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Artificial Production and Natural Breeding of the Endangered Frog Species *Odorrana ishikawae*, with Special Reference to Fauna Conservation in the Laboratory

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***Odorrana ishikawae* is listed as a class IB endangered species in the IUCN Red List and is protected by law in both Okinawa and Kagoshima Prefectures, Japan. Here, in an effort to help effectively preserve the genetic diversity of this endangered species in the laboratory, we tested a farming technique involving the artificial breeding of frogs, and also promoted natural breeding in the laboratory. Field-caught male/female pairs of the Amami and Okinawa Island populations were artificially bred using an artificial insemination method in the 2004, 2006, and 2008 breeding seasons (March to April). Although fewer than 50% of the inseminated eggs achieved metamorphosis, approximately 500, 300, and 250 offspring from the three respective trials are currently being raised in the laboratory. During the 2009 and 2010 breeding seasons, second-generation offspring were produced by the natural mating activities of the first offspring derived from the two artificial matings in 2004. The findings and the methods presented here appear to be applicable to the temporary protection of genetic diversity of local populations in which the number of individuals has decreased or the environmental conditions have worsened to levels that frogs are unable to survive by themselves.**

Key words: artificial insemination, natural breeding, second generation, endangered frog, protected species, IUCN Red List, *Odorrana ishikawae*

INTRODUCTION

Odorrana ishikawae (sensu lato: see below), a species endemic to the Okinawa and Amami Islands, Japan (Figs. 1A, 2), has been described as the most beautiful frog in Japan (Maeda and Matsui, 1999). Regrettably, over-hunting and environmental destruction over the last several decades have devastated the populations of this species. *Odorrana ishikawae* is therefore listed as a class IB endangered species (Environmental Agency, 2000; IUCN, 2010), and is protected by law in both Okinawa and Kagoshima Prefectures (Oumi, 2006; Iwai and Watari, 2006). Thus, it is urgently necessary to protect the local populations in which the number of individuals has decreased. Moreover, environmental conditions have worsened to levels that frogs are unable to survive by themselves.

The morphological characteristics and natural breeding habitats of *O. ishikawae* have been observed in detail in the Okinawa and Amami Islands (e.g., Utsunomiya et al., 1979, 1983). According to these studies, a male frog typically inhabits a hole along a stream during the breeding season, and invites females to this hole by calling, resulting in non-synchronous spawning by females. The natural breeding of *O. ishikawae* has been attempted previously in the laboratory using four wild males and females, but was unsuccessful (Shiihara and Samejima, 1995). In the present study, we performed artificial breeding of frogs, examined a rearing technique, and promoted the natural breeding of frogs in the laboratory in efforts to help preserve the genetic diversity of this endangered species.

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Fig. 1. *Odorrana ishikawae* brood frogs. **(A)** *Odorrana ishikawae* from Amami Island. Scale bar = 3 cm. **(B)** An artificial breeding pair from Amami Island. Scale bar = 3 cm. **(C)** Four-year-old frogs derived from artificial breeding in 2004. Scale bar = 5 cm. **(D)** Natural breeding in the laboratory. Scale bar = 6 cm.

