Genetic Diversity in Three Forms of *Anabas testudineus* Bloch

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Summary Karyotype and RAPD analysis were studied in 3 forms of climbing perch, *Anabas testudineus* viz. wild (native, non-spotted), Thai (introduced from Thailand, spotted) and Thai (a spotted-released form from local hatcheries). $2n=46$ chromosomes were found in the spotted-released form. The total length of $2n$ chromosome complement was $89.96\mu\text{m}$ with a chromosomal length ranging from 0.92 and $2.96\mu\text{m}$. The centromeric formula of this spotted-released form was $15m+9sm+22t$. This form did not show any CMA-bands. The karyotypic features of this spotted-released form were totally different from those given by previous reports on spotted forms. Four primers, namely OPA-2, OPA-4, OPA-7 and OPA-8, were tested on these 3 forms. The wild form showed unique bands in the OPA-2 (1300, 550, 350 bp) and OPA-7 (280 bp) primers. The spotted and spotted-released form showed different RAPD banding patterns in primers OPA-4, OPA-7 and OPA-8. The wild form was found to be separate from the other 2 forms at 13.5 linkage distance. The 2 Thai forms have 11.0 linkage distances indicating that these 2 forms are not genetically very close. The karyotype and RAPD results indicate that either the Thai spotted-released form is not developed exactly from the same stock as the Thai spotted form or that due to the application of physical stress or chemical treatment this spotted-released form has been modified.

Key words Fluorescent banding, Karyotype, RAPD, *Anabas testudineus*.

Anabas testudineus (locally known as “Koi”) belongs to the family Anabantidae. This species has a wide range of distribution from Africa to South-East Asia especially in the Indian subcontinent, Thailand and China (Talwar and Jhingran 1991). Being a freshwater fish, in Bangladesh it inhabits fresh and brackish water mostly in canals, lakes, ponds, ditches, floodplains, haors, baors and swamps (Talwar and Jhingran 2001). In Bangladesh, mainly 2 morphological forms of *Anabas testudineus* are present. The 2 forms are—i) Wild koi (native, non-spotted) and ii) Thai koi (introduced from Thailand, spotted). In addition, another form of *Anabas testudineus* is also present. This form is a result of different breeding programs on the Thai spotted form carried out by different hatcheries. These hatcheries have claimed to develop XX males, polyploid and high yielding varieties of this form (Nurul Haque, Manager, Brammaputra hatchery, Mymensingh, Bangladesh; personal communication 2009). These hatcheries have already released the above-mentioned germplasms of *Anabas testudineus*. Unfortunately, they do not have any genetical data for these XX males, polyploidy and high yielding varieties. Even they do not have the $2n$ chromosome numbers (the basic genetic information) of these released forms.

Tinni *et al.* (2007) tried to characterize the 2 forms of *Anabas testudineus* (native and spotted) with the help of fluorescent banding. They found differences in the fluorescent banding patterns of these 2 forms. However, they did not study the karyotype of the other spotted-released forms (XX-male, polyploid, high yielding varieties *etc.*).

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Genetic information is the basic resource for any successful fish-breeding programme. Information on genetic variation within hatchery stocks indicates the level of success in their management and also the status of their brood stock. Genetic diversity between stocks is also critical when one considers hatcheries as gene banks for conserving genetic resources (Allendorf and Phelps 1980, Cross and King 1983, Kincaid 1983). Proper utilization of the gene pool of an organism requires utilization of biochemical genetic markers to monitor stock purity *i.e.* to quantify the genetic variability, to identify parents and progeny in single pairs or complex crosses (Moav *et al.* 1976) and to monitor introgression (Mostafa *et al.* 2009).

DNA finger printing by Random Amplified Polymorphic DNA (RAPD) is another method for characterizing germplasms authentically. The term DNA fingerprinting/profiling describes the combined use of several single locus detection systems. This method has been used as versatile tool for investigating various genomic aspects of organisms. RAPD markers generated by the polymerase chain reaction (PCR) have been widely used since late 80s of the last century to assess intra-specific genetic variation on the molecular level (Welsh and McClelland 1990, Williams *et al.* 1990). RAPD technique has been used for taxonomic and systematic analysis of various organisms and has provided important applications in catfish (Bartish *et al.* 2000).

