Examples include the characinid fish *Astyanax fasciatus* Cuvier, 1819 and its cave relative *Anoptichthys jordani* Hubbs & Innes, 1936, that have recently been shown to be completely interfertile (Culver, 1982).

Unfortunately, in *Proteus* crossing experiments pose the greatest of technical difficulties and to test for interfertility will be almost impossible. So far both morphs have in nature only been documented in allopatric conditions. The observed genetic distance of $D_{\mathrm{Nei}} = 0.24$ between the black *Proteus* and the white population from Stična is similar to the distances found for the various forms within the *Triturus cristatus* superspecies, that to a large extent are genetically isolated from one another (Wallis & Arntzen, 1989; Arntzen, in prep.).

According to the results of the allozyme study, the black morph is phylogenetically closer to the populations of Stična than to the population of Planina. In view of them being morphologically similar, the large genetic distance between Planina and Stična populations is remarkable. If a study of populations that are geographically intermediate would fail to indicate a pattern of clinal variation, we would identify the populations of Stična as a species separate from *P. anguinus*. Nomenclatorial priority would go to *Proteus zoisii* (Fitzinger, 1850) of which Rupnica is the type locality. The morphologically indistinguishable and biochemically similar population from Vir should be attributed to the

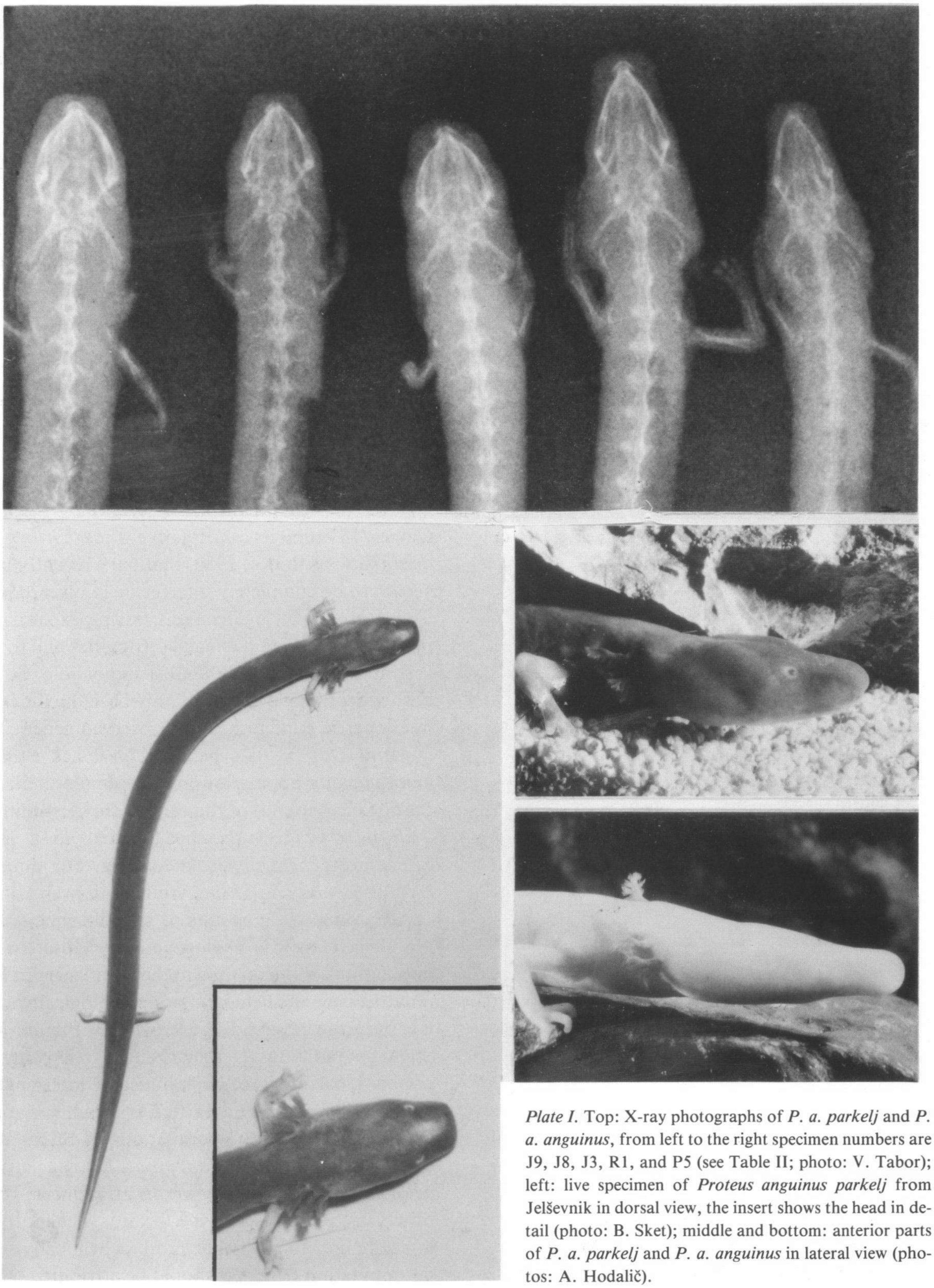

Plate I. Top: X-ray photographs of *P. a. parkelj* and *P. a. anguinus*, from left to the right specimen numbers are J9, J8, J3, R1, and P5 (see Table II; photo: V. Tabor); left: live specimen of *Proteus anguinus parkelj* from Jelševnik in dorsal view, the insert shows the head in detail (photo: B. Sket); middle and bottom: anterior parts of *P. a. parkelj* and *P. a. anguinus* in lateral view (photos: A. Hodalič).