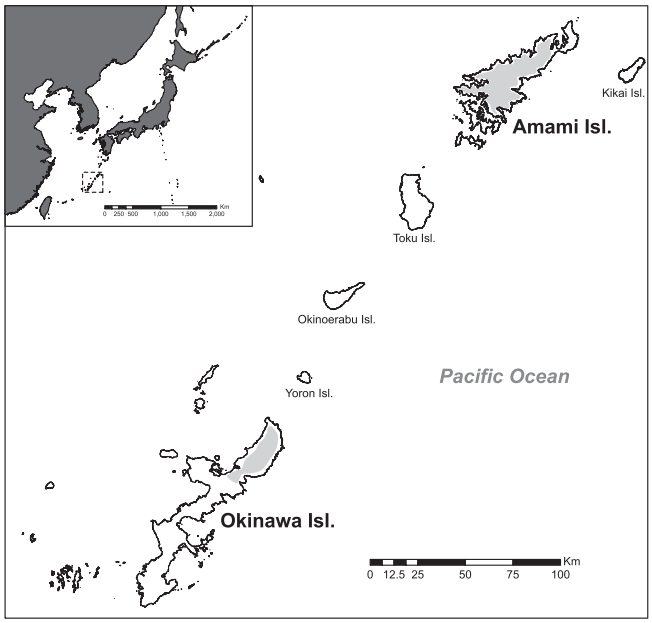

Fig. 2. Distribution map of *Odorrana ishikawae.* The distribution areas are indicated by grey color.

MATERIALS AND METHODS

Four male/female pairs obtained from Amami Island (Fig. 1B) and three pairs from Okinawa Island were bred using an artificial insemination method slightly modified from Nishioka (1982). Ovulation was accelerated by the injection of 0.5 ml frog Ringer's solution containing one bullfrog pituitary into the body cavity of each of Amami and Okinawa female frogs, which were maintained at 18 and 20°C, respectively. For in vitro ovulation experiments, standard frog Ringer's solution was used as the medium for pituitary suspensions. Anterior lobes of pituitaries were obtained from adult *Lithobates catesbeianus* (generally called *Rana catesbeiana*) and dried with acetone. Each pituitary suspension was prepared by grinding a single dried pituitary in 0.5 ml frog Ringer's solution. Ovulation was induced by injecting 0.5 ml pituitary suspension per frog.

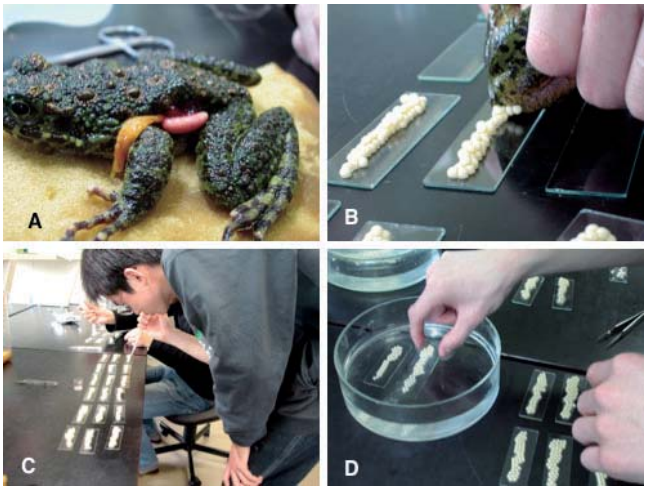

Fig. 3. In vitro artificial insemination method. **(A)** Testes were removed from male abdomens. **(B)** Eggs were stripped from females and placed on slide glasses. **(C)** Eggs were artificially inseminated with a sperm suspension. **(D)** After fertilization, eggs were developed in glass Petri dishes.

Sperm suspensions were prepared from a small piece of testis taken from each male under anesthetized conditions, without sacrificing them (Fig. 3A). After eggs were stripped from females and placed on slide glasses (Fig. 3B), they were artificially inseminated with the sperm suspensions (Fig. 3C). Following fertilization, eggs were reared in glass Petri dishes, and were stored in an incubator at 18 and 20°C for the Amami and Okinawa eggs, respectively (Figs. 3D, 4A, B). Hatched tadpoles were transferred into concrete tanks and fed a diet of boiled spinach (Fig. 4C, D). Metamorphosed frogs were then moved to the frog room at 25°C and fed a diet of crickets (Fig. 4E, F). At each stage, the normally developed embryos and tadpoles were counted, and the developmental capacity (survival rate) was calculated.

