

Full Length Research Paper

Coupled biochemical genetic and karyomorphological analyses for taxonomic classification - A case study of *Schizothorax* species complex (Teleostei: Cyprinidae)

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Biochemical genetic and karyomorphological studies were evaluated for taxonomic importance. Five *Schizothorax* species namely: *Schizothorax niger*, *Schizothorax curvifrons*, *Schizothorax esocinus*, *Schizothorax labiatus* and *Schizothorax plagiostomus* were studied in this experiment as a model for serum proteins and chromosomal analysis. Serum protein band patterns of *Schizothorax* species complex were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Their electrophoretograms revealed similarities as well as differences in the number and molecular weight of protein bands. *S. esocinus* showed five bands, *S. curvifrons* five, *S. niger* seven, *S. labiatus* and *S. plagiostomus* each showed six bands; they also showed species characteristic bands. Karyotypic study of these was carried out. The diploid chromosome numbers recorded were 98 in *S. niger* (24 m + 32 sm + 22 st + 20 t), 98 in *S. esocinus* (30 m + 22 sm + 10 st + 36 t), 98 in *S. labiatus* (24 m + 20 sm + 2 st + 52 t), 96 in *S. plagiostomus* (24 m + 18 sm + 5 4t) and 94 in *S. curvifrons* (26 m + 20 sm + 20 st + 28 t). Coupled biochemical genetic and karyomorphological analysis proved a good taxonomic tool as the results were decisive in establishing the species status of these species despite their overlapping morphological characters.

Key words: *Schizothorax*, serum proteins, karyotype, taxonomy, biochemical.

INTRODUCTION

Taxonomy provides a vocabulary to discuss the world (Knapp et al., 2002). Most morphological features are plastic and have the potential of being modified by the environmental conditions (Svardson, 1965; Fowler, 1970).

Due to the existence of morphological plasticity among

individuals, conventional morphological characters are often found to be deceiving in exact detection of a species (Menon, 1989). Hence, a detailed knowledge of cytogenetic make up is necessary to establish evolutionary relationships between various species, genera, families or orders of animals and plants (Farooq et al.,

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2011).

The comparison about morphological characters is often not adequate for taxonomists to make decisions. By using electrophoretic methods, taxonomic activities have increased rapidly in animal systematics. Proteins are the products of gene action (Crick, 1963; Nirenberg et al., 1963; Ochoa, 1963) and are used as genetic markers which play a significant role in assigning taxonomic status. Genetic markers are superior to artificial markers and tags as they are natural and can be found in all the stages of animal (Kapila and Kapila, 1996). Blood serum proteins have been found to be reasonably conservative among the characteristics of taxonomic importance. Serum proteins can present taxonomic values when serum proteins of different fish species are examined electrophoretically. Therefore, the discrimination between related taxa can be easily made by their electrophoretic results of serum proteins (Theophilus and Rao, 1998).

A morphotaxonomic classification should be replaced by karyotaxonomic classification, for latter being least affected by environmental distortions (Campos, 1972). Genetic information assists in solving problems of identity and defining conservation units for species. Cytogenetic analysis in fish have allowed to determine sex chromosomes (Moreira-Filho et al., 1993; Devlin and Nagahama 2002; Molina and Galetti, 2007) and to perform inferences on cytotaxonomic (Bertollo et al., 2000; Bertollo et al., 2004) and evolutionary issues (Demirok and Unlu, 2001). Karyological studies have also provided basic information on the number, size and morphology of chromosomes (Tan et al., 2004) which is important to undertake chromosome manipulation in fish (Khan et al., 2000).

Schizothorax heckel (Cypriniformes: Cyprinidae) comprises many species that inhabit the reservoirs of Central Asia from Turkmenistan and Eastern Persia in the West to the far reaches of Mekong and Yangtze in the East. Taxonomic status of fishes of the subfamily Schizothoracinae has remained highly controversial, though creditable work has been done by the earlier workers on Kashmir Valley fishes, prominent among them has been that of Hora (1934), Mukerji (1936), Heckel (1838), Silas (1960), Das and Subla (1963, 1964), Qadri et al. (1983), Yousuf et al. (1990), Yousuf (1995), Kullander et al. (1999) etc.