same taxon (although previously it has been given a name of its own: *Hypochthon schreibersii* Fitzinger, 1850). If such a revision would be followed, the black morph would be attributed to *P. zoisii* as the subspecies *P. z. parkelj*. An osteological synapomorphy in the shape of a less equally segmented tail skeleton, such as found in the populations of Jelševnik and Vir (Fig. 6) might speak in favour of a taxonomic separation of the Planina population.

Recognition of two species of *Proteus* would raise the question whether or not the black morph would also deserve specific status. A genetic distance of $D_{\text{Nei}} = 0.24$ is not a priori incompatible with this view, while the morphological distinctiveness of the black morph speaks in its favour. The finding of genetically isolated sympatric black and white *Proteus* would help proving the point. In the absence of firm phylogenetic, ecological, and genetic data we as yet refute such taxonomic revisions in favour of conservatively recognising a single species rather than two or three species.

4.2 *Biogeographic and evolutionary history*

At present the regions of Planina, Stična, and Jelševnik drain towards the Sava and the Danube, but little doubt exists about the mutual isolation of their hypogean habitats (cf. Gams, 1965; Novak, 1990). The genetic data suggest that the western *Proteus* population from Planina has become isolated from the eastern Stična and Jelševnik populations earlier than the Stična and Jelševnik populations with regard to each other.

In amphibians the "molecular clock" has been calibrated as one unit of D_{Nei} reflecting 14 My. of lineage separation (Maxson & Maxson, 1979). For the genus of aquatic salamanders *Triturus* it has been shown that this calibration fits with independently derived estimates of speciation times; on that basis a consistent biogeographic scenario could be put forward, which corresponds to the vicariance biogeography of other amphibian and non-amphibian taxa (Oosterbroek & Arntzen, 1992). Applying this calibration to *Proteus*, while keeping the standard error to the estimate of genetic distance in mind, this would mean that the first documented separation of *Proteus* lineages took place some 9–5 My. ago, which corresponds to the upper Miocene or lower Pliocene. Following the same line of argumentation, the Stična and Jelševnik populations show lineage independence for 4.5–1.1 My. This coincides with the upper Pliocene to the lower Pleistocene.

It is generally agreed that the prekarstic Miocene-Pliocene surface streams of the Stična region and the Bela Krajina flew in approximately the same direction as they do today. There is, however, some controversy concerning the situation around Postojna (cf. Melik, 1951, 1952; Jenko, 1959). Some zoogeographic data, including the distribution of the isopod crustaceans *Asellus aquaticus cavernicolus* Racovitza, 1925 and *Proasellus istrianus* (Stammer, 1932) speak in favour of an ancient hydrographic connection between the Postojna region and the Gulf of Trieste (Sket, in prep.). At the end of the Miocene, southern Slovenia became tectonically active which resulted in the presence of large depressions with lakes (Prelogović et al., 1975) and in changes in the direction of the flow of rivers. We suggest that *Proteus* may have formed a series of taxa in surface rivers and lakes before the Pliocene, with morphological differentiation developing between them. *Proteus* appears to be another example of a cave species in which the distribution and phylogenetic relationships relate to the prekarstic paleohydrology, rather than to the present-day situation (cf. Sket, 1970, 1986; Sket & Bole, 1982).

With the karstification, a new, hypogean habitat became available to *Proteus*. Governed by environmental conditions, the phenotypical differentiation may locally have become obscured through evolutionary convergent, troglomorphic adaptations. Why the black *Proteus* has not undergone such adaptive change remains an open question. The retention of non-troglomorphic traits may be associated to weak selection (cf. Sket, 1985) or to a relatively recent colonization of the hypogean habitat.

The Pleistocene has seen several glaciations but no evidence is available to document that these extended onto Slovene territory before 1 My. ago (cf. Bowen et al., 1986; V. Pohar, pers. comm.).

Intensive karstification, moving surface streams underground, caused complex hydrographical changes. With the karstification proceeding, the *Proteus* populations from Stična and Jelševnik may have become isolated from one another, but not necessarily in caves. In a time span similar to their isolation or less, some stocks within the genus *Triturus* developed into clearly distinct species (Rafinski & Arntzen, 1987; Oosterbroek & Arntzen, 1992).

