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Postmating Isolation in Six Species of Three Genera (*Hoplobatrachus*, *Euphlyctis* and *Fejervarya*) from Family Dicroglossidae (Anura), with Special Reference to Spontaneous Production of Allotriploids

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In light of reproductive isolation being a fundamental aspect of the biological species concept, we performed crossing experiments using six species from three genera (*Hoplobatrachus*, *Euphlyctis* and *Fejervarya*) of family Dicroglossidae to explore postmating isolation in dicroglossid frogs. Our results revealed gametic isolation among these genera, although the intergeneric hybrids between female *E. cyanophlyctis* and male *H. chinensis* were not viable at the tadpole stage, while the hybrids between female *E. cyanophlyctis* and male *H. tigerinus* were inviable at the hatching stage. These results showed complete hybrid inviability between the two genera. Almost all interspecific hybrids between female *H. tigerinus* and male *H. chinensis* died of underdevelopment at the tadpole stage, whereas several hybrids developed normally and survived to maturity. Chromosomal observations and mtDNA and allozyme analyses confirmed that these mature hybrids were allotriploid, with two maternal genomes and one paternal genome. The present results suggest that the allotriploids were produced spontaneously, and histological observations confirmed their sex as sterile males. We also investigated the molecular relationships between *H. tigerinus*, *H. chinensis*, and the interspecific allotriploids by mitochondrial Cyt*b*, 12S and 16S rRNA gene analyses. The maternal inheritance mode of mitochondrial genomes was retained in the hybrids. Finally, the present results suggest that the degree of postmating isolation reflects phylogenetic relationship. In addition, we speculate that allotriploids may be produced via hybridization among cryptic species.

Key words: crossing experiment, postmating isolation, interspecific allotriploid, intergeneric hybrid, dicroglossid frogs

INTRODUCTION

The definition of the taxonomic unit "species" is still controversial and has been debated for a long time (Coyne and Orr, 2004; de Queiroz, 1998, 2005; Frost and Hills, 1990; Hey, 2006; Issac et al., 2004). According to Mayr (1942) "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups". Despite the wide acceptance of Mayr's species definition, many contemporary species concepts have been reviewed (Coyne and Orr, 2004; de Queiroz, 1998; Harrison, 1998; Mayden, 1997), and the problem concerning the nature of species remains unresolved (de Queiroz, 2007; Mallet, 2008). The biological species concept emphasizes the property of reproductive isolation and that the integrity of a sympatric species is maintained over time by reducing or directly impeding gene flow between individuals of different species. When populations are isolated geographically and fail to exchange gene flow through interbreeding, the accumulation of intraspecies variation leads to speciation (allopatric speciation) (Mayr, 1963; Hoskin et al., 2005). The evolution of reproductive isolation is a crucial part of the speciation process. An understanding of the types of barriers responsible for reproductive isolation will help to elucidate the conditions under which speciation is likely to occur. Several factors affect isolating mechanisms, among them is postmating isolation, which can be intrinsic (including low hybrid viability and fertility), or extrinsic (including hybrid ecological inferiority and lower mating suc-

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cess). It is therefore necessary to evaluate each direction separately, with each species acting as the female or male parent in a potential hybridization event to identify the traits responsible for reproductive isolation. While extensive empirical studies of reproductive isolation are involved, artificial crossing experiments among anurans using available live samples might be helpful for understanding their postmating isolating natures.

Genus *Hoplobatrachus* (Anura, Dicroglossidae) has four species, three of which are distributed in South and Southeast Asian countries, while the other is distributed in Africa. Genus *Euphlyctis* is considered a sister taxon to genus *Hoplobatrachus* which has six species, as listed by Frost et al. (2011). Alam et al. (2008) also discovered several

Table 1. List of frogs used for crossing experiments.

Genus	Species	Country	Location	No. of frogs			Abbreviation
				Total	♀	♂	
Hoplobatrachus	*H. tigerinus*	Bangladesh	Mymensingh	6	4	2	tigr.
	H. chinensis	Thailand	Chachoengsao	2	0	2	chin.
Euphlyctis	*E. cyanophlyctis*	Bangladesh	Narsingdi	3	2	1	cyan.
Fejervarya	*F.* sp. (L)	Bangladesh	Chittagong	1	0	1	limn. (L)
	F. sp. (S)	Bangladesh	Mymensingh	1	0	1	limn. (S)
	F. moodei	Thailand	Trad	1	0	1	mood.
Total				14	6	8	

Table 2. Developmental capacity of control and hybrids within and between the three genera *Hoplobatrachus*, *Euphlyctis* and *Fejervarya*.

