

**Reproductive ecology of two species of the mudskippers,
Periophthalmodon septemradiatus and *Periophthalmus modestus***

マッドスキッパー2種 *Periophthalmodon septemradiatus* と *Periophthalmus modestus* の再生産生態

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**Reproductive ecology of two species of the mudskippers,
Periophthalmodon septemradiatus and *Periophthalmus modestus***

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Abstract

Chapter 1 General introduction

Mudskippers are amphibious fish in the subfamily Oxudercinae of the family Gobiidae. Mudskippers typically live in intertidal habitats and exhibit unique adaptations to this environment. Mudskippers are quite active when out of the water, feeding and interacting with one another, for example, to defend their territories and court potential partners. Studies of mudskippers could provide important insight into the vertebrate transition from water to land, which is believed to have occurred in the Devonian period, one of the most important evolutionary innovations of the vertebrates.

Chapter 2 Reproductive ecology of *Periophthalmus modestus* in the Ariake Sea, Japan

Periophthalmus modestus breeds from May to August, in the Ariake Sea. The reproductive behaviors of the species were partly reported earlier, but there are still uncertainties including how *P. modestus* spawns and fertilizes the eggs in a burrow. This study was conducted in the Fukushoe River, Saga Prefecture in 2016. Reproductive behaviors from the burrow entrance of a pair to the departure of a female were recorded and analyzed on five pairs of *P. modestus*. In those cases where spawning was suspected from a drastic reduction in the distension of the female's belly (N = 4), the female remained inside the burrow for 4-6 hours without emersion. In contrast, the male often but irregularly emerged from his burrow while the female was in his burrow. When outside his burrow, the male mostly remained around the burrow openings and protected the burrow from conspecifics and crabs. During the burrow cohabitation of the pair, which started soon after mudflat emersion, burrow openings were often clogged by mud from inside. After the female left the burrow, the male remained inside the burrow for variable periods (several minutes to longer than 1 hour). Then, the male guarded the burrow for about

one week. Upon termination of the one-week burrow guarding, the male began excavating a new burrow near the previous one or refurbish the previous burrow.

Chapter 3 Reproductive ecology of *Periophthalmodon septemradiatus* in the Mekong Delta, Vietnam

Since 2015 I have conducted field surveys along the Hau River and Tien-Co Chien River, two major tributary of the Mekong River, from the river mouth up to the border between Vietnam and Cambodia. Through the field survey, I found that one species of mudskippers, *Periophthalmodon septemradiatus*, inhabits over a 150 km stretch of the riparian zones along the Mekong River in Vietnam. *Periophthalmodon septemradiatus* was mainly distributed in tributaries and rarely found in the mainstream of the Hau River and Tien-Co Chien River, except in the most downstream reach. Adult *Periophthalmodon septemradiatus* is highly terrestrial and has not been observed to venture into the water during my survey. Courtship behavior was observed, and fertilized eggs were recovered from burrows in both brackish and freshwater environments. The smallest fish collected at 12, 96, and 148 km from the river mouth were juveniles shortly after starting an amphibious life. These findings suggest reproduction in both brackish and freshwater environments. In contrast, the otolith Sr:Ca ratio indicates larval hatching only in brackish water. Analysis of a 940-base pair (bp) segment of the mitochondrial cytochrome c oxidase subunit II and a 934-bp segment of the mitochondrial D-loop demonstrated no genetic segregation between populations.

Chapter 4 General discussion and future direction

Reproduction in mudskippers is enigmatic because it occurs within burrows excavated in the mudflat to which is quite difficult for a researcher to access.

There are many unanswered questions on the reproductive ecology of mudskippers. *P. modestus* could be a model species in this respect because of relatively easy accessibility and ample background information. Three important points during burrow resident time of *P. modestus* include:

(1) Why female fish did not come out from the burrow for extended periods?

One possibility is that females were not ready for spawning when they entered a burrow. An unpublished study by Prof. Takita suggested that eggs were not ovulated in females foraging on the mudflat surface (but see page 20). A sampling of female fish is needed at various times after the fish enter the burrow to clarify this point.

(2) Why they clog the burrow opening(s) during spawning?

This could be to avoid the invasion of other animals living in the mudflat, including conspecifics. I observed in some cases that another non-burrow guarding male entered a burrow possibly to consume or fertilize the laid eggs, and in these cases the female left the burrow. We need to analyze the behavior of a male and a female fish within the burrow.

(3) Do male fish add air before or after spawning?