Therefore, a combination of karyotype and RAPD analysis would be helpful to provide sufficient data of an individual fish germplasm. In this study, 3 forms of *Anabas testudineus* viz. i) wild form (native) ii) Thai form (spotted) and iii) Thai form (spotted-released) were studied cytogenetically and by RAPD markers. The aims of this study were—i) to compare the Giemsa- and CMA-stained karyotypes of the claimed Thai spotted-released form with those reported earlier by Tinni *et al.* (2007), ii) to determine the germplasm specific RAPD markers and iii) to provide authentic genetic information of these 3 forms of *Anabas testudineus* for application in successful breeding programs.

Materials and methods

The wild and spotted forms of *Anabas testudineus* were collected from the Fisheries Research Institute, Mymensingh, Bangladesh. However, the spotted-released form of *Anabas testudineus* was collected from Brammaputra hatchery, Mymensingh, Bangladesh. These were then reared in a well-aerated aquarium in the Department of Fisheries, University of Dhaka. Cytogenetical and RAPD analysis were carried out in the Cytogenetics Laboratory, Department of Botany, University of Dhaka, Bangladesh.

Cytogenetical study

Chromosome preparations and Giemsa-staining were made by the “Flame drying” standard methods using kidney and gill cells following the procedure of Pandey and Lakra (1997) with slight modifications. Briefly the air-dried slides were dipped in a coupling jar with 6% Giemsa solution at pH 6.8 for 1 h. The slides were then rinsed in running tap water for 20–25 s and air-dried for a few hours. The air-dried slides were then mounted by DPX with cover slips and observed under microscope.

For CMA banding, the method proposed by Alam and Kondo (1995) was followed with minor modifications. After 48 h of air-drying the slides were first pre-incubated in McIlvaine's buffer (pH 7.0) for 30 m. A drop of 0.1 mg/ml Distamycin A was added to the materials on slides and a cover glass placed on each slide. The slides were rinsed mildly in McIlvaine's buffer supplemented with 5 mM MgSO₄ for 15 m. One drop of Chromomycin A₃ (0.1 mg/ml) was added to the materials of the slides and a clean cover glass placed on each slide. The slides were kept in humid chamber for 15 m. Then the slides were treated again for 15 m in McIlvaine's buffer with Mg⁺² and McIlvaine's buffer without Mg⁺². The slides were mounted in 50% glycerol and kept at 4°C for overnight before

observation. They were observed under a fluorescent microscope (Hund, Germany) with blue violet (BV) filter cassette.

DNA isolation

Caudal fins were collected and total genomic DNA was extracted by using a modified CTAB method (Doyle and Doyle 1987). DNA concentration was quantified through a spectrophotometer (Analytikjena, Specord 50, Germany). The A260/280 readings for DNA samples were 1.6–1.8.

PCR amplification and primer survey

The PCR reaction mix for 25 μ l containing template DNA (25 ng) 2 μ l, de-ionized distilled water 18.8 μ l, *Taq* buffer A 10X (Tris with 15 mM $MgCl_2$) 2.5 μ l, primer (10 μ M) 1.0 μ l, dNTPs (2.5 mM) 0.5 μ l, *Taq* DNA polymerase (5 U/ μ l) 0.2 μ l. PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denaturing at 94°C for 5 min, denaturing at 94°C for 1 min, annealing at 36°C for 30 s, extension at 72°C for 3 min and final extension at 72°C for 5 min. Four primers were used from Operon Technologies, USA, namely OPA-2 (TGC CGA GCT C), OPA-4 (AAT CGG GCT G), OPA-7 (GAA ACG GGT G) and OPA-8 (GTG ACG TAG G) series.

Gel electrophoresis

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide 8 μ l and 100 ml 1×TAE buffer. Agarose gel electrophoresis was conducted in 1×TAE buffer at 50 volts and 100 mA for 1.5 h. DNA ladder (1 kb) was electrophoresed alongside the RAPD reactions as marker. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system.

Scoring and data analysis

The PCR products were analyzed after gel electrophoresis. The photographs were critically discussed on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. Tables were drawn up for further investigation. RAPD analysis was then combined to create a single data matrix. This was used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) Dendrogram among the varieties using computer program “Statistica.” Linkage distances were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied 3 forms of *Anabas testudineus* using UPGMA (Lakhanpaul *et al.* 2000). A dendrogram tree was constructed using the computer software “Statistica.”