Parents		No. of eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of 20-day-old tadpoles		No. of metamor-phosed frogs (%)	No. of mature frogs (%) (3 years)
Female	Male						Normal (%)	Under-developed (%)		
tigr. 1	tigr. 1	937	604 (64.5)	336 (35.9)	192 (20.5)	175 (18.7)	121 (12.9)	2 (0.2)	100 (10.7)	5
tigr. 1	tigr. 2	79	75 (94.9)	7 (8.9)	3 (3.8)	3 (3.8)	3 (3.8)	0 (0)	3 (3.8)	0
tigr. 2	tigr. 2	467	458 (98.1)	416 (89.1)	411 (88.0)	387 (82.9)	356 (76.2)	7 (1.5)	208 (44.5)	1
tigr. 3	tigr. 2	189	54 (28.6)	10 (5.3)	8 (4.2)	2 (1.1)	2 (1.1)	0 (0)	2 (1.1)	2
Total		1672	1191 (71.2)	769 (46.0)	614 (36.7)	567 (33.9)	482 (28.8)	9 (0.5)	313 (18.7)	8 (0.5)
tigr. 1	chin. 1	729	482 (66.1)	229 (31.4)	120 (16.5)	82 (11.2)	9 (1.2)	66 (9.1)	3 (0.4)	1
tigr. 2	chin. 1	237	128 (54.0)	19 (8.0)	19 (8.0)	18 (7.6)	5 (2.1)	13 (5.5)	1 (0.4)	0
tigr. 3	chin. 2	164	19 (11.6)	7 (4.3)	5 (3.0)	4 (2.4)	2 (1.2)	2 (1.2)	2 (1.2)	2
Total		1130	629 (55.7)	255 (22.6)	144 (12.7)	104 (9.2)	16 (1.4)	81 (7.2)	6 (0.5)	3 (0.3)
tigr. 2	cyan. 1	193	20 (5.7)	0 (0)	-	-	-	-	-	-
tigr. 3	cyan. 1	155	0 (0)	-	-	-	-	-	-	-
Total		348	20 (10.4)	0 (0)	-	-	-	-	-	-
tigr. 2	*F.* sp. (L)	112	24 (21.4)	0 (0)	-	-	-	-	-	-
tigr. 3	*F.* sp. (L)	183	9 (4.9)	0 (0)	-	-	-	-	-	-
Total		295	33 (11.2)	0 (0)	-	-	-	-	-	-
tigr. 2	*F.* sp. (S)	217	59 (27.2)	0 (0)	-	-	-	-	-	-
tigr. 3	*F.* sp. (S)	222	0 (0)	-	-	-	-	-	-	-
Total		439	59 (13.4)	0 (0)	-	-	-	-	-	-
tigr. 2	mood.	159	0 (0)	-	-	-	-	-	-	-
tigr. 3	mood.	251	0 (0)	-	-	-	-	-	-	-
Total		410	0 (0)	-	-	-	-	-	-	-
cyan. 1	cyan. 1	142	7 (4.9)	6 (4.2)	4 (2.8)	4 (2.8)	4 (2.8)	0 (0)	4 (2.8)	4
cyan. 2	cyan. 1	96	78 (81.3)	55 (57.3)	55 (57.3)	55 (57.3)	50 (52.1)	0 (0)	31 (32.3)	22
Total		238	85 (35.7)	61 (25.6)	59 (24.8)	59 (24.8)	50 (21.0)	0 (0)	35 (14.8)	26 (11.0)
cyan. 1	tigr. 2	174	11 (6.3)	0 (0)	-	-	-	-	-	-
cyan. 2	tigr. 2	107	40 (37.4)	14 (13.1)	0 (0)	-	-	-	-	-
Total		281	51 (18.1)	14 (14.5)	0 (0)	-	-	-	-	-
cyan. 1	chin. 2	114	78 (68.4)	22 (19.3)	21 (18.4)	20 (17.5)	0 (0)	20 (17.5)	0 (0)	-
cyan. 2	chin. 2	77	58 (75.3)	41 (53.3)	16 (20.8)	13 (16.9)	0 (0)	5 (6.5)	0 (0)	-
Total		191	136 (71.2)	63 (33.0)	37 (19.4)	33 (17.3)	0 (0)	5 (2.6)	0 (0)	-
cyan. 1	*F.* sp. (L)	145	0 (0)	-	-	-	-	-	-	-
cyan. 2	*F.* sp. (L)	74	17 (23.0)	0 (0)	-	-	-	-	-	-
Total		219	17 (7.8)	0 (0)	-	-	-	-	-	-
cyan. 1	*F.* sp. (S)	158	0 (0)	-	-	-	-	-	-	-
cyan. 2	*F.* sp. (S)	59	0 (0)	-	-	-	-	-	-	-
Total		217	0 (0)	-	-	-	-	-	-	-
cyan. 1	mood.	155	0 (0)	-	-	-	-	-	-	-
cyan. 2	mood.	97	0 (0)	-	-	-	-	-	-	-
Total		252	0 (0)	-	-	-	-	-	-	-

cryptic species among *Hoplobatrachus* and *Euphlyctis* genera (Anura, Dicroglossidae) from Bangladesh and neighboring countries. Several species (e.g., *E. aloysii* and *E. mudigere*) are also described from genus *Euphlyctis* (Joshy et al., 2009), and recently a new species in Bangladesh from genus *Hoplobatrachus* has also been described (Hasan et al., 2012). Furthermore, current nucleotide sequences of mitochondrial genomes of *Hoplobatrachus tigerinus* (Indian bullfrog) and *Euphlyctis hexadactylus* (Indian green frog) have revealed novel gene rearrangements among them (Alam et al., 2010). Gene rearrangement and duplication is considered an important step in speciation in plants (Considine et al., 2012), and such aspects have been discussed in amphibians and fishes by Mable et al. (2011). Genus *Fejervarya* is recognized as a sister taxon to *Hoplobatrachus* and *Euphlyctis*, and reproductive isolation experiments have been conducted between *Fejervarya* species; subsequently, the genetic relationships and taxonomic status of cryptic species have been discussed in relation to artificial crossing experiments (Djong et al., 2007; Islam et al., 2008; Kurniawan et al., 2011; and Sumida et al., 2003, 2007). In the present study, we performed artificial crossing experiments between available *Hoplobatrachus*

Table 3. List of enzymes and blood proteins, analyzed for *Hoplobatrachus tigerinus*, *H. chinensis* and the interspecific hybrids.

Enzymes or blood proteins	E. C. No.	Abbreviation	No. of locus	Locus
Aspertate aminotransferase	2.6.1.1	AAT	2	AAT-1 AAT-2
Adenylate kinase	3.5.4.4	AK	1	AK
Creatine kinase	2.7.4.3	CK	1	CK
Fumarate hydratase	4.2.1.2	FUM	1	FUM
α-Glycerophosphate dehydrogenase	1.1.1.18	α-GDH	1	α-GDH
Isocitrate dehydrogenase	1.1.1.42	IDH	2	IDH-1 IDH-2
Lactate dehydrogenase	1.1.1.27	LDH	2	LDH-A LDH-B
Malate dehydrogenase	1.1.1.37	MDH	2	MDH-1 MDH-2
Malic enzyme	1.1.1.40	ME	2	ME-1 ME-2
Mannose phosphate isomerase	5.3.1.8	MPI	1	MPI
6-Phosphogluconate dehydrogenase	1.1.1.44	6-PGD	1	6-PGD
Peptidase	3.4.3.1	PEP	3	PEP-A PEP-B PEP-D
Phosphoglucomutase		PGM	1	PGM
Superoxide dismutase	1.15.1.1	SOD	2	SOD-1 SOD-2
Serum albumin	-	Alb	1	Alb
Hemoglobin	-	Hb	2	Hb-I Hb-II

