A Ploidy Difference Represents an Impassable Barrier for Hybridisation in Animals. Is There an Exception among Botiid Loaches (Teleostei: Botiidae)?

Jörg Bohlen*, Vendula Šlechtová, Vlastimil Šlecht, Vera Šlechtová, Alexandr Sember, Petr Ráb

Institute of Animal Physiology and Genetics AS CR, v.v.i., Rumburská 89, 277 21 Liběchov, Czech Republic

* bohlen@iapg.cas.cz

Abstract

One of the most efficient mechanisms to keep animal lineages separate is a difference in ploidy level (number of whole genome copies), since hybrid offspring from parents with different ploidy level are functionally sterile. In the freshwater fish family Botiidae, ploidy difference has been held responsible for the separation of its two subfamilies, the evolutionary tetraploid Botiinae and the diploid Leptobotiinae. Diploid and tetraploid species coexist in the upper Yangtze, the Pearl River and the Red River basins in China. Interestingly, the species 'Botia' zebra from the Pearl River basin combines a number of morphological characters that otherwise are found in the diploid genus Leptobotia with morphological characters of the tetraploid genus Sinibotia, therefore the aim of the present study is to test weather 'B.' zebra is the result of a hybridisation event between species from different subfamilies with different ploidy level. A closer morphological examination indeed demonstrates a high similarity of 'B.' zebra to two co-occurring species, the diploid Leptobotia guilinensis and the tetraploid Sinibotia pulchra. These two species thus could have been the potential parental species in case of a hybrid origin of 'B.' zebra. The morphologic analysis further reveals that 'B.' zebra bears even the diagnostic characters of the genera Leptobotia (Leptobotiinae) and Sinibotia (Botiinae). In contrast, a comparison of six allozyme loci between 'B.' zebra, L. guilinensis and S. pulchra showed only similarities between 'B.' zebra and S. pulchra, not between 'B.' zebra and L. guilinensis. Six specimens of 'B.' zebra that were cytogenetically analysed were tetraploid with 4n = 100. The composition of the karyotype (18% metacentric, 18% submetacentric, 36% subtelocentric and 28% acrocentric chromosomes) differs from those of L. guilinensis (12%, 24%, 20% and 44%) and S. pulchra (20%, 26%, 28% and 26%), and cannot be obtained by any combination of genomes from L. guilinensis and S. pulchra. Phylogenetic reconstructions based on sequence data of the mitochondrial cytochrome b gene and the nuclear RAG-1 gene invariably places 'Botia' zebra as sister species to S. pulchra, while L. guilinensis is only distantly related. The presented combination of genetic data demonstrates that 'B.' zebra is not the result of a hybridisation, but a species of tetraploid genus Sinibotia with a striking morphological evolution towards
an enormous similarity with a co-occurring, but not directly related species. The complete lack of knowledge of the ecology of these species, their main predators or their ecological interactions hampers any conclusion regarding the evolutionary advantage of such adaptation.

Introduction

One of the most efficient barriers for horizontal gene flow between vertebrate animals is a difference in ploidy level [1,2]. While it might not prevent an original hybridisation event and in many cases not the viability of the F1-offspring, it generally terminates the reproduction line of such hybrids by sterility of the offspring [3,4,5]. In some exceptional cases, the resulting hybrids can make it through with clonal and/or asexual reproduction [1], but due to the absence of gene flow and recombination, offspring of such lineages resemble F1 hybrid individuals and such lineages often are not long lasting. This general rule has been observed in plants as well as in animals, and exceptional cases are very rare, especially among animals. Therefore, any evolutionary successful case of a hybridisation between parental species that differ in ploidy level would provide an interesting model to study the limits of polyploidy as barrier for horizontal gene flow.

Freshwater fishes of the family Botiidae (Cobitoidea: Cypriniformes) are widespread across East, Southeast and South Asia [6,7]. Many species are valued as ornamental fishes worldwide and as tasty food fishes in the area of occurrence. The monophyly of the family has been demonstrated by morphological as well as genetic data [7–11]; and phylogenetic reconstructions of the family revealed two major, long-time separated lineages, which are referred to as subfamilies Leptobotiinae and Botiinae [11,12]. The most remarkable difference between the two subfamilies comes from cytogenetics: all studied Leptobotiinae are diploid with a chromosome number of 2n = 50, while all Botiinae are tetraploid with 4n = 98–100 [12,13]. It has been hypothesised [12] that the difference in ploidy level has played an important role in the separation of the two lineages, since it represents an efficient barrier for hybridisation between the lineages. Both subfamilies have a similar number of species, which was used to claim that there is no obvious difference in the evolutionary success of diploid or tetraploid animals [14]. Leptobotiinae occur in the northern half of the total distribution area (north of the Mekong basin—China, Japan, eastern Russia, northern Vietnam), while most Botiinae live in the southern half of the total distribution area (Mekong and areas south and west of Mekong—from Pakistan to Laos, Malay Peninsula, Indonesia) [7]. However, Leptobotiinae and Botiinae co-occur in the upper Yangtze, the Pearl and the Red River basins, where the genus Sinibotia (belonging to Botiinae) is distributed with five recognised species in the area that otherwise is inhabited by Leptobotiinae (a sixth species of Sinibotia in the upper Mekong lives outside the range of Leptobotiinae) [7,15].