RESULTS

Development of artificially inseminated eggs

The early development of an artificially inseminated *O. ishikawae* egg is illustrated in Fig. 5. Eggs of this species are white and comparatively large with a diameter of 4 mm (Fig. 5A). The first cleavage event occurred five hours after insemination, which represented the initiation of the early development stages (Fig. 5B–I). At six and 11 days after insemination, tail-bud embryos (Fig. 5J) and hatched tadpoles (Fig. 5K), respectively, were formed, with the tadpoles developing into feeding tadpoles by day 19 (Fig. 5L). The tadpoles reached 35 mm in total body length 60 days after insemination (Fig. 5M), and completed metamorphosis within 3–4 months of the initial artificial insemination (Fig. 5N).

Developmental capacity

The developmental capacity of artificially inseminated eggs was evaluated by determining the number of eggs that formed normally developed tadpoles. Among the 3,078 eggs derived from four mating pairs of Amami frogs in 2004 and 2006, 2,742 (89.1%) cleaved normally, 2,536 (82.4%) developed into normal tail-bud embryos, 1,761 (57.2%) became normal 30-day-old tadpoles, and 1,390 (45.2%) metamorphosed normally (Table 1, Fig. 6A). A similar developmental capacity was observed for the artificially bred Okinawa

Fig. 4. Post-breeding rearing facilities in the laboratory. **(A, B)** Embryos (150–200 individuals) were reared in a glass Petri dish (18 cm × 5 cm) and maintained in a temperature-controlled incubator at 18 and 20°C for eggs of Amami and Okinawa frogs, respectively, with lights on from 8:00 to 18:00. **(C, D)** Tadpoles (around 100 individuals) were transferred to a concrete tank (70 cm × 80 cm × 20 cm) in a glass-made green house kept between 20 and 25°C, and were fed a diet of boiled spinach. **(E, F)** Metamorphosed frogs (around 50 individuals) were housed in the terrarium (40 cm × 60 cm × 15 cm) of the frog room controlled at 25°C with lights on from 8:00 to 18:00, and were fed a diet of crickets.