However, most of the above work was based on morphometric and meristic characters. The occurrences of overlapping external characters and the possibility of hybrids have caused further complications. These inspired us to work on SDS-PAGE of blood serum proteins and karyomorphology for comparative taxonomic study of Kashmir valley *Schizothorax* and also evaluate the role of the present study as a taxonomic tool. The present study has unravelled their real taxonomic status and has paved the way for their evaluation for other studies viz. nutritional biochemistry, aquaculture, etc. The study has also shown that the biochemical, gene-

genetic and cytogenetic methods are corroborating and a good taxonomic tool to solve taxonomic problems.

MATERIALS AND METHODS

Collection of fish

Sample fishes were caught alive with an electric fisher and were also bought from the local fish markets. They were transported to the limnology and fisheries laboratory of CORD, University of Kashmir, where they were kept in fully aerated aquaria.

Serum protein analysis

Blood was taken by cardiac puncture of fish with syringe (40 fishes in total; eight of each species, Wt. 200 g \pm 10/fish). In order to prevent haemolysis, blood was poured into centrifugation tubes after removing the needle. The blood sample was allowed to clot and thereafter centrifuged at 5000 rpm for 10 min. Separated serum was used for protein estimation and further for electrophoresis (SDS-PAGE). Protein concentration was determined by using the method of Lowery et al. (1952) with BSA as a standard protein.

SDS-PAGE was performed according to the Laemmli (1970) and O'Farrell (1975) methods. Proteins were separated on 12 - 8 cm and 1 mm thick slab gel. Slab gel consists of stacking gel in which proteins are stocked and running gel part in which proteins are resolved. Running gel contained 10% and stacking gel contained 5% polyacrylamide. Each sample was mixed with a sample buffer which consisted of 0.5 M Tris-Cl (PH 6.8), 10% SDS, BPB, 10% glycerol, beta-mercaptoethanol. The sera were mixed with sample buffer as described by Laemmli (1970). Equal amount of sample was loaded in each lane. Electrode buffer solution was made from 0.025 M Tris, 0.192 M glycine and 0.1% SDS at pH 8.3. A current of 70 V was applied to stacking gel. After tracking dye reached separating gel, voltage was adjusted at 110 V. After electrophoresis, gels were stained with 0.25 g Coomassie Brilliant Blue R250 in a solution of methanol (45 ml), ddH₂O (45 ml) and glacial acetic acid (10 ml) and destained with a solution of glacial acetic acid (10 ml), methanol (10 ml), ddH₂O (80 ml). Gel was scanned in a densitometer.

Cytogenetic analysis:

Air dried chromosome preparation method with some modifications was used as described by Thorgaard and Disney (1990). Fish (30 specimens, six of each species) received two doses of phytohemagglutinin (PHA) injections (4 μ g g⁻¹ BW), in a 20 h interval at 20°C. 8 h after the second PHA injection, colchicine was injected intraperitoneally, 0.05% at 0.1 ml/100 g BW to depress the mitotic division at the metaphase stage and left for 2 to 3 h before sacrificing.

The fish were anesthetized by 300 ppm clove oil for 40 s, their anterior kidney was removed, homogenized and hypotonised simultaneously by potassium chloride, 0.56% for 35 min at room temperature. Suspensions were centrifuged at 1000 rpm for 10 min. Supernatant was removed and the cells were fixed by cold fresh Carnoy fixative (3:1 methanol and glacial acetic acid). This fixation process was repeated three times and the cold fresh Carnoy was replaced at 30 min intervals. Smears were prepared on cold lamellae using splash method from 1 m height and air dried for 24 h, then stained with 2% Giemsa.