At least seven species of the genera Leptobotia, Parabotia and Sinibotia occur in the River Li, a northern tributary of the River Xi, Pearl River basin, in southern China [16,17], with two of them being endemic to this river: Leptobotia guilinensis Chen, 1980 and 'Botia' zebra Wu, 1939. Since the latter is bearing the diagnostic character of the genus Leptobotia, a simple suborbital spine (versus bifid in all other genera of Botiidae), and generally shows a close similarity to the sympatric Leptobotia guilinensis, 'Botia' zebra was placed into Leptobotia [16]. However, in a phylogenetic analysis basing on the mitochondrial cytochrome b gene, 'B'. zebra was found to be more closely related to the genus Sinibotia, especially to a species that occurs in the River
Li, S. pulchra [18]. One of the possible explanations for a strong discrepancy between morphological and mitochondrial characters, respectively, is mitochondrial introgression, a process where an initial hybridisation is followed by repeated back-crossing events with the paternal species; leading to a morphology that is closer to the paternal species, but a mitochondrial genome that is close to the maternal species. In 'Botia' zebra the morphology is similar to Lepobotia guilinensis, but the mitochondrial genome close to Sinibotia pulchra and all three species co-occur in the upper River Li (Fig 1). We therefore hypothesise that the evolutionary history of 'Botia' zebra included a hybridisation event between L. guilinensis and S. pulchra and test this hypothesis in the present study. Such a hybridisation event between a diploid and a tetraploid species would refute the general assumption that differences in ploidy level represent an efficient barrier against hybridisation and would be of general interest for evolutionary biology.

In the present study, we compare L. guilinensis, S. pulchra and 'Botia' zebra using morphologic, cytogenetic, allozyme variability as well as mitochondrial and nuclear DNA sequence characters to test if the later reveals any trace of a past hybridisation between the first two species.

Material & Methods
Specimens
Live individuals of L. guilinensis (eight individuals), S. pulchra (two individuals) and S. zebra (six individuals) were obtained together in one mixed group from an import for the ornamental fish trade (Aquarium Glaser, 63110 Rodgau, Germany). Live fishes were kept in the fish housing facilities in Institute of Animal Physiology and Genetics, 277 21 Liběchov, Czech Republic in 60 l tanks with flow-through water of 22°C at a light;dark cycle of 10:14 h. Fishes were fed ad libitum with life Tubifex worms. Ethanol or formalin fixed specimens were obtained from local food markets in Guilin (25°16′N, 110°17′E), Mengshan (24°12′N, 110°31′E), Fuzhou (26°04′N, 119°18′E) and Nanning (22°48′N, 108°21′E) in the provinces Guangxi and Fuxien in southern China. A total number of 108 individuals of the three species from 9 localities across the whole distribution area of the species has been analysed (Table 1). Additional 33 specimens of 26 other species as comparative material were obtained from the ornamental fish trade (AquaGlobal, 16356 Werneuchen, Germany). Vouchers are deposited in the collection of the Laboratory of Fish Genetics, IAPG AS CR, Liběchov. All experimental procedures involving fishes during this study were approved by the Institutional Animal Care and Use Committee of the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic, according with directives from the State Veterinary Administration of the Czech Republic, permit number 155/2012, and by permit number CZ 02386 from the Ministry of Agriculture of the Czech Republic.

Morphology
Morphological measurements were taken from 26 specimens of L. guilinensis, S. pulchra and S. zebra with digital callipers point-to-point according [19]. Important morphologic characters and pigmentation were estimated from nearly all specimens either directly in the case of fixed specimens or from photos taken of live specimens. Preparations of suborbital spines of nine specimens were carried out under an Olympus SZX7 stereomicroscope equipped with a u-Eye camera.

Chromosome analysis
Mitotic chromosomes were obtained from regenerated fin tissue as described by [20,21] with slight modifications. Briefly, the posterior margin of the caudal fin was cut off and three weeks...
later, the regenerated tissue of the fin was collected to be incubated in Ringer solution with 0.025% colchicine for about two hours at room temperature. Cells were fixed in a mixture of methanol and acetic acid (3:1) at 4°C for 25 min. This step was repeated three times. The fixed tissue was minced in 50% acetic acid and drops of the resulting suspension were placed on pre-heated slides (50°C) and sucked back after 20 sec. The slide was dried at room temperature and stained for 10 min in 5% Giemsa solution (pH 6.8) (Merck, Darmstadt, Germany) before examination of metaphase plates with an Olympus AX70 light microscope. From 17 live individuals that were available for the analyses, results with satisfying quality were obtained from nine individuals (five *L. guilinensis*, two *S. pulchra*, two 'B.' *zebra*). The number of chromosomes of at least 20 metaphase plates per individual was counted. Chromosome morphology was classified as m—metacentric, sm—submetacentric, st—subtelocentric, a—acrocentric [22].

**Allozyme analysis**

Fin tissue was homogenised with an equal amount of buffer (0.1 mol/l Tris-HCl pH 8.5) and centrifuged for clarifying. All manipulations with tissue were carried out on ice. Electrophoresis on starch gel was carried out in a refrigerator. Six allozyme loci (glucosephosphate isomerase Gpi-A, aspartate amino transferase s-Aat, malate dehydrogenase s-Mdh A, lactate dehydrogenase Ldh A and Ldh B, phosphoglucomutase Pgm) were stained [23,24]. Altogether 18
individuals were studied. Loci Gpi-A and Pgm were analysed in three and two buffer systems, respectively (F [25], MC2 [26], V [27]).

DNA sequence analyses

Genomic DNA was isolated from fin tissue samples using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. The mitochondrial cytochrome \( b \) (cyt \( b \)) was amplified and sequenced using the primers Glu-L.Ca1337-14359: 5' - GAA GAA CCA CCG TTG TTC AA- 3' and Thr-H.Ca15568-15548: 5' - ACC TCC RAT CTY CGG ATT ACA - 3' [12]. An approximately 970 bp long portion of RAG-1 was amplified using the primers RAG-1F (5' - AGCTGTAGTCAGTAYCACAARATG-3' [28]) and RAG-RV1 (TCCTGRAAGATTTGTTAGA-3' [10]) or RAG-8R (5' - CGC CAC ACA GGY TTC ATC T-3' [28]). Same primers were used also for sequencing reactions. PCR amplifications were performed in 25 \( \mu \)l reaction volumes of 10 mM Tris-HCl, 50 mM \((\text{NH}_4)_2\)SO\(_4\), 0.1% of Triton X-100, 1.5 mM Mg\(_2\)Cl\(_2\), 2 mM TMA oxalate (PCR enhancer), containing 5 nmol of each nucleotide, 1.25 U of Taq polymerase (all chemicals Top-Bio, Prague, Czech Republic) and 12.5 pmol of each primer.