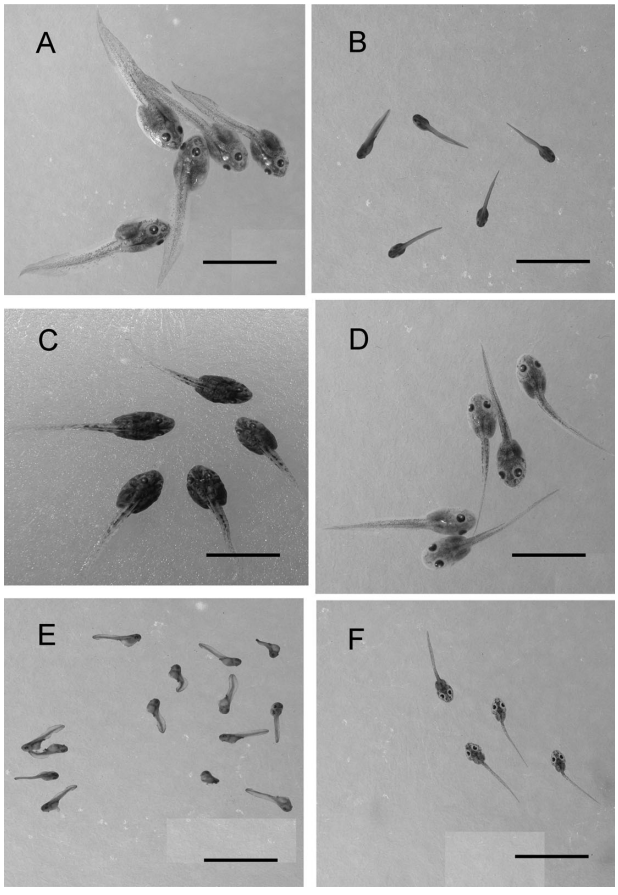

Fig. 1. Interspecific and intergeneric hybrids of *H. tigerinus*, *H. chinensis* and *E. cyanophlyctis* and controls at different stages. **(A)** tigr. ♀ 1 × tigr. ♂ 1 (20 days). **(B)** cyan. ♀ 2 × cyan. ♂ 1 (5 days). **(C)** cyan. ♀ 2 × cyan. ♂ 1 (20 days). **(D)** tigr. ♀ 3 × chin. ♂ 2 (20 days). **(E)** cyan. ♀ 2 × tigr. ♂ 2 (5 days). **(F)** cyan. ♀ 1 × chin. ♂ 2 (20 days). Scale bar = 10 mm.

Table 4. Samples used for mitochondrial Cyt *b*, 12S and 16S rRNA genes analyses.

Species/hybrid	Collection station		Haplotypes accession number		
	Country	(Locality)	Cyt*b*	12S	16S
Hoplobatrachus chinensis	Thailand	(Chachoengsao)	AB636597	AB636606	AB636615
H. chinensis	Thailand	(Chachoengsao)	AB636598	AB636607	AB636616
H. chinensis	Thailand	(Chachoengsao)	AB636599	AB636608	AB636617
H. chinensis	Thailand	(Nong khai)	AB636600	AB636609	AB636618
H. tigerinus		Laboratory	AB636601	AB636610	AB636619
H. tigerinus		Laboratory	AB636602	AB636611	AB636620
Hybrid 1 (tigr. ♀ 3 × chin. ♂ 2)		Laboratory	AB636603	AB636612	AB636621
Hybrid 2 (tigr. ♀ 1 × chin. ♂ 1)		Laboratory	AB636604	AB636613	AB636622
Hybrid 3 (tigr. ♀ 3 × chin. ♂ 2)		Laboratory	AB636605	AB636614	AB636623

and allied genera and elucidated the degree of reproductive isolation between the three dicroglossid genera *Hoplobatrachus*, *Euphlyctis*, and *Fejervarya*. Interestingly, we obtained several mature allotriploids between two *Hoplobatrachus* species (*H. tigerinus* and *H. chinensis*). In the present paper, we focus on the remarkable survival of allotriploids and their nature by performing chromosomal and histological observations, as well as allozyme and mitochondrial analyses.

MATERIALS AND METHODS

Frog samples

Frogs from genera *Hoplobatrachus*, *Euphlyctis*, and *Fejervarya* were collected from Bangladesh and Thailand. The live frogs were reared until a mature stage at the Institute for Amphibian Biology in Hiroshima University, and were then used for crossing experiments. Detailed information on frog collection is given in Table 1.

Artificial crossing experiments

Artificial insemination was conducted for crossing experiments according to the procedure by Sumida et al. (2011) with a slight modification. Mature frogs were selected during the breeding sea-

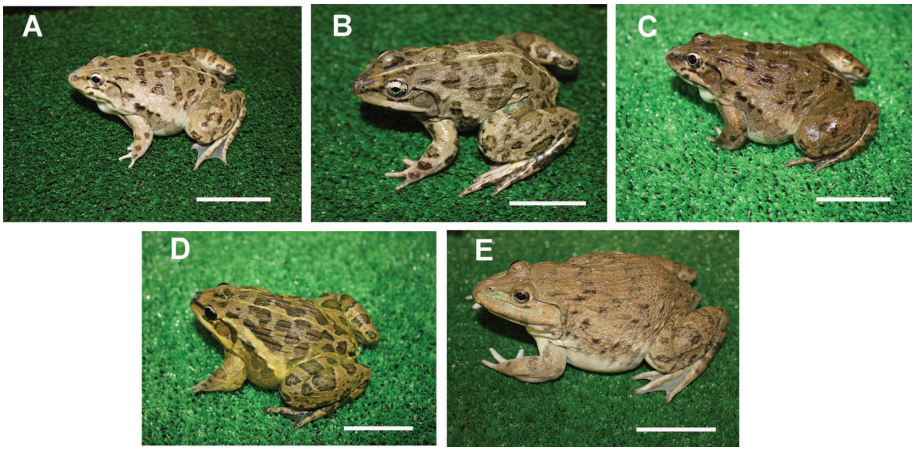

Fig. 2. Three-year-old interspecific hybrids between female *H. tigerinus* and male *H. chinensis* and controls. **(A)** Hybrid 1 (tigr. ♀ 3 × chin. ♂ 2). **(B)** Hybrid 2 (tigr. ♀ 1 × chin. ♂ 1). **(C)** Hybrid 3 (tigr. ♀ 3 × chin. ♂ 2). **(D)** Control *H. tigerinus* (tigr. ♀ 1 × tigr. ♂ 1). **(E)** *H. chinensis*. Scale bar = 10 mm.