Chromosomal analysis

Leica DM LS2 trinocular photomicroscope with 1000 X magnification

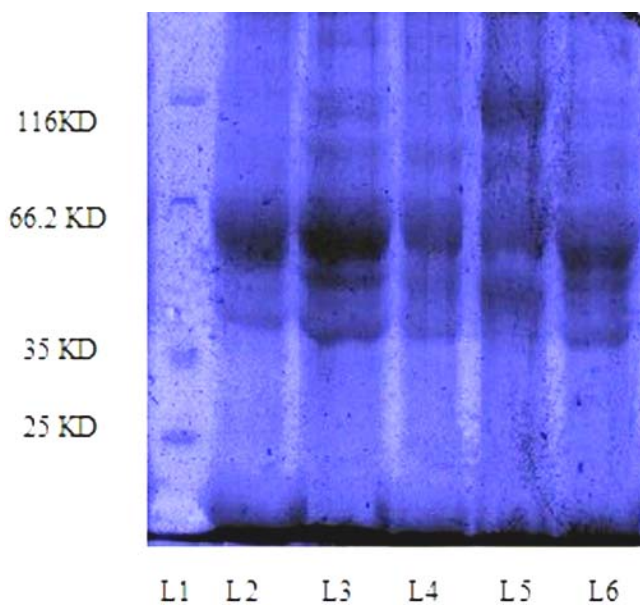


Figure 1. SDS-PAGE (10% gel) showing electrophoretic pattern of serum proteins observed in *S. curvifrons* (L2), *S. labiatus* (L3), *S. niger* (L4), *S. esocinus* (L5) and *S. plagiostomus* (L6). L1 shows the separation of marker protein.

lens oil immersion was used for taking the photographs and analyzing the chromosomes. Eighty metaphase plates were counted per species and a proper plate was selected to obtain karyotype formulae. Microsoft Excel 2010 software was used to calculate the centromeric indices and to draw ideogram. For each chromosome, centromeric index, arm ratio and total length were calculated as described by Levan et al. (1964) and the fundamental arm number was also calculated. Chromosomes were classified into metacentric, sub-metacentric, sub-telocentric and telocentric following the method of Levan et al. (1964).

RESULTS

Biochemical genetic analysis

SDS-PAGE patterns of serum proteins of five species of *Schizothorax* studied are shown in Figure 1. In the SDS-PAGE electrophoretograms, *S. niger*, *S. curvifrons*, *S. esocinus*, *S. labiatus* and *S. plagiostomus* showed 7, 5, 5, 6 and 6 bands, respectively. Molecular weights of protein bands estimated (Table 1) were as 121, 117.5, 97.5, 83, 66.2, 52.8 and 41.5 KDa in *S. niger*, 121, 97.5, 66.2, 52.8 and 45.8 KDa in *S. curvifrons*, 121, 115, 83, 66.2 and 52.8 KDa in *S. esocinus*, 121, 97.5, 83, 66.2, 52.8 and 41.5 KDa in *S. labiatus*, 120, 96, 83, 64, 52.8 and 45.8 KDa in *S. plagiostomus*.

Karyomorphological study

Schizothorax niger revealed a diploid number of 98 comprising 12 metacentric pairs, 16 sub-metacentric pairs, 11

sub-telocentric pairs and 10 telocentric pairs of chromosomes (Figure 2). *Schizothorax esocinus* showed a diploid complement of 98 comprising 15 metacentric chromosome pairs, 11 sub-metacentric pairs, 5 sub-telocentric pairs and 18 telocentric pairs (Figure 3). *Schizothorax labiatus* also revealed a diploid complement of 98 that comprised 12 metacentric pairs, 10 sub-metacentric pairs, 1 sub-telocentric pair and 26 telocentric pairs (Figure 4). *Schizothorax plagiostomus* showed a somatic complement of 96 chromosomes comprising of 12 metacentric pairs, 9 sub-metacentric pairs and 27 telocentric pairs (Figure 5). The diploid chromosomal complement of *Schizothorax curvifrons* comprised 94 chromosomes; 13 metacentric pairs, 10 sub-metacentric pairs, 10 sub-telocentric pairs and 14 telocentric pairs (Figure 6).