The PCR reaction profile (M) Research thermocycler) included 5 min of initial denaturation at 95°C, touch-down profile of 1 min at 94°C, 1 min 30 s at 60–55°C (1°C/cycle) and 2 min at 72°C, followed by 30 cycles with annealing temperature held at 54°C. The reaction was completed by final extension at 72°C for 7 min.

PCR products were purified by QIAquick PCR Purification Kit (Qiagen). Forward and reverse sequencing reactions were performed with BigDye™ Terminator Cycle Sequencing Kit.
Hybridisation between Diploid and Tetraploid Fishes?

Results

Morphology

Morphometry. - In 13 out of 33 morphometric and meristic characters there was no overlap of measurements between *L. guilinensis* and *S. pulchra* (Category A, Table 2), while in further 12 characters, the overlap was small (Category B). In the remaining eight characters the overlap was large (Category C); therefore these characters were unsuited to evaluate a morphological similarity between ‘*B.’ zebra* and the two potential parental species. However, in one of these ‘uninformative’ characters in Category C (Number of branched dorsal-fin rays), seven out of eight specimens of ‘*B.’ zebra’ showed a character state that was observed in neither *L. guilinensis* nor *S. pulchra*, indicating an autapomorphy of ‘*B.’ zebra’. When comparing ‘*B.’ zebra’ with *L. guilinensis* and *S. pulchra*, it shared the range of measurements with *S. pulchra* in two characters of Category A and in two characters of Category B, with *L. guilinensis* in six characters of Category A and in eight characters of Category B, while its range was intermediate between the two species in five characters of Category A and in two characters of Category B.

Pigmentation pattern. - The pigmentation pattern of *S. pulchra* is much like that of all other members of the genus *Sinibotia*: Broad dark brown bars run from one body side across the back to the other side, reaching nearly always below lateral midline and regularly to level of pelvic fin origin (Fig 2). In small and medium sized individuals 6–10 bars are present, much broader than interspaces, but in larger individuals each bar might split into two. On the dorsal side of the head run two dark stripes from the snout to the neck and one on each side of the head from the snout through the eye. Between the dark stripes are two prominent light stripes, a long one from the snout to the end of the operculum and a short along the dorsal midline of the head. In *Leptobotia guilinensis*, body and head are homogenously light to dark brown with a lighter belly. Prominent light blotches are present along the dorsal midline, but often only visible behind the base of the dorsal fin. Dark saddles are sometimes visible between the light
blotches, but usually too faint to figure out the precise number and outline. A thin black stripe runs from the snout to the eye, but no light stripes are present. In ‘B. zebra’ the body sides are uniformly brown like in L. guilinensis, but usually in lighter brown. On the back, faint saddles are sometimes visible, often hard to see, never reaching to lateral midline. In some specimens the saddles are present only in the anterior part of the body, but if present along whole back their number is higher than ten. Between the saddles are light blotches, very similar to the light

Table 2. Morphometric comparison of Leptobotia guilinensis, Sinibotia pulchra and ‘Botia’ zebra.

|                         | Leptobotia guilinensis | ‘Botia’ zebra | Sinibotia pulchra | Comparison        |
|-------------------------|------------------------|---------------|-------------------|-------------------|
| **A. Characters without overlap between Leptobotia guilinensis and Sinibotia pulchra** |                        |               |                   |                   |
| Pre-pelvic length       | 51–54                  | 56–59         | 55–59             | zebra = pulchra   |
| Preanal length          | 74–77                  | 78–80         | 78–80             | zebra = pulchra   |
| Dorsal head length      | 16–20                  | 20–22         | 21–24             | zebra intermediate|
| Snout length            | 6–8                    | 10–11         | 12–14             | zebra intermediate|
| Pre-anus length         | 63–71                  | 70–73         | 73–76             | zebra intermediate|
| Lateral head length     | 21–24                  | 24–26         | 27–30             | zebra intermediate|
| Head depth at eye       | 8–9                    | 10–11         | 11–12             | zebra intermediate|
| Head depth at nape      | 11–13                  | 12–14         | 15–16             | zebra = guilinensis|
| Maximum body depth      | 11–16                  | 13–17         | 19–23             | zebra = guilinensis|
| Body depth at dorsal origin | 11–17               | 13–17         | 18–23             | zebra = guilinensis|
| Maximum head width      | 8–10                   | 8–10          | 11–13             | zebra = guilinensis|
| Head width at nares     | 4–5                    | 4–6           | 6–9               | zebra = guilinensis|
| Body width at anal origin| 4–7                   | 5–6           | 8–10              | zebra = guilinensis|
| **B. Characters with slight overlap between Leptobotia guilinensis and Sinibotia pulchra** |                        |               |                   |                   |
| Predorsal length        | 49–58                  | 55–60         | 55–62             | zebra = pulchra   |
| Number of pectoral-fin rays | 11–13               | 13–15         | 13–15             | zebra = pulchra   |
| Interorbital width      | 3–4                    | 4–4           | 4–6               | zebra intermediate|
| Length of caudal peduncle | 14–18              | 13–16         | 12–14             | zebra intermediate|
| Length of upper caudal lobe | 16–21             | 18–20         | 20–25             | zebra = guilinensis|
| Length of pectoral fin  | 11–14                  | 12–14         | 14–19             | zebra = guilinensis|
| Length of lower caudal lobe | 18–21             | 18–21         | 20–26             | zebra = guilinensis|
| Body width at dorsal origin | 6–10                | 6–9           | 10–15             | zebra = guilinensis|
| Depth of caudal peduncle | 10–13                 | 10–13         | 13–14             | zebra = guilinensis|
| Length of pelvic fin    | 10–12                  | 10–11         | 12–15             | zebra = guilinensis|
| Length median caudal rays | 7–10                | 7–9           | 9–15              | zebra = guilinensis|
| Total length            | 116–121                | 116–120       | 119–127           | zebra = guilinensis|
| **C. Characters with broad overlap between Leptobotia guilinensis and Sinibotia pulchra** |                        |               |                   |                   |
| Eye diameter            | 2–3                    | 2–3           | 3–3               |                   |
| Depth of anal fin       | 13–15                  | 12–15         | 14–17             |                   |
| Postorbital length      | 12–14                  | 13–14         | 13–15             |                   |
| Height of dorsal fin    | 11–16                  | 11–14         | 12–18             |                   |
| Branched dorsal-fin rays | 8 ½                 | 7(8) ½       | 8 ½               | zebra speciality  |
| Branched caudal-fin rays | 9+8                 | 9+8           | 9+8               |                   |
| Branched anal-fin rays  | 5                      | 5             | 5                 |                   |
| Number of pelvic-fin rays | 8                    | 8             | 8                 |                   |