Table 5. Morphometric measurements of three years old interspecific hybrids and controls of *H. tigerinus*. The detail abbreviations are described by Islam et al. (2008).

Morphological characters	Measurements (mm)				
	Hybrid 1 (tigr. ♀ 3 × chin. ♂ 2)	Hybrid 2 (tigr. ♀ 1 × chin. ♂ 1)	Hybrid 3 (tigr. ♀ 3 × chin. ♂ 2)	Control 1 (tigr. ♀ 1 × tigr. ♂ 1)	Control 2 (tigr. ♀ 1 × tigr. ♂ 1)
SVL (Snout-vent length)	63.2	86.0	83.2	82.5	58.2
HL (Head length)	23.4	31.2	32.1	30.5	27.0
HW (Head width)	21.5	32.4	33.4	28.0	23.6
STL (Snout-tympanum length)	16.7	20.4	21.1	21.5	19.8
MSL (Mouth-angle-snout length)	19.9	24.6	26.4	30.8	20.8
NS (Nostril-snout length)	4.3	5.5	5.0	6.2	5.0
SL (Snouth length)	9.4	12.4	12.8	12.8	11.4
NTL (Nostril-tympanum length)	11.5	15.3	14.4	16.0	14.0
EN (Distance from front of eyes to nostril)	5.0	6.2	6.5	5.8	5.8
TEL (Tympanum eye elngth)	1.7	2.0	2.1	1.2	1.2
TD (Tympanum diameter)	4.9	6.6	6.0	7.1	5.3
MN (Distance from back of mendible to nostril)	21.4	26.9	25.6	25.5	21.2
MFE (Distance from back of mandible to front of eye)	16.0	22.3	23.0	18.8	16.1
MBE (Distance from back of mandible to back of eye)	10.3	17.7	18.1	12.8	11.2
IN (Internarial space)	3.2	3.2	3.4	4.7	3.5
EL (Eye length)	6.0	9.4	7.8	8.5	7.2
IOD (Interorbital distance)	1.9	3.2	2.7	4.0	3.1
UEW (Maximum width of upper eyelid)	4.5	5.4	4.8	4.8	4.4
HAL (Hand length)	13.3	16.2	16.2	17.4	14.7
FLL (Fore arm length)	12.7	17.6	15.7	15.6	14.1
LAL (Lower arm length)	11.0	12.1	11.5	13.5	7.9
HLL (Hind limb length)	95.8	116.9	118.2	121.0	101.6
THIGHL (Thigh length)	31.0	37.5	37.9	35.0	29.0
TL (Tibia length)	35.8	40.7	41.0	44.0	35.0
FOL (Foot length)	30.5	41.8	37.8	33.0	32.0
TFOL (Length of tarsus and foot)	45.3	57.0	55.3	25.4	20.0
3FL (Third finger length)	7.1	10.6	9.2	12.0	10.5
1FL (First finger length)	6.5	8.5	6.7	8.4	8.8
4TL (Fourth toe length)	18.6	24.3	23.9	30.0	24.0
IMTL (Inner metatersal tubercle length)	3.3	5.2	5.1	4.8	4.3
ITL (Inner toe length)	6.9	7.1	7.7	9.9	8.2
Sex	Male	Male	Male	Male	Female

son (June to August) by observation of external body features. Mature female frogs were then injected with saline solution containing pituitary of *Lithobates catesbeiana* into the body cavity at a dose of one and half pituitary gland per frog. The injected female frogs were checked periodically to verify ovulation. We found that the ovulation time for *H. tigerinus* was about 12 h. Eggs were stripped and spread on a series of glass slides. Poor quality eggs were not used for fertilization and discarded. Side by side sperm suspension was made by crushing a testis removed from each male with a small amount of distilled water. Eggs were fertilized with normal sperm suspension by gentle mixing, after checking the motility of the sperm. Then the glass slides of fertilized eggs were submerged in petri-dishes containing Cl-free water with an appropriate marking. We maintained water temperature at around 24°C. The developmental capacity of the fertilized eggs was monitored from the cleavage stage to the subsequent embryonic stages (Table 2). The hatched tadpoles were reared around three weeks in petri-dishes and fed boiled spinach in an incubator (24°C) before being moved to a cement tank and reared for about two months until metamorphosis. The room temperature was always monitored and maintained at around 24°C. The frogs were transferred to the facilitated frog room at the Institute for Amphibian Biology, Hiroshima University and reared by feeding live crickets for further study.

Morphological measurements and sex

Morphological measurements of the three interspecific hybrids and the controls were obtained to investigate potential variation. The method used for morphological measurements followed that of Islam et al. (2008). We also checked the sexes of the interspecific hybrids.

Chromosomal and histological preparations

Chromosomes were observed in metaphase plates of bone marrow cells of 3-year-old mature interspecific 3 hybrids (tigr. × chin.; Table 2), 2 controls (tigr. × tigr.; Table 2), as well as their parent's (*H. tigerinus* and *H. chinensis*) by Omura's method (1967). The same samples were also used for histological observation. The testis was fixed in Navashin's solution, sectioned at 10 μm and stained with Heidenhain's iron hematoxylin for histological observation.