Results and discussion

Giemsa stained karyotypes

The Thai spotted-released form was found to possess $2n=46$ chromosomes. The same $2n$ chromosome number of this species was reported earlier (Manna and Prashed 1974, Tinni *et al.* 2007). Therefore, the present finding regarding the $2n$ chromosome number of the Thai spotted-released form confirmed earlier reports. However, huge genetical differences were found regarding the other karyotypic parameters between the Thai spotted form (reported by Tinni *et al.* 2007) and the spotted-released form of the present investigation. These are: i) in this investigation, the centromeric formula of the Thai spotted-released form was $15m+9sm+22t$ (Fig. 1c, Table 1). Tinni *et al.* (2007) reported $6m+40t$ for the Thai spotted form. The present centromeric formula was more heterogeneous since it possesses 3 types of chromosomes on the basis of centromeric position and thus shows a more asymmetric nature of karyotype; ii) in the present investigation, heteromorphicity in respect

Figs. 1a–g. Mitotic metaphase chromosomes and RAPD analysis with 4 primers on 3 forms of *Anabas testudineus*. a. Giemsa-stained mitotic metaphase chromosome of *Anabas testudineus* (Thai spotted-released form), b. CMA-stained mitotic metaphase chromosomes of Thai spotted-released form, c. Giemsa-stained karyotype prepared from mitotic metaphase chromosome of Thai spotted-released form (Bar=5 μ m). d. Primer OPA-2, e. Primer OPA-4, f. Primer OPA-7 and g. Primer OPA-8. M=1 kb DNA ladder, TR=Thai spotted-released form, W=wild (native, non-spotted) and TS=Thai spotted form.

of chromosome length was found in pairs X and XIV (Fig. 1c, Table 1). One member of pair X was telocentric and the other metacentric. On the other hand, 1 chromosome of pair XIV was submetacentric and the other telocentric. The probable reason for heteromorphism might be due to structural aberration *i.e.* deletion or pericentric inversion of chromosomal segments that change the shape of respective chromosomes. No such heteromorphism was found by Tinni *et al.* (2007); iii) the total length of $2n$ chromosome complement of the Thai spotted-released form studied in this investigation was 89.96 μ m and the chromosomal length ranged from 0.92–2.96 μ m (Table 1).

Table 1. Comparative Giemsa and CMA-analysis of spotted-released form and spotted form (from Tinni *et al.* 2007) of *Anabas testudineus*

Feature	Present investigation on <i>Anabas testudineus</i> (spotted-released form)	Earlier report on <i>Anabas testudineus</i> (spotted form) by Tinni <i>et al.</i> (2007)*
Centromeric formula	15m+9sm+22t	6m+40t
Heteromorphism	Was found in pair x and xiv	No heteromorphism was found
Total length of 2n chromosome complement	89.96 μ m	146.44 μ m
Range of chromosomal length	0.92–2.96 μ m	1.06–5.32 μ m
CMA-band	Not found	4 CMA- band

m=metacentric chromosome, sm=submetacentric chromosome and t=telocentric chromosome.

* Data were taken with prior permission.

Table 2. RAPD analysis of 3 forms of *Anabas testudineus*

Forms	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	% of polymorphism in the form	Average % of polymorphism in the variety
Wild (native)	13	9	4	4	69.23	69.29
Thai spotted-released form	11	7	4	1	63.63	
Thai spotted form	16	12	4	5	75.00	

However, according to Tinni *et al.* (2007), the 2n length of chromosome complement was 146.44 μ m and the range of chromosomal length 1.06–5.32 μ m. The present results indicated that the length of 2n chromosome complements of Thai spotted-released form was much less than that reported by Tinni *et al.* (2007); iv) no CMA-band was found in any chromosome of the Thai spotted-released form in the present investigation (Fig. 1b), although, Tinni *et al.* (2007) reported 4 CMA-bands in the spotted Thai forms. Since the classical Giemsa karyotype indicated the presence of deletion, the lack of CMA-bands in the Thai spotted-released form might be a case of deletion of the banded portions.