After the female fish left the burrow, the male fish stayed inside the burrow for a while. What did male fish do during this time? It is hard to observe inside mudskipper burrows but we may be able to observe the spawning chamber by setting up an endoscope. By chance, I observed in a laboratory aquarium that a male *P. variabilis* added air only after spawning but this may be species-specific (Rupp, unpublished data). To confirm this behavior, we need to excavate and sample the air in the spawning chamber right after the female fish leaves the burrow or to observe with an endoscope installed in a spawning chamber with minimal disturbance to the natural behavior of the fish within the burrow.

Our finding of *Pn. septemradiatus* inhabiting and spawning in the freshwater river banks have added further complicacy to the question of mudskipper reproduction. Two important questions about *Pn. septemradiatus* reproduction include:

(1) What limits the distribution of *Periophthalmodon septemradiatus*?

The distribution of *Pn. septemradiatus* is unique not only because of its extended range into the upstream direction but also restricted occurrence along the coastal mudflats. To gain a clue to understanding factors limiting the upstream distribution of *Pn. septemradiatus*, I measured the most basic environmental parameters within and beyond the distribution range of the species. None of the measured parameters seems to be responsible for preventing the fish from migrating beyond the observed distribution range. Some limitations that restrict successful reproduction might be involved. Larval hatching, growth, and survival at various salinity conditions must be tested in controlled conditions in the laboratory. The swimming ability of the larvae and juveniles must also be investigated.

(2) Where does the fish reproduce, in freshwater, in coastal areas, or both?

The courtship display was found from all observation sites from the coast to the upper limit of distribution. These results support that the fish reproduce in both freshwater and coastal areas. On the other hand, the Sr:Ca ratio indicated that all the samples (coastal and freshwater samples) were spawned in high salinity conditions because Sr:Ca is much higher than the seawater value of 8.4 (mmol/mol, de Villiers et al., 1994). This suggests that all larvae hatched in freshwater sites failed. More field sampling on the distribution of larvae and juveniles needs to be done together with environmental conditions.

Pn. septemradiatus is probably one of the most terrestrial fishes living today. During four years of our field survey in the Mekong Delta, I did not see *Pn. septemradiatus* ventures into

the water. The high terrestriality of *Pn. septemradiatus* is further supported by the finding that ants, *Dolichoderus* sp., constituted a substantial portion of the total gut content (> 80% by biovolume analysis, Dinh et al., 2020). This agrees with the hypothesis that the fish comes out of the water to find/explore new food resources. A detailed investigation into the feeding ecology is necessary to reveal the resource utilization of the fish in the terrestrial environment near the river.

Chapter 1

General introduction

The earliest transition of vertebrates from aquatic to terrestrial habitat is inferred to have occurred during the Devonian period, from paleontological investigations. In transitional environments, the animals have to face new environmental conditions and they need to develop novel body morphology, physiology, and behavior to survive. Discoveries of new fossils of transitional animals, e.g. elpistostegalian fishes (the fishes most closely related to tetrapods) and early tetrapods, during the last few decades, have refined our knowledge on the process considerably, especially on how body structure changed accompanying the habitat transition. These palaeontological studies have also given important clues to unveiling the environmental settings and ecosystem structures of the sites where the transition might have occurred. Even though these palaeontological studies provide the most direct evidence for the process of land invasion by early vertebrates, the fossil records are inherently fragmentary, and some aspects of their ecology remain difficult to reconstitute from these materials. Some of these knowledge gaps may, therefore, be complemented by studying extant animals that are showing such a transition today.

Mudskippers are amphibious fish in the subfamily Oxudercinae within the family Gobiidae. Mudskippers typically live in intertidal habitats of the tropics and subtropics and exhibit unique adaptations to this environment. Mudskippers are quite active when out of the water, feeding and interacting with one another, for example, to defend their territories and court potential partners. Studies of mudskippers could provide important insights into the vertebrate transition from water to land, in particular with respects to behavior, ecology, soft body morphology and physiology, all of which are hardly reconstituted from fossil materials.

Chapter 2

Reproductive ecology of *Periophthalmus modestus* in the Ariake Sea, Japan

2.1 Introduction

Reproduction at the air-water interface is a formidable task for animals that are in the process of habitat expansion from water to land. Although amphibians show an impressive variety of reproductive strategies from laying eggs in subterranean, terrestrial, or arboreal nests to oviposition to female's back or eggs retained in the oviduct (Wells, 2007), reproduction at the air-water interface is known in only 104 among over 35,000 species of extant fishes (Ishimatsu et al., 2018). It requires the preparation of a nest or a burrow, or exploitation of a spawning site that protects small anamniotic eggs from desiccation while securing a sufficient supply of oxygen.