Allozyme and mitochondrial DNA analyses

A total of 25 frogs were used for allozyme electrophoresis. Of them, 20 were *H. chinensis* from Chachoengsao, Thailand, three were interspecific hybrids (tigr. × chin.; Table 2) and two were control *H. tigerinus* (tigr. × tigr.; Table 2). Fourteen enzymes and two blood proteins were analyzed by the method of horizontal starch-gel electrophoresis (Table 3). The details of the electrophoretic method have been reported previously by Nishioka et al. (1980). Detection of each enzyme was carried out by the agar-overly method outlined by Harris and Hopkinson (1976). Detection of blood proteins was made by the amido-black-staining method. A total of nine individuals were used for mitochondrial Cyt*b*, 12S and 16S rRNA gene analysis, and a list is given in Table 4. The primers used for PCR amplification and sequencing are described in Alam et al. (2010). The resultant sequences were deposited in the gene bank under Accession Nos. AB636597 to AB636623. The nucleotide sequences of each gene (Cyt*b*, 12S and 16S rRNA) were aligned using the ClustalW program (Thompson et al., 1994). Gaps and obscure areas were excluded using Gblocks Ver. 0.91b (Castresana, 2000) with default parameters. To confirm species level variation, we constructed the NJ tree using *F. limnocharis*, as an outgroup.

RESULTS

Developmental capacity

We used female *H. tigerinus* and *E. cyanophlyctis* for crossing experiments with male *H. tigerinus*, *H. chinensis*, *E. cyanophlyctis*, *Fejervarya* sp. (Large type BD), *Fejervarya* sp. (Small type BD) and *F. moodiei* (Table 2). The controls, *H. tigerinus* (Table 2; 4 series female tigr. 1, 2, 3 with male tigr. 1, 2) and *E. cyanophlyctis* (Table 2; 2 series female cyan. 1, 2 with male cyan. 1) showed normal development and metamorphosis. The rate of metamorphosed frogs was 18.7% and 14.8% in *H. tigerinus* and *E. cyanophlyctis*, respectively (Fig. 1A, B, C). We faced difficulty with mass rearing of metamorphosed controls to mature adult in the laboratory, and this aspect requires improvements in captive rearing and feeding strategies for better survival. By contrast, interspecific hybrids produced from female *H. tigerinus* and male *H. chinensis* showed lower levels of developmental capacity (Table 2; 3 series female tigr. with male chin. 1, 2) (Table 2; Fig. 1D), and although most were underdeveloped (7.2%), some metamorphosed (0.5%) and matured, which took about three years. Finally, we found three mature hybrids that were similar to *H. tigerinus* in external characters (Fig. 2), and there was no significant difference in morphological measurements between hybrids and controls (Table 5). We compared the developmental capacity data of control *H. tigerinus* (tigr. × tigr.) and interspecific hybrids (tigr. × chin.) by t-test and found statistical significance at the 5% level. The intergeneric hybrids produced by female *E. cyanophlyctis* and male *H. tigerinus* (Table 2; 2 series female cyan. 1, 2 with male tigr. 2) survived until the

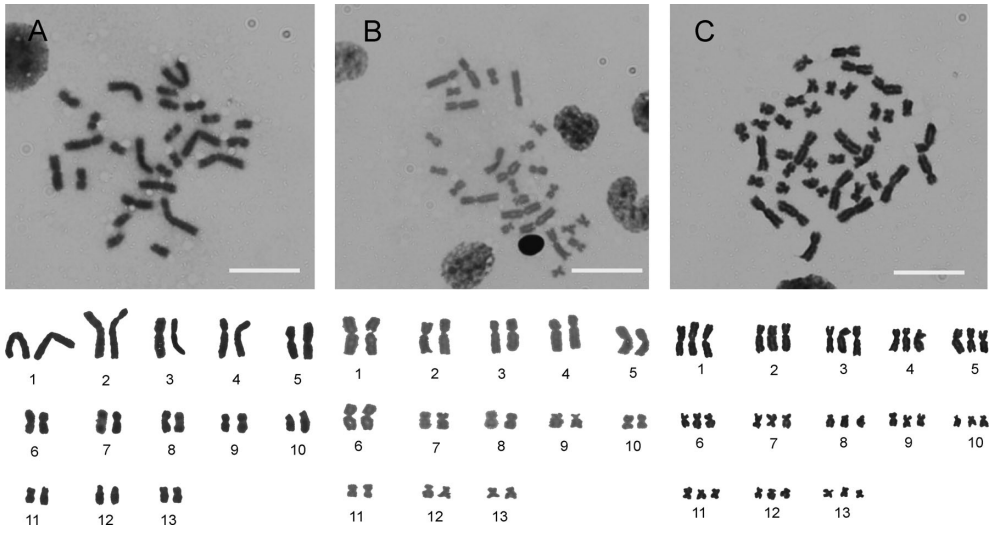

Fig. 3. Metaphase spreads and karyotypes of bone marrow cells from *H. tigerinus*, *H. chinensis* and the interspecific hybrid. **(A)** *H. tigerinus* (tigr. ♀ 1 × tigr. ♂ 1). **(B)** *H. chinensis*. **(C)** Hybrid (tigr. ♀ 3 × chin. ♂ 2) . Scale bar = 10 μm.

tail-bud embryonic stage (14.5%) and were abnormal (Fig. 1E), whereas those of female *E. cyanophlyctis* and male *H. chinensis* (Table 2; 2 series female cyan. 1, 2 with male chin. 2) survived until the feeding tadpole stage (17.3%) but developed abnormally (Fig. 1F). Besides, normally cleaved eggs were extremely few in the crosses of female *H. tigerinus* with male *E. cyanophlyctis*, *F.* sp. (L) and *F.* sp. (S) and crosses of female *E. cyanophlyctis* with *F.* sp. (L), and all died before the tail-bud embryo stage (Table 2). On the other hand, no cleavage was found in any combination with *F. moodei*. We found complete gametic isolation between the female *H. tigerinus* and male *Fejervarya* group and between the female *E. cyanophlyctis* and male *Fejervarya* group.