Values give range as % of standard length. Under ‘Comparison’ is indicated if ‘Botia’ zebra has values like one of the potential parental species or if it is intermediate.

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blotches in *L. guilinensis*, often merging into a line in the anteriormost part of the dorsum. On the head are dark stripes from the snout to the neck and from the snout through the eye two and prominent light stripes between them as described for *S. pulchra*. In general, *'B.' zebra* combines the head pigmentation of *S. pulchra* with the body pigmentation of *L. guilinensis*

**Suborbital spine.** – The suborbital spine is an erectable spine formed by the lateral ethmoid bone and located in a skin pocket below each eye. It is present in all members of the family Botiidae as well as in the distantly related families Cobitidae and Serpenticobitidae and its shape is of taxonomical value. In all species of *Leptobotia*, including *L. guilinensis*, this spine is simple, meaning it has a single branch and tip [7]. In all other Botiidae, including *S. pulchra*, the spine is double, meaning it has a main and a side branch and two tips [7]. In *'B.' zebra*, the spine turned out also to be simple, like in *Leptobotia* (Fig 3).

**Mental lobes.** – In many species of Botiidae the lower lip develops two median extensions, called mental lobes, and presence and shape of these extensions are important taxonomic characters [7]. In all species of *Sinibotia* the extensions are present, large and of oval or kidney-like shape [7,15]. This shape of the mental lobes is considered a diagnostic character for the genus *Sinibotia* [7]. In all analysed specimens of *S. pulchra* the mental lobes were present, large and had the shape characteristic for *Sinibotia*, while in all analysed specimens of *L. guilinensis* no mental lobes were present. In all analysed specimens of *'B.' zebra* mental lobes were present, large and had the shape characteristic for *Sinibotia* (Fig 4).

The literature names additional characters to distinguish between *Leptobotia* and *Sinibotia*, namely the presence of scales on the cheeks and of a pario-frontal fontanelle in *Leptobotia* (vs. both characters absent in *Sinibotia*) [7,33,34]. Since both turned out to be absent in five
dissected specimens of *L. guilinensis*, these characters are not truly diagnostic and were unsuited for the comparison in the given case.

**Chromosome analysis**

Metaphases of suited quality for further analyses were obtained from five individuals of *L. guilinensis*, two *’B. zebra* and two *S. pulchra*. The diploid chromosome number of all analysed *L. guilinensis* was 2n = 50, proving the diploid status of these individuals, while all individuals of *’B. zebra* and *S. pulchra* were tetraploid with a chromosome number of 4n = 100 (Table 3, Fig 5). Karyotypes of all analysed species were composed of comparatively small chromosomes, slightly decreasing in size.
Especially in the tetraploid species chromosomes were generally very small, with their centromere positions gradually ranging from median to nearly terminal making the borderlines between formal chromosomal categories questionable in a small subset of chromosomal pairs.

**Allozyme analysis**

In three (s-Aat, Ldh A, Ldh B) of the six analysed loci alleles were shared between the three analysed taxa and therefore were not informative for the given study. In all informative loci (Gpi-A, s-Mdh A, Pgm), *S. pulchra* shared alleles with *’Botia’ zebra*, but both did not share alleles with *L. guilinensis* (Table 4). Therefore the allozyme data suggest a high similarity between *S. pulchra* and *’Botia’ zebra*, while *L. guilinensis* appears to be more distantly related.

**DNA sequence analysis**

Table 3 summarises the species and individuals analysed in this study including the novel sequences generated as well as those that were obtained from GenBank.

Altogether we have analysed 102 specimens of Botiidae including 49 and 59 novel sequences of cyt b (1121 bp) and RAG1 (971 bp), respectively. Into the cytochrome b dataset, 14 sequences of *L. guilinensis*, 19 sequences of *S. pulchra* and 12 sequences of *’Botia’ zebra* were included; in the RAG dataset it were 14, 10 and six sequences, respectively.

The models selected for each partition (codon position) based on BIC score were following: TN93+G+I, HKY+G and GTR+G for the 1st, 2nd and 3rd codon positions of cyt b, respectively, and HKY+G, JC+I and JC for the 1st, 2nd and 3rd codon positions of RAG 1, respectively. Those were taken into account for the subsequent Bayesian analyses.

Phylogenetic reconstructions of both analysed genes provide generally congruent genealogies: the major split within Botiidae separated the diploid subfamily Leptobotiinae from the tetraploid subfamily Botiinae (Figs 6 and 7). Both datasets identified all described genera as monophyletic lineages with high statistic support except *Leptobotia* in the RAG dataset. These observations are well in agreement with former observations [11,12]. In general, the slower evolving RAG gene brought a better resolution at the older genealogic events, while the faster evolving cytochrome b gene had a better resolution around the tips of the trees, which is a well-known characteristics of these two genes. No discrepancies that would indicate a potential hybridisation event were detected.

All specimens of *’Botia’ zebra*, *L. guilinensis* and *S. pulchra* form own monophyletic groups, confirming that the three species are unambiguously identifiable by these markers. The lineages of *’Botia’ zebra* and *S. pulchra* show a sister relationship and together are embedded into the comparative material of Sinibotia, while the lineage of *L. guilinensis* is closely related to all comparative samples of *Leptobotia*, but only distantly related to the lineages formed by *’Botia’ zebra* and *S. pulchra*.