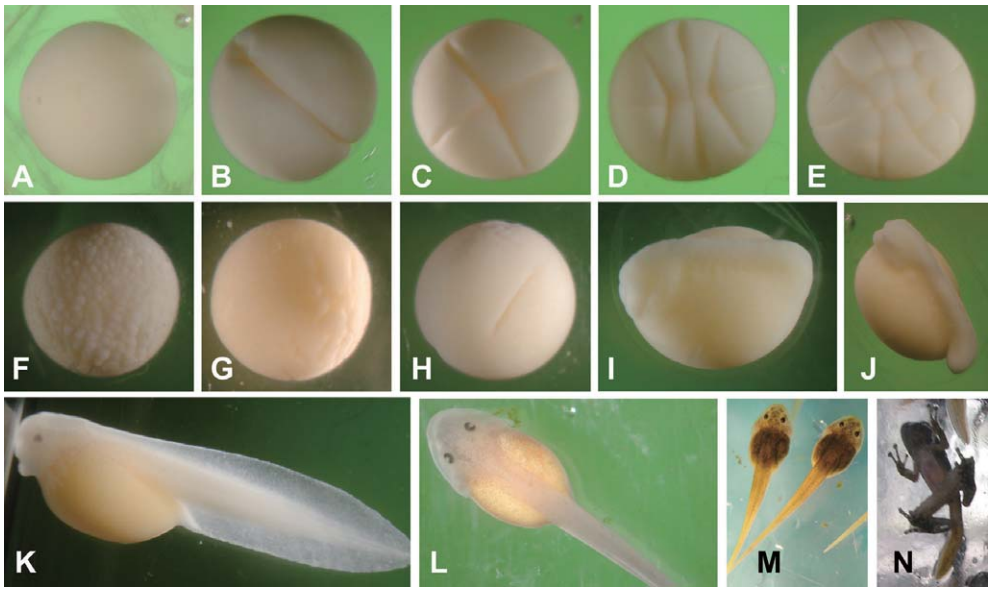

Fig. 5. Early developmental stages of an artificially inseminated egg. **(A)** Fertilized eggs (0 h, egg diameter = 4 mm). **(B)** 2-cell stage (5 h). **(C)** 4-cell stage (6 h). **(D)** 8-cell stage (8.5 h). **(E)** 16-cell stage (9.5 h). **(F)** Morula stage (17.5 h). **(G)** Blastula stage (29 h). **(H)** Gastrula stage (68 h). **(I)** Neurula stage (106 h). **(J)** Tail-bud stage (6 days). **(K)** Hatched tadpole (11 days). **(L)** Feeding tadpole (19 days). **(M)** 60-day-old tadpoles (60 days). **(N)** Metamorphosed froglet (96–120 days).

frogs. Among the 647 eggs derived from three mating pairs of Okinawa frogs in 2008, 537 (83.0%) cleaved normally, 457 (70.6%) formed normal tail-bud embryos, 352 (54.4%) became normal 30-day-old tadpoles, and 313 (48.4%) metamorphosed normally (Table 1, Fig. 6B). From the above mating pairs of Amami and Okinawa frogs, approximately 500 six-year-old, 300 four-year-old, and 250 two-year-old frogs successfully produced via artificial breeding in 2004, 2006, and 2008, respectively, are currently being reared in the laboratory (Fig. 1C).

Natural breeding of *O. ishikawae* in the laboratory

In an attempt to further propagate frogs in the laboratory, the natural mating activities of five- and six-year-old Amami frogs were promoted during the 2009 and 2010 breeding seasons, respectively (Tables 2, 3). In the 2009 breeding season, 21 egg masses were naturally deposited by female frogs produced artificially in 2004 (Fig. 1D). Among 7,643 eggs derived from the 21 egg masses, 2,462 (32.2%) formed normal tail-bud embryos, 1,607 (21.0%) became normally hatched tadpoles, 821 (10.7%) developed into normally feeding tadpoles, and 588 (7.7%) metamorphosed normally (Table 2). In the 2010 breeding season, the identical group of female frogs naturally deposited 77 egg masses containing a total of 18,405 eggs, of which 4,485 (24.4%) formed normal tail-bud embryos, 2,198 (11.9%) became normally hatched tadpoles, 1,840 (10.0%) developed into normally feeding tadpoles, and 1,113 (6.0%) metamorphosed normally (Table 3).

DISCUSSION

The artificial insemination technique described by Nishioka (1982), which involves the injection of a bullfrog pituitary into the female body cavity to induce ovulation, has been applied to numerous species, including toads (Kawamura et al., 1980), brown frogs (Kawamura et al., 1981; Sumida et al., 2003), pond frogs (Kawamura and Nishioka, 1986), stream frogs (Nishioka et al., 1987), and hylid frogs (Kawamura et al., 1990). In this study, we demonstrated that this technique is also applicable to the artificial breeding of *O. ishikawae*. After rearing artificially bred frogs in the laboratory, second-generation frogs were successfully produced through natural mating activities.

We identified differences in the adaptation temparature for egg maturation and embryonic development between the

Table 1. Developmental capacity of offspring produced by artificial breeding.