Interspecific hybrids of *H. tigerinus* and *H. chinensis*

We could not identify the sex of hybrids by external morphology, so we examined the gonads of matured interspecific hybrids after dissection. The 3-year-old hybrids were found to have underdeveloped or abnormal testes. We then proceeded with further karyological and histological studies to elucidate the details of hybrid gonads. The *H. tigerinus* and *H. chinensis* controls had 26 diploid chromosomes, comprising five pairs of large chromosomes and eight pairs of small chromosomes (Fig. 3A, B). Several recent and earlier studies found the chromosome number of *Hoplobatrachus* spp. to be 26 (Chakrabarti et al., 1983; Joshy and Kuramoto, 2008; Shanthi et al., 2010; and Yadav and Pillai, 1975). Interestingly, 39 chromosomes were found in the hybrids (Fig. 3C), and thus we confirmed the hybrids as allotriploid. In addition, we also found more normal sperm bundles in the control *H. tigerinus* and *H. chinensis* (Fig. 4A) than in the hybrids (Fig. 4B). The formation of normal sperm bundles is a key characteristic of the normal maturation process of sperm. Thus, the results

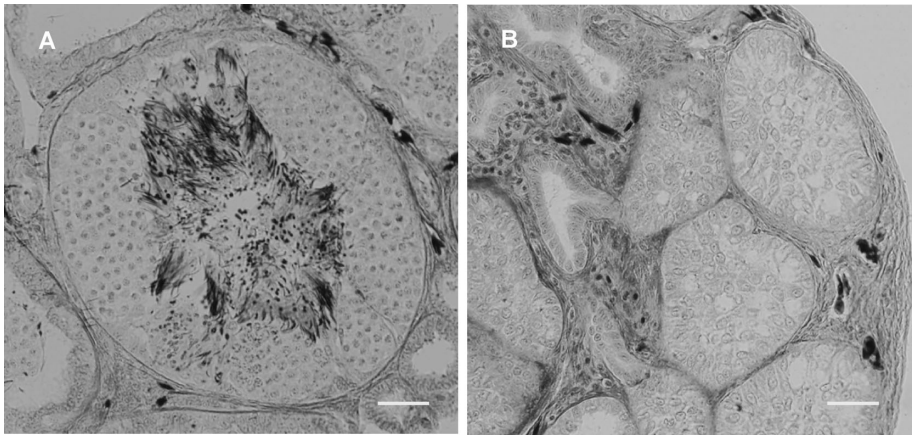

Fig. 4. Histological observations of testes cross sections. **(A)** Control *H. tigerinus* (tigr. ♀ 1 × tigr. ♂ 1). **(B)** Hybrid (tigr. ♀ 3 × chin. ♂ 2). Scale bar = 10 μm.

Table 6. Genotype at 24 enzymes and blood proteins loci in *H. tigerinus* control and *H. chinensis*, their hybrids.

Enzymes or blood proteins	Locus	*H. tigerinus* (control)		Hybrids			*H. chinensis*	
		1 (tigr. ♀ 1 × tigr. ♂ 1)	2 (tigr. ♀ 1 × tigr. ♂ 1)	1 (tigr. ♀ 3 × chin. ♂ 2)	2 (tigr. ♀ 1 × chin. ♂ 1)	3 (tigr. ♀ 3 × chin. ♂ 2)	1	2
Aspertate aminotransferase	AAT-1	AB	BB	BBB	BBB	BBB	BB	BB
	AAT-2	AA	AA	AAA	AAA	AAA	AA	AA
Adenylate kinase	AK	AA	AA	AAA	AAA	AAA	AA	AA
Creatine kinase	CK	AA	AA	AAA	AAA	AAA	AA	AA
Fumarate hydratase	FUM	AA	AA	AAA	AAA	AAA	AA	AA
α-Glycerophosphate dehydrogenase	GDH	BB	BB	ABB	ABB	ABB	AA	AA
Isocitrate dehydrogenase	IDH-1	AC	BB	ACC	BCC	AAB	AA	AA
	IDH-2	AA	AA	AAA	AAA	AAA	AA	AA
Lactate dehydrogenase	LDH-A	BB	BB	ABB	ABB	ABB	AA	AA
	LDH-B	BB	BB	ABB	ABB	ABB	AA	AA
Malate dehydrogenase	MDH-1	BB	BB	ABB	ABB	ABB	AA	AA
	MDH-2	BB	BB	ABB	ABB	ABB	AA	AA
Malic enzyme	ME-1	BB	BB	ABB	ABB	ABB	BC	BC
	ME-2	AA	AA	ABB	ABB	ABB	BB	BB
Mannose phosphate isomerase	MPI	BB	BB	BBB	BBB	BBB	BB	BB
6-Phosphogluconate dehydrogenase	6PGD	AA	AA	AAA	AAA	AAA	AA	AA
Peptidase	PEP-A	AA	AA	AAA	AAA	AAA	AA	AA
	PEP-B	BB	BB	ABB	ABB	ABB	AA	AA
	PEP-D	AA	AA	AAC	AAB	AAB	BC	CC
Phsphoglucomutase	PGM	AA	AA	AAB	AAB	AAB	AB	BB
Superoxide dismutase	SOD	AA	AA	AAA	AAA	AAA	AA	AA
Serum albumin	Alb	BB	AB	ABB	ABB	BBB	AB	BB
Hemoglobin	Hb-1	AA	AA	AAA	AAA	AAA	AA	AA
	Hb-2	BB	BB	ABB	ABB	ABB	AA	AA