Table 3. Chromosome numbers and karyotype composition of *Leptobotia guilinensis*, *Sinibotia pulchra* and *’Botia’ zebra*.

|                          | n | m | sm | st | a |
|--------------------------|---|---|----|----|---|
| *Leptobotia guilinensis* | 50 | 6 | 12 | 10 | 22 |
| *Sinibotia pulchra*      | 100 | 20 | 26 | 28 | 26 |
| *’Botia’ zebra*          | 100 | 18 | 18 | 36 | 28 |

Chromosomal characteristics of *Sinibotia pulchra*, *’Botia’ zebra* and *Leptobotia guilinensis* from the upper Li River (Pearl River basin) including diploid chromosome number (2n) and karyotype description.

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Discussion

Our results demonstrate that 'B.' zebras show a high morphological similarity to *L. guilinensis*, but also shares characters with *S. pulchra* and in some characters presents an intermediate
Table 4. Allozymes of Leptobotia guilinensis, Sinibotia pulchra and 'B.' zebra.

| Species                  | n   | Gpi-A | Gpi A | Gpi A | s-Aat | s-Mdh A | Ldh-A | Ldh-B | Pgm | Pgm |
|--------------------------|-----|-------|-------|-------|-------|---------|-------|-------|-----|-----|
| Buffer                   |     | V     | MC 2  | F     | MC 2  | MC 2    | V     | V     | F   |     |
| Sinibotia pulchra        | 4   | 055   | 037   | 039   | 096   | A, C    | 030, 136 | 053, 067 | 083 | 080 |
| 'Botia' zebra            | 6   | 055   | 037   | 039   | 096   | A       | 030   | 053, 067 | 083 | 080 |
| Leptobotia guilinensis   | 8   | 065, 070, 083, 099 | 050, 064, 078, 095 | 060, 078, 096 | 084, 096 | B | 136 | 053, 067 | 107 | 100 |

Presence of six allozymes in Sinibotia pulchra, 'Botia' zebra and Leptobotia guilinensis.

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morphotype. Moreover, except having 7½ - 8½ branched dorsal-fin rays (versus 8½ in to L. guilinensis and S. pulchra) it reveals only characters that are also found in either of the two species. The prevalence of synapomorphies with either one of the two other species or an intermediate character state strongly supports the hypothesis of a hybrid origin of 'B.' zebra. Most important in this respect are the diagnostic characters: the diagnostic character for the genus Leptobotia is the simple suborbital spine. In 'B.' zebra, the spine also is simple; therefore it bears the diagnostic character of the genus Leptobotia (Fig 3). Consequently, 'B.' zebra was placed into Leptobotia [7,16,35,36]. The diagnostic character of the genus Sinibotia is the presence of a pair of mental lobes in a button-like; and 'B.' zebra bears these buttons, meaning it also carries the diagnostic character of the genus Sinibotia (Fig 4). This result offers two potential explanations: either 'B.' zebra is of hybrid origin or the described characters are not diagnostic. As mentioned above, the pigmentation pattern of 'B.' zebra includes the head pigmentation of Sinibotia and the body pigmentation of L. guilinensis, further strengthening the assumption of a hybrid origin (Fig 2). Therefore all morphological data suggest that 'B.' zebra is a mixture of these two species; that means the product of a hybridisation. As stated above, the different ploidy level of the diploid L. guilinensis and the tetraploid S. pulchra should represent an efficient barrier against any horizontal gene flow between these two lineages.

However, a first hybridisation step would be possible, but potentially formed F1 hybrids would be excluded from further reproduction. In order to test the F1 hybrid status of 'B.' zebra, their ploidy level was investigated. All six analysed individuals of 'B.' zebra were tetraploid with a chromosome number of 4n = 100. Consequently, these individuals were no F1 hybrids, inducing strong doubts against the postulate of the efficiency of ploidy level differences as barrier against gene flow. Moreover, the composition of the karyotype of 'B.' zebra turned out to be very similar to that of S. pulchra, but did not reveal any trace of introduction of one or two chromosome sets of L. guilinensis into its karyotype. Due to the high number of uni-armed chromosomes the number of uni-armed chromosomes in L. guilinensis the number of uni-armed chromosomes in 'B.' zebra would have elevated considerably in comparison to S. pulchra. Nevertheless, the number of uniarmed chromosomes is slightly increased in 'B.' zebra when compared to S. pulchra. Theoretically, this could be the result of a number of back-crossings of the original hybrid with S. pulchra that brought the karyotype composition of 'B.' zebra closer to that of S. pulchra, while some chromosomes of L. guilinensis are still present, but not distinguishable from Sinibotia chromosomes with the given Giemsa staining technique. In such case, comparisons of proteins and molecular genetic markers could still reveal a genetic introgression by L. guilinensis.

However, the allozyme comparison did not reveal any sign of L. guilinensis genome, but all analysed specimens of 'B.' zebra were in all of the informative proteins undistinguishable from S. pulchra. These results provide evidence that no genetic introgression by L. guilinensis has occurred.