RAPD analysis-wild versus the other 2 forms

The primer combinations were tried on 3 forms of *Anabas testudineus*. Although the 3 forms showed a few common bands in different primer combinations, each form had a different DNA fingerprinting pattern. In primer OPA-2, 3 unique bands of 1300, 550 and 350bp were found in the wild form which were absent in the other 2 forms. In addition, a fragment of 520bp was missing in the wild form which was present in the other 2 forms (Fig. 1d). These banding patterns separated the wild form from the other 2. In primers OPA-4, a band of 400bp was found in both the Thai form which was absent in the wild form (Fig. 1e). In OPA-8, fragments of 11000bp and 200bp were present in both the Thai forms whereas they were absent in the wild form (Fig. 1g). Therefore, the RAPD fingerprinting was able to characterize the wild form.

RAPD analysis-spotted versus spotted-released form

In OPA-4, fragments of 1450bp and 500bp were found in the spotted form whereas no such fragments were present in spotted-released form. A fragment of 1000bp was found in the spotted-released form which was absent in the spotted form in the same primer (Fig. 1e, Table 2). In primer OPA-7, the spotted form had 5 fragments of 2000, 1300, 850, 600 and 410bp which were absent in the spotted-released form. On the other hand, a fragment of 12000bp was present in the spotted-re-

Fig. 2. Cluster analysis by unweighted pair group method of arithmetic means (UPGMA) for 3 forms of climbing perch (*Anabas testudineus*).

leased form but absent in the spotted form (Fig. 1f, Table 2). In primer combination OPA-8, a fragment of 400bp was found in the spotted form but not in the spotted-released form. In addition, the spotted-released form had a fragment of 300bp which was absent in the spotted form (Fig. 1g). The above results clearly showed different RAPD fingerprinting pattern in these 2 forms.

Cluster analysis

On the basis of RAPD fingerprinting pattern, the wild form separated at 13.5 linkage distance from the 2 Thai forms. The 2 Thai forms separated at 11.0 linkage distance. Although the 2 Thai form were placed in a cluster, the linkage distance was not narrow (Fig. 2). It indicated that these 2 forms were not genetically very close.

What actually is the spotted-released form?

Different hatcheries claimed to produce polyploidy, XX-male, high yielding varieties *etc.* from the spotted climbing perch (Nurul Haque, Manager, Bhrammaputra Hatchery, Mymensingh, Bangladesh; personal communication). Data on the karyotype (Giemsa- and CMA-) of the spotted form was available in the Cytogenetics laboratory, Department of Botany, University of Dhaka, Bangladesh (Tinni *et al.* 2007). In this experiment, both the Giemsa- and CMA-karyotypes of the claimed spotted-released form were prepared to make a comparative study with that reported by Tinni *et al.* (2007). The comparative results indicated that—i) the claimed Thai released form was found to possess $2n=46$ chromosome (Fig. 1a, Table 1) as reported by Tinni *et al.* (2007). It revealed that the present specimen was not a polyploid of the spotted form. If it was a polyploid, it should have had either $2n=3X=69$ or $2n=4X=92$ chromosomes; ii) the claimed spotted-released form had no CMA band (Fig. 1b). Tinni *et al.* (2007) found 4 CMA-bands in the spotted form. If the specimen was an XX male of the Thai spotted form, it should have had 4 CMA-bands (because CMA-bands were very stable and specific); iii) the RAPD DNA-fingerprinting patterns with primer OPA-4, OPA-7 and OPA-8 were totally different for these 2 forms. If these 2 specimens were from the same stock, they should have shown similar RAPD patterns. On the basis of karyotype and RAPD analysis, 2 possibilities arise namely such as—i) either the spotted-released form is not exactly the same stock as the Thai spotted form or ii) due to application of physical stress or chemical treatment, this spotted-released form has been modified. Whether this genetic modification is a threat to human health should be investigated.

Genetic diversification

Karyotypically, the claimed Thai spotted-released form is quite different from the spotted and wild form. This form has its own characteristic karyotypic features. The 3 forms showed RAPD fin-

gerprinting specific to each form. The present data revealed the genetic diversity of these 3 forms. This genetic information will be useful in developing future successful breeding programmes for these 3 forms of *Anabas testudineus*.

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