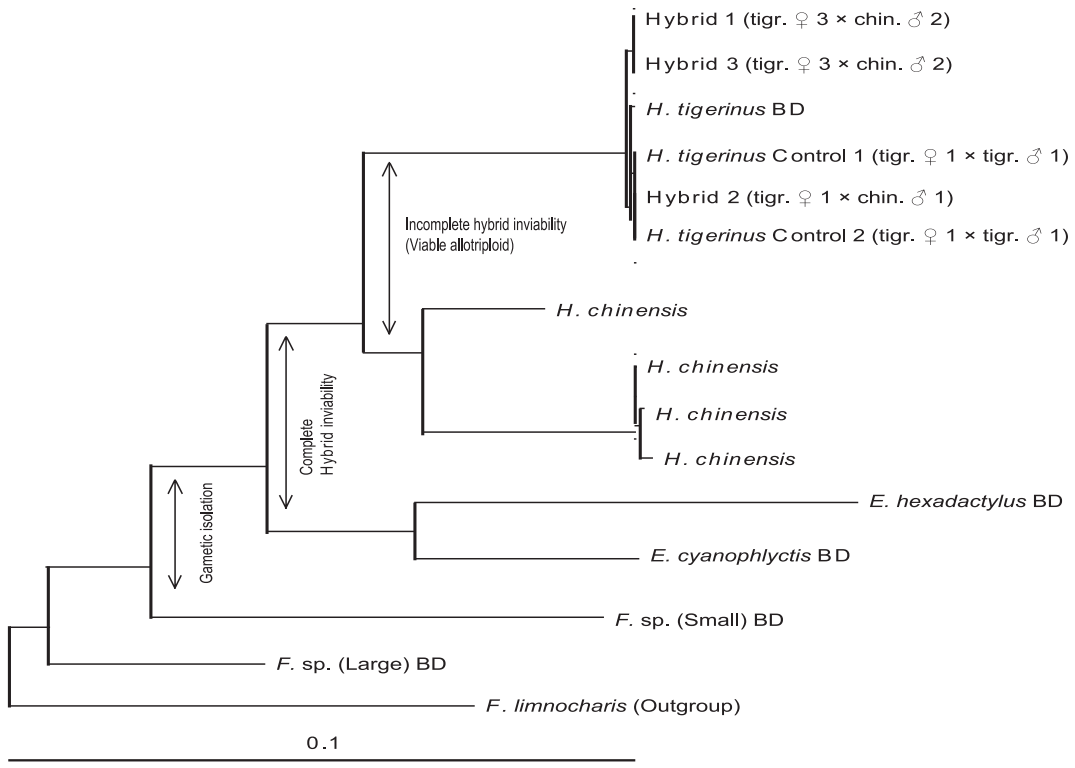

Fig. 5. Phylogenetic tree and isolating mechanisms of genera *Hoplobatrachus*, *Euphlyctis* and *Fejervarya* and the interspecific hybrids (see Table 2) based on 16S rRNA gene sequences. The sequence data of *Hoplobatrachus tigerinus*, *Euphlyctis hexadactylus*, *Euphlyctis cyanophlyctis*, *Fejervarya* sp. (Large type BD) and *Fejervarya* sp. (Small type BD) were collected from the DDBJ data bank under accession numbers of AP011543, AP011544, AB27260, AB372009 and AB372012, respectively.

suggest hybrid spermatogonia cannot mature. Finally, we distinguished the spermatogenetic cell as an undifferentiated cell that become sterile.

Allozyme and mitochondrial DNA analysis

In the allozyme analysis, enzymes and blood proteins were found to be controlled by 24 loci; 12 of these were diagnostic between *H. tigerinus* and *H. chinensis* (Table 6). The allozyme data of interspecific hybrids (allotriploids) suggest that the two genomes have the same maternal origin but different paternal origin. The nucleotide sequences of the mitochondrial Cyt*b*, 12S and 16S rRNA genes of the hybrids were found to be identical to those of the maternal parent *H. tigerinus*. A neighbor joining (NJ) tree was constructed using the mitochondrial Cyt*b*, 12S and 16S gene sequences (Table 4) with *F. limnocharis* as an outgroup that shows a similar topology found by Alam et al. (2008). The hybrids (tigr. × chin.; Table 2) made a cluster with the maternal parent *H. tigerinus* (tigr. × tigr.; Table 2) (Fig. 5). Furthermore, postmating isolation can be explained in the phylogenetic tree by mitochondrial gene sequences. *Hoplobatrachus tigerinus* was isolated from *H. chinensis* by incomplete hybrid inviability (viable allotriploid), genus *Hoplobatrachus* was isolated from genus *Euphlyctis* by complete hybrid inviability, and genus *Fejervarya* was isolated from *Hoplobatrachus* and *Euphlyctis* by gametic isolation (Fig. 5).

DISCUSSION

Postmating isolation in intergenus and interspecies level

Crossing of allopatric species is a useful method for evaluating reproductive isolation processes. Information regarding artificially crossed intergenus postmating isolation between anurans is limited. Dubois (1988, 2007, 2008), and Dubois and Raffaëlli (2009) discussed a mixogenus as a taxon of nomenclatural rank genus that includes at least some taxonomic species in which true diploid hybrid adult (not polyploid, gynogenetic, or androgenetic offspring) are known to have been produced, either naturally or artificially, between specimens belonging to two distinct taxa. This is the first report of crossing between genera *Hoplobatrachus*, *Euphlyctis*, and *Fejervarya*. We found gametic isolation among intergenus species of *Hoplobatrachus*, *Euphlyctis*, and *Fejervarya* in most of the cases of the present study. Interestingly, we found that hybrid tadpoles of female *E. cyanophlyctis* and male *H. chinensis* survived to around twenty days, whereas hybrid embryos of female *E. cyanophlyctis* and male *H. tigerinus* died at around five days. This intergenus reproductive isolation could be regarded as complete hybrid inviability, and the results suggest a closer relationship between *E. cyanophlyctis* and *H. chinensis* than between *E. cyanophlyctis* and *H. tigerinus*, indicating a transitional stage with respect to reproductive isolation between *Hoplobatrachus* and *Euphlyctis* genera. Perhaps, the structure and physiological matter of the eggs and sperm of

these intergeneric groups hindered zygote formation. Kawamura et al. (1981) also observed and reported gametic isolation among indigenous species of brown frogs due to the physiological properties of egg and sperm. However, Dubois (1988, 2004) described that crossability between species is not an intrinsic ability of either species but rather a "relational taxonomic criterion" or "relacter" between them and that its use does not rely on bearing information on cladistics relationships, but on the overall genetic divergence between each species after separation. The most intriguing finding in the present study is the production of interspecific sterile hybrids between female *H. tigerinus* and male *H. chinensis*. Our results are in agreement with those of Sasa el al. (1998) who found that hybrid sterility in anurans appears to evolve more rapidly than inviability. As the spontaneous production and remarkable survival of these hybrids was found to be due to allotriploidy; we focus our discussion on this matter in a later section. However, there has also been some debate that ploidy elevated taxa (e.g., tetraploid and triploid) should be excluded from the phylogenetic tree (Mable et al., 2011). In contrast, Sumida et al. (2007) found that the phylogenetic relationships among taxa are closely related to the degree of reproductive isolation, and the present results also suggest the close relationships between the phylogenetic trees and crossing experiments (Fig. 5). New species that evolved from a single common ancestor are thought to be monophyletic, whereas allotriploid species with polyphyletic origins often exhibit coherent phenotypes and occupy distinct ecological niches (Vrijenhoek, 2006). We believe that the present findings of the hybrid sterility of allotriploids can be used as a reference in future integrative taxonomical studies.