Finally, both phylogenetic reconstructions, one on base of a mitochondrial gene and the other on base of a nuclear gene, suggested with high statistical support that 'B.' zebra is the sister lineage to S. pulchra, while all specimens of Leptobotia were only distantly related.
| Species              | ID    | Cyt b     | RAG     |
|---------------------|-------|-----------|---------|
| *Ambastaia nigrolineata* | A0031 | AY887845  | EF056329 |
| *Ambastaia sidthimunki*  | A0183 | AY887842  | KU517025 |
|                      | KP319024 |         |         |
| *Botia dario*         | A7553 | KU517084  | KU517026 |
|                      | EU409614 |       | EU409614 |
| *Botia histrionica*   | A0041 | AY887794  | KU517027 |
| *Botia lohachata*     | A0426 | KUS17085  | KUS17028 |
| *Botia striata*       | A0011 | AY887783  | KUS17029 |
|                      | EU711109 |      | EU711109 |
| *Chromobotia macracanthus* | A0178 | AY887840  | KUS17030 |
|                      | A0179 | AY887841  | KUS17031 |
|                      | EU711137 |       | EU711137 |
| *Leptobotia elongata* | A0214 | AY887779  | KUS17032 |
|                      | A8392 | KUS17086  | KUS17033 |
|                      | JN177196 |    | JN177196 |
| *Leptobotia guilinensis* | A0124 | AY887780  | KUS17034 |
|                      | A0205 | AY887781  | KUS17035 |
|                      | A1799 | KUS17087  | KUS17036 |
|                      | A5267a, k | KUS17089  | KUS17038 |
|                      | A5268 a  | KUS17090  | KUS17039 |
|                      | A5269 a, k | KUS17091  | KUS17040 |
|                      | A5270 a, k | KUS17092  | KUS17041 |
|                      | A5271 a  | KUS17093  | KUS17042 |
|                      | A5273 a, k | KUS17094  | KUS17043 |
|                      | A5277 a, k | KUS17095  | KUS17044 |
|                      | A5279 a  | KUS17096  | KUS17045 |
|                      | A8569    | KUS17097  | KUS17046 |
|                      | A8570    | KUS17098  | KUS17047 |
| *Leptobotia microphthalmia* | A5283 | KUS17099  | KUS17048 |
|                      | A5285    | KUS17100  | KUS17049 |
| *Leptobotia pellegrini* | A1459 | KUS17101  | KUS17050 |
|                      | A1813    | KUS17102  | KUS17051 |
|                      | EU292683 |        | EU292683 |
| *Leptobotia taeniops* | A8544    | KUS17103  | KUS17052 |
|                      | A8545    | KUS17104  | KUS17053 |
|                      | JN177193 |        | JN177193 |
|                      | JN177194 |        | JN177194 |
| *Parabotia banarescui* | A0217    | AY887782  | KUS17054 |
| *Parabotia bimaculata* | JN177197 |        | JN177197 |
| *Parabotia fasciata*  | A8391    | KUS17105  | KUS17055 |
| *Parabotia lijiangensis* | JN177199 |         | JN177199 |
| *Parabotia mantschuricus* | EU711138 |       | EU711138 |
| *Sinibotia pulchra*   | A0015    | AY887800  |         |
|                      | A0016    | AY887801  |         |
|                      | A0121    | AY887802  |         |

(Continued)
### Table 5. (Continued)

| Species          | ID     | Cyt b     | RAG      |
|------------------|--------|-----------|----------|
|                  | A0396  | AY887803  | KU51705  |
|                  | A0397 a| AY887804  | -----    |
|                  | A1782  | KU517106  | -----    |
|                  | A1783  | KU517107  | KU517057 |
|                  | A1785  | KU517109  | KU517058 |
|                  | A1786  | KU517110  | KU517059 |
|                  | A1787  | KU517111  | KU517060 |
|                  | A3681 a| -----     | -----    |
|                  | A3682 a, k| -----     | -----    |
|                  | A5287 a, k| KU517112  | KU517061 |
|                  | A8397  | KU517113  | KU517062 |
|                  | A8398  | KU517114  | KU517063 |
|                  | A8615  | KU517115  | KU517064 |
|                  | A8616  | KU517116  | KU517065 |
|                  | A0243  | KU517117  | -----    |
|                  | AY625705| AY625705  | -----    |
|                  | AY625706| AY625706  | -----    |
|                  | EU282332| EU282332  | -----    |
| Sinibotia robusta| A0024  | AY887805  | EF056333 |
|                  | A2226  | KU517118  | KU517066 |
|                  | A2227  | KU517119  | KU517067 |
|                  | A2228  | -----     | KU517068 |
|                  | A5852  | KU517120  | KU517069 |
|                  | A5853  | KU517121  | KU517070 |
|                  | A0242  | KU517122  | -----    |
|                  | JN177191| -----     | JN177191 |
|                  | AY625707| AY625707  | -----    |
|                  | AY625708| AY625708  | -----    |
|                  | DQ105208| DQ105208  | -----    |
| Sinibotia supercillaris| JN177190| -----     | JN177190 |
|                  | AY625702| AY625702  | -----    |
|                  | AY625703| AY625703  | -----    |
|                  | AY625704| AY625704  | -----    |
| Sinibotia zebra   | A5272 a, k| KU517123  | KU517071 |
|                  | A5274 a| KU517124  | -----    |
|                  | A5275 a, k| KU517125  | KU517072 |
|                  | A5276 a| KU517126  | KU517073 |
|                  | A5278 a| KU517127  | KU517074 |
|                  | A5280 a| KU517128  | KU517075 |
|                  | A8614  | KU517129  | KU517076 |
|                  | DQ105206| DQ105206  | -----    |
|                  | DQ105207| DQ105207  | -----    |
|                  | EU282333| EU282333  | -----    |
|                  | EU282334| EU282334  | -----    |
|                  | EU282335| EU282335  | -----    |
| Syncrossus beauforti| A0059  | AY887816  | KU517077 |
|                  | FJ650411| -----     | FJ650411 |
At the end, we report a strong discrepancy between morphological and genetic data with the former suggesting gene flow between the diploid *Leptobotia* and the tetraploid *Sinibotia* in the upper River Li basin, while the later did not reveal any sign of genetic introgression of *Leptobotia* into the evolutionary history of *B*. *zebra*. Since the amount of information taken from the genetic analyses was high and the different methods that have been applied in the present study have analysed a wide range of genetic data (chromosomes, allozymes, mitochondrial and nuclear DNA sequences), it is very unlikely that a hybridisation event would have stayed undetected.