Date	Parents		No. of eggs	Normally cleaved eggs (%)	Normal tail-bud embryos (%)	Normally hatched tadpoles (%)	Normally feeding tadpoles (%)	30-day-old tadpoles (%)	Metamorphosed frogs (%)
	Female	Male							
2004/4/1	Amami ♀ 1	Amami ♂ 1	849	587 (69.1)	565 (66.5)	548 (64.5)	541 (63.7)	490 (57.7)	300 (35.3)
2004/4/7	Amami ♀ 2	Amami ♂ 2	1,284	1,238 (96.4)	1,116 (86.9)	1,041 (81.1)	965 (75.2)	813 (63.3)	680 (53.0)
2006/3/1	Amami ♀ 3	Amami ♂ 3	484	473 (97.7)	448 (92.6)	417 (86.2)	286 (59.1)	215 (44.4)	187 (38.6)
2006/3/23	Amami ♀ 4	Amami ♂ 4	461	444 (96.3)	407 (88.2)	396 (85.9)	372 (80.7)	243 (52.7)	223 (48.4)
Total			3,078	2,742 (89.1)	2,536 (82.4)	2,402 (78.0)	2,164 (70.3)	1,761 (57.2)	1,390 (45.2)
2008/3/18	Okinawa ♀ 1	Okinawa ♂ 1	304	277 (91.1)	270 (88.8)	245 (80.6)	240 (78.9)	224 (73.7)	218 (73.1)
2008/3/18	Okinawa ♀ 2	Okinawa ♂ 1	117	106 (90.6)	93 (79.5)	77 (65.8)	73 (62.4)	38 (32.5)	35 (29.9)
2008/3/18	Okinawa ♀ 3	Okinawa ♂ 1	226	154 (68.1)	94 (41.6)	90 (39.8)	86 (38.1)	63 (27.9)	60 (26.5)
Total			647	537 (83.0)	457 (70.6)	412 (63.7)	412 (63.7)	352 (54.4)	313 (48.4)

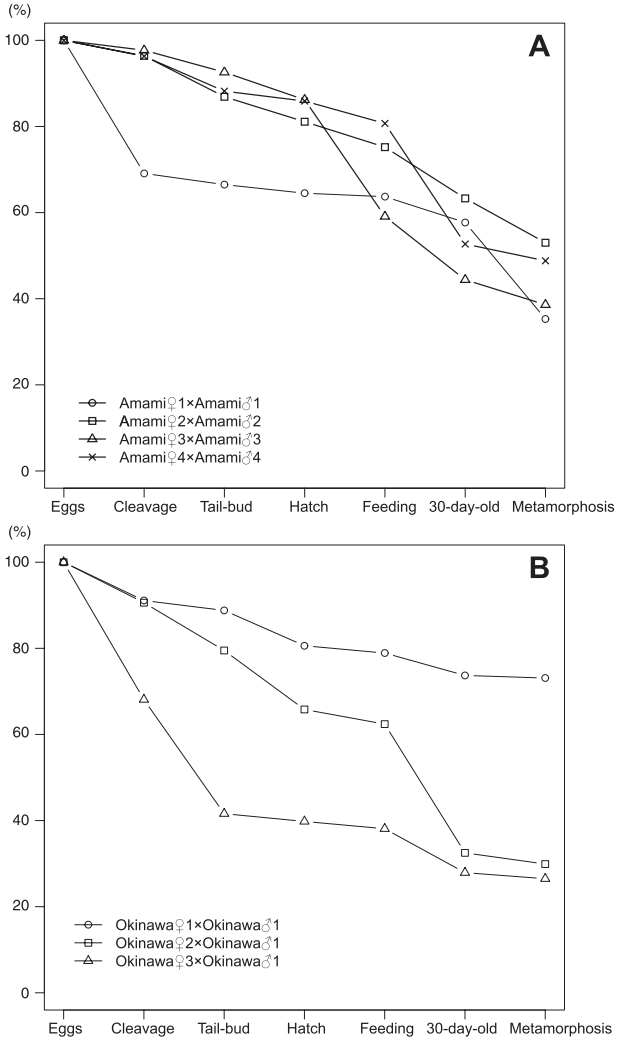

Fig. 6. Survival curve of the offspring of the Amami **(A)** and Okinawa **(B)** populations produced through artificial breeding at 18 and 20°C, respectively. Beginning with the first cleavage, viability at six early developmental stages was monitored to evaluate developmental capacity.