Mechanism of the production of allotriploids

The existence of triploid vertebrates is thought to be a rare phenomenon in nature, although some reports explain the intentional production of allotriploids by crossing experiments (Sumida et al., 2003; Ohtani, 1993; and Kashiwagi, 1993). However, in the present study, allotriploids were unexpectedly found in crosses between two species of genus *Hoplobatrachus*, but all such diploid hybrids eventually died before metamorphosis. The production of unreduced gametes can cause the production of ploidy individuals, and several factors may be involved in the production of allotriploids. Establishment and survival of these organisms depends on several reproductive modes such as parthenogenesis or gynogenesis, the polyspermy mechanism, and autopolyploidy. At present, our allozyme data suggest that two genomes were of maternal, and one of paternal origin. The spontaneous production of triploids is possibly due to the over-ripening of eggs and that facilitated the incorporation of the second polar body, which gave the zygote three sets of chromosomes. Sumida et al. (2007) also discussed similar patterns of triploid production in cases of hybrid allotriploids between *R. catesbeiana* and *R. clamitans*, as described by Elinson and Briedis (1981). Another possibility of the formation of allotriploids is that the previous ancestor (parent) is tetraploid, but our kariological studies confirmed that the parents are in fact, diploid.

Viability of allotriploids

Generally speaking, triploid tadpoles are more viable than diploid ones of the same species (Kawamura, 1952; Nishioka, 1971, 1983; Sumida and Nishioka, 1993). Bogart (1972) also reported that triploid hybrids between distantly related species of *Bufo* were more viable than diploids. Sasa et al. (1998) conferred hybrid sterility appears to evolve more quickly than inviability in anurans. In our crosses, all diploid hybrid tadpoles produced between *H. tigerinus* and *H. chinensis* became underdeveloped and died before metamorphosis, unlike triploid tadpoles. The closely related species of *Hoplobatrachus* may have allowed further survival of their hybrids than other distantly mating species through the production of triploids, as we have not yet found a diploid hybrid. It can be suggested that the sperm and egg of closely related species are prone to zygote formation and that some cases the hybrids become more stable, survive, and metamorphose through the production of triploid instead of diploid states. Polyploids have been thought to persist when they can exploit new habitats to avoid competition with their diploid progenitors. It is often assumed that polyploids spread because they can tolerate and invade harsher environments than their diploid counterparts owing to increased hardiness and/or increased genetic buffering provided by having "extra" genome copies (Mable, 2003). However, it remains unclear how general this pattern is. By contrast, retention of the same body size by reducing the number of cells in some animals means that tetraploids are often quite morphologically indistinguishable from their diploid progenitors. The present allotriploids were also similar to the normal diploid controls in body size, suggesting the possibility of positive assortative mating among closely related species around genera *Hoplobatrachus* and *Euphlyctis*.

Testes and reproductive capacity of allotriploids

Triploid males of several species often develop testes and successfully complete spermatogenesis but are in general sterile due to the production of aneuploid and/or abnormally shaped spermatozoa, although some may produce functional sperm and generate embryos with an extremely low survival rate (Sousa-Santos et al., 2007). In contrast, unreduced sperm production by allotriploid is also theoretically possible, which would give rise to a viable tetraploid offspring after fertilization of a haploid egg. Generally, allotriploidy is coupled with infertility, since normal meiosis is likely to be disrupted by cytological mechanisms that preclude synapsis between heterospecific chromosomes (Dawley, 1989). Our histological study failed to find spermatogonia in the hybrid allotriploid; rather undifferentiated cells were observed, signifying disruption of further sperm maturation. Nishioka and Ohtani (1984) reported thin seminiferous tubules without germ cells in the testes of allotriploids between *R. nigromaculata* and *R. lessonae*. Kashiwagi (1993) also reported that artificially produced triploid frogs of *R. rugosa* were males or hermaphrodites which had transformed into males. Our results finally show that the allotriploids become degenerative sterile male.

Significance of allotriploids

It has been reported that the production of bisexual allotriploid vertebrates has been possible by mating three pairs of triploid Batura toads (Stöck et al., 2002, 2010).

Sumida and Nishioka (1993) reported that triploids between *R. tsushimensis* and *R. japonica* developed normally and reached maturity and that some triploids reproduced by "hybridogenesis" in which the *R. japonica* genome was eliminated during spermatogenesis. We found sterile allotriploids between female *H. tigerinus* and male *H. chinensis* by artificial crossing; whereas it has been reported that diploid and tetraploid populations were found in African *H. occipitalis* (*Dicroglossus occipitalis*) populations in nature (Bogart and Tandy, 1976). However, Mable et al. (2011) explained in detail genome duplication in amphibians and fish, and they suggested that the polyploids could be formed in nature during times of climatic instability. We also found duplication events in the mt genome of *Hoplobatrachus* and *Euphlyctis* genera in our previous paper (Alam et al., 2010). At present, we would like to avoid further discussion about the possibility of a past evolutionary route of tetraploid formation via the triploid stage in nature. In future studies, cryptic species of *Hoplobatrachus*, *Euphlyctis*, and *Fejervarya* genera need to be studied more closely to understand the polyploidization that might occur during the formation of species using multiple genetic markers.

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