We finally conclude that *B*. *zebra* is not the result of a hybridisation event, but a species of *Sinibotia* that underwent an outstanding example of evolution that has changed its morphology in the way that it strikingly matches the morphology of the co-occurring species *L. guilinensis*. This seems at least surprising, since evolutionary theory pronounces that a strong selection exists against the co-occurrence of highly similar species (competitive exclusion, Gaus’s law) [37,38]. We therefore have to assume that there exists an evolutionary advantage for *S. zebra* in looking so similar to *L. guilinensis*. The most common mechanism to achieve such an advantage is mimicry; which helps to reduce the predation pressure on one (Batesian mimicry) or both (Mullerian mimicry) similar species [39,40]. Since all species of Botiidae have a suborbital spine as anti-predator weapon and six out of eight species of Botiidae in the River Li share a pattern of broad bands on the body, the possibility exists that they represent a case of Mullerian mimicry. The only species with a different pattern are *L. guilinensis* and *B. zebra*; which does not fit to the assumption of Mullerian mimicry. Nothing is known about ecology and micro-habitat of *S. zebra* and *L. guilinensis*, but the their frequent occurrence in the same lot on local markets and ornamental fish imports indicate that they live very close to each other, making their case of ‘mimicry’ an interesting topic for further research.

Another result of the present study is the first record of *S. zebra* from outside the upper River Li basin and even outside the Pearl River basin. Specimen A8614 was found among specimens of *S. pulchra* from the Min River in Fujian province (Fig 1). It bears the characteristic pigmentation of *S. zebra* (head like *S. pulchra*, body like *L. guilinensis*) as well as the diagnostic combination of

| Species                      | ID    | Cyt b    | RAG    |
|------------------------------|-------|----------|--------|
| *Syncrossus berdmorei*       | A0277 | AY887823 | KU517078 |
| *Syncrossus helodes*         | A0574 | KU517130 | KU517079 |
| *Yasuhikotakia eos*          | A0062 | AY887829 | KU517080 |
| *Yasuhikotakia lecontei*     | A0568 | KU517131 | KU517081 |
| *Yasuhikotakia modesta*      | A0200 | AY887833 | KU517082 |
| *Yasuhikotakia morleti*      | A0067 | AY887835 | KU517083 |
| *Gyrinocheilus aymonieri*    | A0256 | KU517132 | EF056390 |

Species, number code, and Genbank accession numbers of Botiidae used in the DNA sequence analyses, cytogenetic analysis and allozyme analysis. Individuals with GenBank accession number have been included in the DNA sequence analyses, those marked a were used in allozyme analyses and those marked with k were karyotyped.

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mental lobes and a simple suborbital spine and has the *S. zebra* specific character state of 7½ branched dorsal-fin rays. Interestingly, no species of *Leptobotia* has been recorded from this basin up to now; therefore no partner for any co-evolution as discussed above would be available. However, our phylogenetic reconstructions based on the mitochondrial and nuclear genes show...
that specimen A8614 from the Min basin is very closely related to their conspecifics from the Li basin. The same is true for \textit{S. pulchra}; the specimens from the Min basin bear very similar haplotypes as specimens from the Li River. We consequently assume their presence in the Min basin to be the result of a very recent range extension. Range extensions along the southeaster Chinese
coast were possible during the Pleistocene glacial maxima, when the lowered global sea level led to prolongation and joining of coastal rivers. However, no botiid species is known to occur on Taiwan, which also was connected to the Chinese coast during the glacial maxima in Pleistocene and therefore shares several freshwater species with the coastal rivers of China [41,42]. It is possible that the presence of botiid fishes in the Min River basin is even younger than the last glacial maximum and might be the result of human activity.

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Author Contributions
Conceived and designed the experiments: JB Vendula Šlechtova PR. Performed the experiments: JB Vendula Šlechtova V. Slechta Vera Slechtova AS. Analyzed the data: Vendula Šlechtova V. Slechta Vera Slechtova AS. Contributed reagents/materials/analysis tools: JB Vendula Šlechtova V. Slechta Vera Slechtova AS. Wrote the paper: JB Vendula Šlechtova V. Šlechta AS.

References
1. Comai L. The advantages and disadvantages of being polyploidy. Nat Rev Genet. 2005; 6: 836–846. PMID: 16304599
2. Otto SP, Whitton J. Polyploid incidence and evolution. Ann Rev Genet. 2000; 34, 401–437. PMID: 11092833
3. Arnold ML. Natural hybridization and evolution. New York: Oxford University Press; 1997.
4. Benfey TJ. The physiology and behaviour of triploid fishes. Rev Fish Sci 1999; 7: 39–67.
5. Pfarrer F, Beaumont A, Falguiere JC, Flajšhans M, Haffray P, Kolombo L. Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture. 2009; 239: 125–156.
6. Bănărescu P. Zoogeography of fresh waters. Volume 2. Distribution and dispersal of freshwater animals in North America and Eurasia. Wiesbaden: Aula-Verlag; 1992.
7. Nalbant TT. Sixty million years of evolution: Part one: Family Botiidae (Pisces: Ostariophysi: Cobitoidae). Trav Mus Nat Hist Nat Grigore Antipa. 2002; 44: 309–333.
8. Saitoh K, Sado T, Mayden RL, Hanzawa N, Nakamura K, Nishida M, et al. Mitogenomic Evolution and Interrelationships of the Cypriniformes (Actinopterygii: Ostariophysi): The first evidence toward resolution of higher-level relationships of the world’s largest freshwater fish clade based on 59 whole mitogenome sequences. J Mol Evol. 2006; 63: 826–841. PMID: 17086453
9. Sawada Y. Phylogeny and zoogeography of the superfamily Cobitoidea (Cyprinoidei, Cypriniformes). Mem Fac Fish Hokkaido Univ. 1982; 28: 65–223.
10. Šlechtová V, Bohlen J, Tan HH. Families of Cobitoidea (Teleostei; Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera Barbutca, Psilorhynchus, Serpenticobitis and Vaillantella. Mol Phylogenet Evol. 2007; 44: 1358–1365. PMID: 17433724
11. Tang QY, Xiong B, Yang X, Liu H. Phylogeny of the East Asian botiine loaches (Cypriniformes, Botiidae) inferred from mitochondrial cytochrome b gene sequences. Hydrobiologia. 2005; 544: 249–258.
12. Šlechtová V, Bohlen J, Freyhof J, Ráb P. Molecular phylogeny of the Southeast Asian freshwater fish family Botiidae (Teleostei: Cobitoidea) and the origin of polyploidy in their evolution. Mol Phylogenet Evol. 2006; 39: 529–541. PMID: 16337410
13. Suzuki A, Taki Y. Tetraploidization in the cobitid subfamily Botiinae (Pisces, Cypriniformes). Cytobios. 1996; 85: 229–245.
14. Zhan SH, Glick L, Tsigenopoulos CS, Otto SP, Mayrose I. Comparative analysis reveals that polyploidy does not decelerate diversification in fish. J Evol Biol. 2014; 27: 391–403. doi: 10.1111/jeb.12308 PMID: 24417407

15. Yang JX, Chen YR. Revision of the subgenus Botia (Sinibotia) with description of a new species (Cypriniformes: Cobitidae). Ichthyol Explor Freshw. 1992; 2: 341–349.