Table 2. Developmental capacity of second-generation offspring in 2009. —, mixed in one container and no data.

Date	Parents		No. of eggs	Normal tail-bud embryos	Normally hatched tadpoles	Normally feeding tadpoles	Metamorphosed frogs
	Female	Male					
2009/3/31	E04 No. 1	E04 No. 1	352	102	13	5	–
	E04 No. 2	E04 No. 2	367	0	0	0	0
	E04 No. 3	E04 No. 3	271	56	9	2	–
	E04 No. 4	E04 No. 4	341	0	0	0	0
2009/4/4	E04 No. 5	E04 No. 5	254	0	0	0	0
	E04 No. 6	E04 No. 6	503	0	0	0	0
	E04 No. 7	E04 No. 7	247	203	101	48	–
	E04 No. 8	E04 No. 8	187	0	0	0	0
	E04 No. 9	E04 No. 9	545	358	230	82	–
	E04 No. 10	E04 No. 10	213	58	25	10	–
	E04 No. 11	E04 No. 11	135	43	12	6	–
2009/4/6	E04 No. 12	E04 No. 12	258	0	0	0	0
	E04 No. 13	E04 No. 13	203	0	0	0	0
	E04 No. 14	E04 No. 14	141	109	64	28	–
	E04 No. 15	E04 No. 15	482	202	156	42	41
2009/4/9	E04 No. 16	E04 No. 16	606	305	184	73	70
	E04 No. 17	E04 No. 17	430	306	205	85	76
	E04 No. 18	E04 No. 18	207	0	0	0	0
	E04 No. 19	E04 No. 19	328	0	0	0	0
2009/4/10	E04 No. 20	E04 No. 20	623	0	0	0	0
	E04 No. 21	E04 No. 21	950	720	608	440	401
Total			7,643	2,462 (32.2%)	1,607 (21.0%)	821 (10.7%)	588 (7.7%)

Amami and Okinawa populations of *O. ishikawae*, which is a similar trend to that found in populations of *Rana pipiens* (Moore, 1946, 1947) and *Buergeria buergeri* (Ueda et al., 1998). Based on our trials (data not shown), it was determined that the ideal temperatures for the ovulation and maturation of eggs, and the rearing of embryos and larvae are 18 and 20°C for frogs of the Amami and Okinawa populations, respectively. From the climate statistics of the Japan Meteorological Agency, the mean annual temperature and precipitation are 22.5°C (19.7–25.4°C) and 2127.3 mm in Nago, Okinawa and 21.5°C (18.6–24.7°C) and 2913.5 mm in Naze, Amami. During the breeding season (December to March), the mean temperature and precipitation are 17.1°C (15.9–18.4°C) and 514.8 mm in Nago, Okinawa and 15.7°C

Table 3. Developmental capacity of second-generation offspring in 2010.

Date	Parents		No. of eggs	Normal tail-bud embryos	Normally hatched tadpoles	Normally feeding tadpoles	Metamorphosed frogs
	Female	Male					
2010/3/10	E04No. 1– 4	E04No. 1– 4	776	37	25	18	3
2010/3/12	E04No. 5–10	E04No. 5–10	1,453	92	4	4	3
2010/3/13	E04No. 11	E04No. 11	401	295	59	18	6
2010/3/14	E04No. 12–17	E04No. 12–17	1,114	692	186	156	108
2010/3/15	E04No. 18–21	E04No. 18–21	530	92	43	40	27
2010/3/16	E04No. 22–33	E04No. 22–33	2,822	1,304	686	639	492
2010/3/17	E04No. 34–37	E04No. 34–37	995	170	87	79	63
2010/3/18	E04No. 38–43	E04No. 38–43	1,605	289	184	155	22
2010/3/19	E04No. 44	E04No. 44	228	0	0	0	0
2010/3/20	E04No. 45–46	E04No. 45–46	319	40	31	31	13
2010/3/21	E04No. 47–53	E04No. 47–53	1,472	151	82	81	78
2010/3/22	E04No. 54–63	E04No. 54–63	3,340	390	265	249	92
2010/3/23	E04No. 64–66	E04No. 64–66	723	230	115	97	61
2010/3/24	E04No. 67–70	E04No. 67–70	750	269	188	164	91
2010/3/26	E04No. 71–72	E04No. 71–72	741	235	98	68	46
2010/3/27	E04No. 73–74	E04No. 73–74	426	105	90	15	3
2010/3/28	E04No. 75–76	E04No. 75–76	573	94	55	26	5
2010/3/30	E04No. 77	E04No. 77	137	0	0	0	0
Total			18,405	4,485 (24.4%)	2,198 (11.9%)	1,840 (10.0%)	1,113 (6.0%)