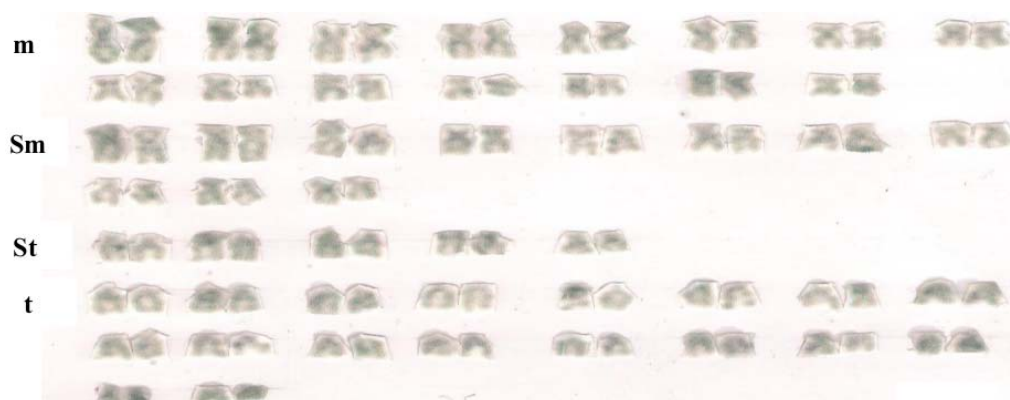
DISCUSSION

Our study on comparative analysis of serum proteins of five species of *Schizothorax* found in Kashmir valley (*S. niger*, *S. curvifrons*, *S. esocinus*, *S. labiatus* and *S. plagiostomus*) revealed that the amount of serum protein differed considerably among them. Diet might be the contributor factor to the variable amount of protein in sera (Badawi, 1971).

The present SDS-PAGE serum protein, study of five species of *Schizothorax* revealed similarities as well as differences in the number and molecular weight of protein bands among the five species studied. Protein differences

Table 1. Molecular weight (in KDa) of protein bands in electrophoretograms of five fishes.

Fraction number/band number	<i>S. niger</i>	<i>S. curvifrons</i>	<i>S. esocinus</i>	<i>S. labiatus</i>	<i>S. plagiostomus</i>
1	121	121	121	121	-
2	-	-	-	-	120
3	117.5	-	-	-	-
4	-	-	115	-	-
5	97.5	97.5	-	97.5	-
6	-	-	-	-	96
7	83	-	83	83	83
8	66.2	66.2	66.2	66.2	-
9	-	-	-	-	64
10	52.8	52.8	52.8	52.8	52.8
11	-	45.8	-	-	45.8
12	41.5	-	-	41.5	-

**Figure 2.** Karyotype of *S. niger* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).**Figure 3.** Karyotype of *S. esocinus*. (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

between species are specific for individuals representing a group. This could elucidate taxonomic controversies in the cases of disputed species (Smith et al., 1979). *S.*

esocinus and *S. curvifrons* showed same number of bands and similarity in molecular weight of certain bands (121, 66.2 and 52.8 KDa) but differed in bands 115 and

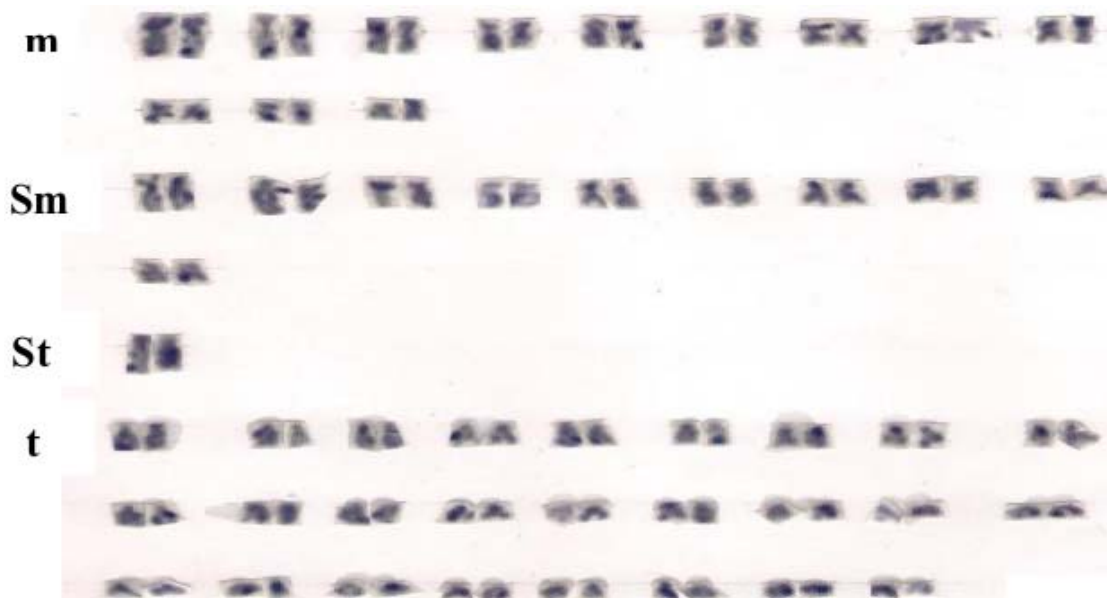


Figure 4. Karyotype of *S. labiatus* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