16. Chen JX. A study on the classification of the botiid fishes of China. Zool Res. 1980; 1: 3–26.

17. Wu HW. On the fishes of Li-Kiang. Sinensia. 1939; 10: 92–100.

18. Tang Q-Y, Yu D, Liu H-Z. Leptobotia zebra should be revised as Sinibotia zebra (Cypriniformes: Cobitidae). Zool Res. 2008; 29: 1–9.

19. Kottelat M. Indochinese nemacheilines. A revision of nemacheiline loaches (Pisces: Cypriniformes) of Thailand, Burma, Laos, Cambodia and southern Viet Nam. München: Pfeil; 1990.

20. Völker M, Sonnenberg R, Ráb P, Kullmann H. Karyotype differentiation in Chromaphyosemion killi-fishes (Cyprinodontiformes, Nothobranchiidae). II: Cytogenetic and mitochondrial DNA analyses demonstrate karyotype differentiation and its evolutionary direction in C. riggenbachii. Cytogenet Genome Res. 2006; 115: 70–83. PMID: 16974086

21. Völker M, Ráb P. Direct chromosome preparation from regenerating fin tissue. p.37

22. Völker M, Sonnenberg R, Ráb P, Kullmann H. Karyotype differentiation in Chromaphyosemion killi-fishes (Cyprinodontiformes, Nothobranchiidae). II: Cytogenetic and mitochondrial DNA analyses demonstrate karyotype differentiation and its evolutionary direction in C. riggenbachii. Cytogenet Genome Res. 2006; 115: 70–83. PMID: 16974086

23. Šlechtová V, Šlechta V, Lusková V, Lusk S, Berrebi P. Genetic variability of common barbel, Barbus barbus populations in the Czech Republic, involving C. elongatoides and C. spp. Allozyme interpopulation and interspecific differences. Folia Zool. 2000; 49 (Suppl. 1): 67–78.

24. Ferguson KA, Wallace ALC. Starch-gel Electrophoresis of Anterior Pituitary Hormones. Nature. 1968; 190: 629–630.

25. Clayton JW, Tretiak DN. Amine-citrate buffers for pH control in starch gel electrophoresis. J Fish Res Board Can. 1972; 29: 1169–1172.

26. Valenta M, Hyldgaard-Jensen J, Jensen SE. Interaction of veronal, pyrophosphate, citrate and protein with lactate dehydrogenase isoenzyme determination and kinetics. Acta Vet Scand. 1971; 12: 15–35. PMID: 4324963

27. Perdices A, Doadrio I, Bermingham E. Evolutionary history of the synbranchid eels (Teleostei: Synbranchidae) in Central America and the Caribbean islands inferred from their molecular phylogeny. Mol Phylogenet Evol. 2005; 37: 460–473. PMID: 16223677

28. Hall T. BioEdit: Biological sequence alignment editor for Win95/98/NT/2K/XP/7. Version 7.2.5; 2003. Available: http://www.mbio.ncsu.edu/BioEdit/bioedit.html

29. Bohlen J, Šlechtová V. Phylogenetic position of the fish genus Ellopostoma (Teleostei: Cypriniformes) using molecular genetic data. Ichthyol Explor Freshw. 2009; 20: 157–162.

30. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19: 1572–1574. PMID: 12912839

31. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGAS: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol. 2011; 28: 2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546353

32. Taki Y. Botia eos, a new spiny loach from Thailand and Laos, with notes on some related forms in Asia. Japanese Journal of Ichthyology. 1972; 19: 63–81.

33. Fang PW. Study on the botoid fishes of China. Sinensia. 1936; 7: 1–49.

34. Li J, Li X-H, Chen X-L. A new species of the genus Leptobotia from Guangxi, China (Cypriniformes Cobitidae). Acta Zootaxon. Sinica 2008; 33: 630–633.

35. Kottelat M. Botia kubotai, a new species of loach (Teleostei: Cobitidae) from the Ataran River basin (Myanmar), with comments on botine nomenclature and diagnosis of a new genus. Zootaxa 2004; 401:1–18.

36. Gause GF. The struggle for existence. Baltimore: Williams & Wilkins; 1934.

37. Hardin G. The Competitive Exclusion Principle. Science. 1960; 131: 1292–1297. PMID: 14399717

38. Edmunds M. Defence in animals: a survey of anti-predator defences. Essex: Longman; 1974.

39. Wickler W. Mimicry in plants and animals. New York: Mc Graw-Hill; 1968.
41. Huang JP, Lin CP. Lineage-specific late pleistocene expansion of an endemic subtropical gossamer-wing damselfly, *Euphaea formosa*, in Taiwan. BMC Evol Biol. 2011; 11: 94. doi: 10.1186/1471-2148-11-94 PMID: 21486452

42. Yang JQ, Tang WQ, Liao TY, Sun Y, Zhou ZC, Han CC, et al. Phylogeographical Analysis on *Squalidus argentatus* Recapitulates Historical Landscapes and Drainage Evolution on the Island of Taiwan and Mainland China. Int J Mol Sci. 2012; 13: 1405–1425. doi: 10.3390/ijms13021405 PMID: 22408398