(14.6–17.0°C) and 739.3 mm in Naze, Amami. The observed difference in optimal temperature for egg maturation and embryonic development is likely a result of habitat differences between the Amami and Okinawa populations, with the more northerly Amami Island subject to cooler temperatures.

To evaluate the feasibility of this artificial breeding approach, the developmental capacity of artificially inseminated eggs was monitored in detail. As a whole, the survival power of *O. ishikawae* during early development was not strong, with fewer than 50% of inseminated eggs on average achieved metamorphosis (Table 1). Notably, the mortality rate was highest during the period from the 30-day-old larval stage to immediately after metamorphosis. One reason for the high mortality during this period was that frogs often suffered from infection during the summer season, when the water temperature was higher. Thus, to limit the risk of infection, it is important to maintain clean water with cool temperatures during the summer season. In the field, certain larvae require up to one year to complete metamorphosis (Utsunomiya et al., 1983); however, all of the tadpoles completed metamorphosis within four months after artificial insemination in the laboratory environment. This finding may be related to temperature and diet, as our larvae were kept in a concrete tank of a glass-made green house and typically fed a diet of boiled spinach to promote healthy growth and development.

The findings presented here regarding artificial insemination and the raising of larvae are useful for the breeding of *O. ishikawae* in a laboratory setting and are applicable for the preservation of genetic diversity in the wild populations of this species. During the 2009 and 2010 breeding seasons, second-generation offspring were produced through the natural mating activities of five- and six-year-old frogs derived from two artificial matings in 2004. This is the first report of the generation of second-generation offspring of *O. ishikawae* by facilitated natural breeding in a laboratory, although the rate of metamorphosis was low (7.7% in 2009 and 6.0% in 2010). This technique can also be used for the temporary protection of local populations, in which the number of individuals has decreased or environmental conditions have worsened to levels that prevent frogs from being able to survive by themselves. However, despite these promising results, it is necessary to improve the rate of normal development of second-generation offspring produced by natural breeding, and evaluate the genetic diversity of artificially and naturally bred individuals in the laboratory to effectively conserve diversity. These studies are now underway in our laboratory.

Odorrana ishikawae is geographically isolated on the Amami and Okinawa Islands, and it is thought that these two populations resulted when these islands were separated in Japan (approximately 3.2–1.5 Ma) (Maeda and Matsui, 1999; Ota, 2003; Matsui et al., 2005). Numerous differences have been observed in the morphological and ecological characters (Utsunomiya et al., 1979), karyotypes (Seto et al., 1984), genetic characters (Matsui et al., 2005; Sumida et al., 2008), and postmating isolation (Kuramoto et al., 2011) of the Amami and Okinawa populations of *O. ishikawae*. Recently, the Amami population of this species has been described as a new species *O. splendida* (Kuramoto et al., 2011). Thus, preservation efforts should be aimed at conserving not only the Amami, but also the Okinawa population of *O. ishikawae* frogs. The successful artificial breeding approach for both the Amami and Okinawa populations described in the present study has the potential to aid in the conservation of genetic diversity of *O. ishikawae*.

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