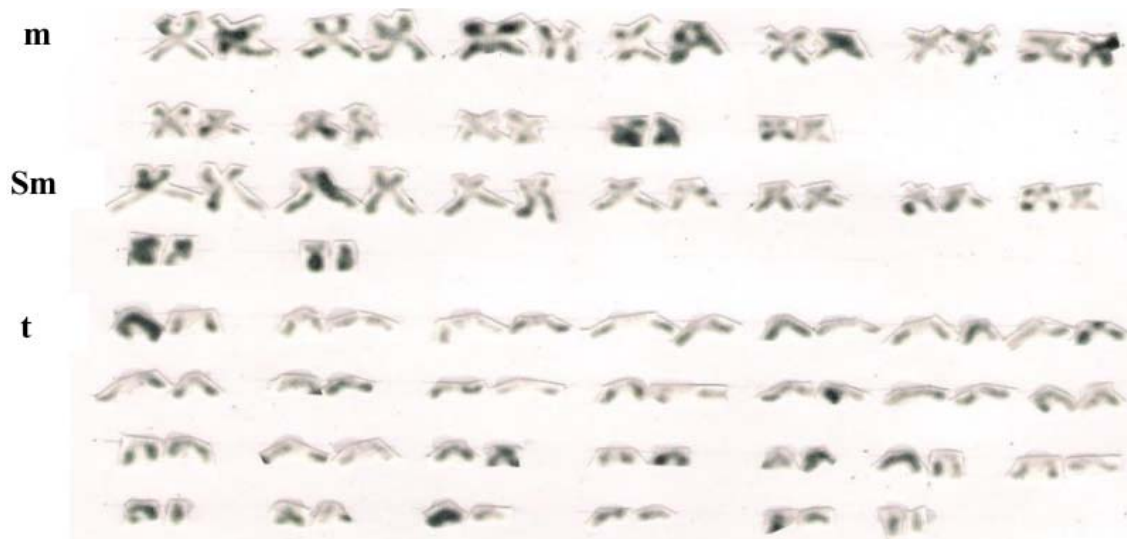


Figure 5. Karyotype of *S. plagiostomus* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

83 KDa (found in *S. esocinus* but absent in *S. curvifrons*) and bands of molecular weight 97.5 and 45.8 KDa (found in *S. curvifrons* but absent in *S. esocinus*). Closely related species not only share many electrophoretic alleles, but also differ at some gene loci at which they are fixed for different alleles (Smith et al., 1990).

S. labiatus and *S. plagiostomus* are similar in having same number of bands that is, 6 and sharing bands of molecular weight 83, and 52.8 KDa, but differed in 121, 97.5, 66.2 and 41.5 KDa (found in *S. labiatus* but absent

in *S. plagiostomus*) and bands of molecular weight 120, 96, 64 and 45.8 KDa (found in *S. plagiostomus* but absent in *S. labiatus*). If two different species have same number of electrophoretic fractions, further close comparison of percentage age relative mobility (Rf) values and corresponding molecular weight of one or more fractions could reveal species-specific differences. Species specificity of a protein should mean primarily the electrophoretic mobility differences of one or more bands (Verghese and Jayasankar, 1999). In our study, although



Figure 6. Karyotype of *S. curvifrons* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

S. curvifrons and *S. esocinus* showed same number of bands that is 5; they differed in the Rf values of individual fractions. Similarly *S. labiatus* and *S. plagiostomus* each depicted 6 bands, but they also differed in the Rf values of individual fractions or bands. Differing in their relative mobilities, they possessed different banding patterns, hence their patterns were species diagnostic. *S. plagiostomus* showed characteristic bands of molecular weight 120, 96 and 64 KDa. Band 115 KDa was found characteristic to *S. esocinus*, while *S. niger* depicted a characteristic band of molecular weight 117.5 KDa. The electrophoretic patterns, therefore, confirm their status as valid species which have undergone speciation long back that is all the five species revealed bands in the range 19.5 to 39 KDa and originated from the same genetic stock. The fractional differences have taxonomic significance at the level of population, sub-species or species.