4.3 *Character evolution*

Within *Proteus*, the presence of externally differentiated eyes seems unique to *P. a. parkelj*. Kammerer (1912), on the basis of experiments that so far have not successfully been repeated (Durand, 1973), claimed that under the influence of light, blind *Proteus* may develop eyes and pigmentation. Occasional observations on captive *Proteus* do suggest that the ability of pigment synthesis is retained indeed (Ehrenberg, 1867 in: Knauer, 1878), but thoroughly conducted experiments have not been carried out in *Proteus*, nor in any other cave amphibian. The newly developed pigmentation may cover the whole body or only part of it (Aljančič et al., 1986), while retaining a white triangle on the snout (Ehrenberg, 1867 in: Knauer, 1878) – perhaps not unlike that found in *P. a. parkelj*.

The Jelševnik population of *Proteus* is pigmented although specimens are normally not exposed to daylight. As in *Proteus*, the atavism of melanin synthesis is found in some cavernicolous fish while others seem to have completely lost the ability (see e.g. Vandel, 1965: 408). The pigmentation of *P. a. parkelj* along with the well-developed eyes, are considered to be plesiomorphic traits. Since the duck-bill shape of the *P. a. anguinus* head is an attribute typical of troglobiont vertebrates (cf. Vandel, 1965; Cooper & Kühne, 1974), occurring in fishes as well as in plethodontid amphibians, it can be regarded apomorphic compared to the plesiomorphic shape of *P. a. parkelj*. The change in head shape seems to be associated with a narrowing of the neurocranial bones and the mandibular arch and a weakening of the major head musculature. The elongation of the snout may be associated with a slight elongation of the mandibular bones and an increase in the number of teeth they carry. The diminution of the eye with the small eye bulb freely below the skin has not affected the skull as in *Astyanax fasciatus* (Breder, 1944; cited by Culver, 1982).

4.4 *Distribution, threats, and legal protection*

Up to the present, Jelševnik and Dobličica are the only localities where *P. a. parkelj* has been found with certainty. No reliable data exist on the finding of *Proteus* in the main spring of Jelševnik. A report on the finding of a black *Proteus* near Mala Lahinja (A. Hudoklin, pers. comm.), located 7 km to the southeast of Dobličica and within the region of low karst, has not been documented. No *Proteus* have been reported from springs south of Dobličica (F. Velkovrh, pers. comm.) or in any of the few active caves south of Črnomelj. Unfortunately, all over the Poljanska Gora the speleobiological sampling conditions are unfavourable.

Black *Proteus* in populations of whites? The only mention of the natural occurrence of "black" specimens by Graf (1882) unfortunately is not accompanied by any morphological data. The particular karst spring in Slovenska Vas (formerly Windischdorf) near Kočevje (Gottschee) in southeastern Slovenia has now been adopted for the town's water supply. In recent years only white specimens have been obtained from here.

White *Proteus* in populations of blacks? Local inhabitants reported on the finding of white *Proteus* in a locality between Jelševnik and Dobličica. Unfortunately no voucher specimens have become available and the reports will have to be confirmed. A voucher specimen does exist for a locality at 12 km to the northeast of Jelševnik (Klepec, 1981; cf. Fig. 1).

If the hypogean waters of the high karst of the Poljanska Gora are its defined habitat, the existence of *P. a. parkelj* may be limited to water channels in the hydrographical background of both springs that cover an area of approximately 55 km^2 (cf. Habič et al., 1990). As has been suggested by

P. Habič (pers. comm.), the occurrence of *P. a. parkelj* may in fact be restricted to the shallow karst of the Bela Krajina while the deep karst of the surrounding mountains is inhabited by the white taxon. The available data do not contradict this hypothesis. Indeed, the hypothesis is in line with (unconfirmed) data about the sporadic occurrence of white *Proteus* in Jelševnik and black *Proteus* in the springs of Lahinja. Because *Proteus* are difficult to find, their perceived absence in any area does not provide a powerful argument.

The water from the Na Trati boiling holes at Jelševnik is polluted. The contamination can be attributed to the dump of a smelting plant in a karst doline uphill the Poljanska Gora, less than a kilometre away. The fine grained and skin damaging sandy debris dumped in the doline is rich in phenols. It has been demonstrated that (white) *Proteus* in captivity did not survive a ten day period when kept in close contact with these sands (T. Valentinčič, pers. comm.). We consider the pollution a serious threat to the survival of *P. a. parkelj*. The boiling holes of Jamnice are hydrologically similar but pollution has not been noticed there.

Since its habitat is not directly accessible to man, a population estimate cannot easily be performed. As it appears to be a rare taxon with a small distribution area, *P. a. parkelj* has been placed on the Slovenian Red List of endangered animal species (Sket, 1992). It is under strict protection of the Slovenian law as a member of the category "permanently cavernicolous animals", as well as in its own right as a subspecies of *P. anguinus*. We discourage collecting for any purpose. The most recent "Ordinance on protection of rare or endangered animal species and their developmental stages" (Brelih & Gregori, 1980) has become operational in 1976 and a new act updating the old one is in preparation.

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