Three out of the five species analysed in the present study namely: *S. niger*, *S. esocinus* and *S. labiatus* revealed a diploid number of 98 and a fundamental arm number (FN) of 154, 150 and 142, respectively. The variation in the fundamental arm number without change in the $2n$ may be attributed to the intra chromosomal changes involving pericentric and paraentric inversion and centromeric shifts (Rishi et al., 1998). Variation in the karyotypic configuration of *S. niger* and *S. esocinus* can be easily explained by centric fusion and fission events. It is evident from the karyotype of these two species that there has been simultaneous fusion of telocentric and fission of metacentric chromosomes in *S. esocinus* which resulted in the karyotype of *S. niger*. This is because *S. niger* is having more biarmed chromosomes than *S. esocinus* and karyotype with biarmed chromosomes are generally regarded to represent a derived condition (Ohno et al., 1968; Ohno 1970; Denton 1973; Gold 1979). Same types of chromosomal rearrangements seem to have framed the karyotype of *S. labiatus*.

Other two species namely: *S. plagiostomus* and *S. curvifrons* revealed a diploid number of 96 and 94 and FN of 138 and 140 respectively. Decrease in the $2n$ in these species may be attributed to the Robertsonian arrangements and change in FN to pericentric inversion (Choudhury et al., 1982).

Highest number of biarmed chromosomes was found in *S. niger* followed by *S. esocinus*, *S. curvifrons*, *S. plagiostomus* and *S. labiatus* whereas highest number of sub-telocentrics were found in the order of *S. plagiostomus* and *S. labiatus* (equal), *S. curvifrons*, *S. esocinus* and *S. niger*. The primitive teleost karyotype is thought to have consisted of 46-48 acrocentrics (Nayyar, 1966; Ohno et al., 1968; Ohno 1970; Fitzsimons, 1972; Legrande, 1975) and the karyotypes with biarmed chromosomes are regarded to represent a derived condition (Ohno et al., 1968; Ohno, 1970; Denton, 1973; Gold, 1979). Keeping this into consideration, *S. plagiostomus* and *S. labiatus* seem to be primitive fishes when compared to *S. niger*, *S. esocinus* and *S. curvifrons* which possess a more derived karyotype. The trend of gradual increase in the FN from 138 in *S. plagiostomus* to 154 in *S. niger* supports the above assertion.

The overall dissimilarity in the $2n$, karyotypic configuration and FN points to the role of almost all types of chromosomal rearrangements in the karyological evolution of Schizothoracinae, however deviation in the chromosome number can be possibly dislocation hypothesis of evolution of chromosomes according to Navashin's (1932).

This hypothesis explains that each chromosome is monocentric and an evolutionary change in the chromosome number must involve duplication of centromere together with a region within it while a decrease in the number must mean a permanent loss from the karyotype of the region containing centromere. Family Cyprinidae points to the role of almost all types of chromo-

somal rearrangements for their karyological evolution (Rishi et al., 1998).

The present study conclusively confirms the specific status of the five species of *Schizothorax* on the basis of their serum proteins and genetic material. The study negates the proposition of Silas (1960) regarding the taxonomic status of *Schizothorax niger* and *Schizothorax curvifrons*, who had combined these two species into a single species *Schizothorax niger* treating *curvifrons* and *niger* as two varieties or sub-species. The present study has clearly shown that these two species of fish be treated as distinct species and not varieties or sub-species of the same species. The present study has also cleared the taxonomic position of *Schizothorax plagiostomus* side by side *S. richardsonii* for a long time; the two species were regarded as synonyms. A comparison of the two species clearly indicates that the two species are distinct from each other as proposed by Kullander et al. (1999).

Coupled biochemical genetic and karyomorphological analyses constitute a good taxonomical tool that the classifications should be based on. In fact genetic changes proceed morphological ones (Howell and Villa 1976), which also render support to this notion and morphological based classifications, should be re-evaluated using these methods.

Conflict of interests

The author(s) have not declared any conflict of interests.

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