Mudskippers (Oxudercinae, Gobiidae) are among those rare examples. They store a volume of air in the spawning chamber of the burrow that they excavate in a muddy substratum so that embryos develop in air, not in water. This is presumably to protect embryos from nearly anoxic environments prevailing in the subsurface sediments of muddy shores (Little, 2000). The air storage in a spawning chamber has been confirmed for only four species of mudskippers, *Boleophthalmus pectinirostris* (Toba and Ishimatsu, 2014), *Periophthalmodon schlosseri* (Ishimatsu et al., 1998, 2009), *Pn. septemradiatus* (Mai et al., 2019), and *Periophthalmus modestus* (Ishimatsu et al., 2007), but it is probably prevalent among other mudskippers too (Ishimatsu and Graham 2011 and Martin and Ishimatsu 2017 for review). Apart from the air-filled spawning chamber, mudskipper burrows are filled with extremely hypoxic water (Gordon, 1995; Ishimatsu et al., 1998,2007).

Periophthalmus modestus is distributed along the Pacific coast from Tokyo Bay to Okinawa in Japan (Takita and Ishimatsu, 2015). In mudflats in the Ariake Sea of the Kyushu Island, the

breeding season of *P. modestus* lasts from May to August (Ishimatsu et al., 2007), during which the fish breeds several times (Soyano et al., 2008a). Males dig J-shaped burrows with two or three openings on the mudflat surface (Kobayashi et al., 1971). To attract females, male fish change body color from grey to pinkish, raise and wave the tails, and make jumps with fins stretched. When a gravid female approaches a male, he leads her to his burrow by slow, wiggling movements (Matoba and Dotsu, 1977; Baeck et al., 2008). The spawning chamber is located at the terminus of a burrow and it is the site of oviposition (Kobayashi et al., 1971; Matoba and Dotsu, 1977; Ishimatsu et al., 2007). After spawning, the female leaves the burrow, but the male guards it for about a week. During low tide when burrow openings are uncovered by water, the burrow-guarding male gulps fresh air and adds it into the spawning chamber. During high tide when the burrow is inundated, the male remains inside the burrow. When the eggs are competent to hatch, the male expels the air from within the spawning chamber at nighttime high tide and submerges the eggs for hatching (Ishimatsu et al., 2007).

One of the major remaining questions regarding mudskipper reproduction is whether the spawning chamber is filled with air or water at the time of spawning and fertilization. Presently, there is conflicting circumstantial evidence in this regard. The facts that support aerial spawning and fertilization include: (1) Air was collected from the burrows of courting male *P. modestus* (Ishimatsu et al., 2007). Because there is only one clutch in the spawning chamber (Kobayashi et al., 1971), these burrows are inferred to contain no eggs; (2) The presence of air was confirmed by direct video-recording in the spawning chambers of *Pn. schlosseri* burrows where no eggs were found (Ishimatsu et al., 2009); and (3) The amphibious blenny *Andamia tetradactyla* spawns and fertilizes eggs in air-exposed rock crevices (Shimizu et al., 2006), the only known example for subaerial piscine reproduction. On the contrary, the following observations are in favor of oviposition in water: (1) A preliminary observation showed that a male *P. variabilis* probably added air only after spawning and fertilization in an

aquarium (Rupp, unpublished observation); and (2) *Pn. schlosseri* can weigh more than 200 g (Ishimatsu et al., 1999), which makes it unlikely for those individuals to cling onto the ceiling of a spawning chamber filled with air for spawning and fertilization.

This study aimed to determine the burrow residence duration of the male and the female of *P. modestus* during spawning and its relationship with the tide, with the hope that the data will benefit future investigations to answer the question. The obtained knowledge would also help us understand the possibilities and limitations of fish reproductive strategies at the air-water interface, and may also shed light on the earliest steps of evolution of vertebrate reproduction from water to land.

2.2 Methods

This study was conducted in the mudflat along the Fukushoe River, Ogi City, Saga Prefecture, Japan (33°12'22.24"N, 130°13'41.97"E) in May and June 2016. Reproductive behavior before, during, and after the cohabitation in a burrow was recorded and analyzed for five pairs of *P. modestus* (4 complete cycles and 1 incomplete cycle, see below). Even though the total number of observations was more than 50, the rest had to be canceled because female fish either did not enter a burrow or stayed inside for only a short period. During daytime low tide, a video camera (Sony FDR-AX100E 4K, Japan) was set on a concrete pier to record the timing of male and female entry into a burrow and their residence time within the burrow, and the frequency of male emerging from the burrow. Video recording started at 1015 to 1330 and lasted for 380 to 410 min, except for one case (Pair 2) in which the recording lasted only 250 min. A digital camera (Nikon D800, Japan) with a telephoto lens (Nikon AF-S VR 70-300mm, Japan) was used for capturing selected events or changes in burrow openings during burrow residence.

The spawning was assumed to have occurred if the following two conditions were satisfied; (1) A male continued guarding his burrow after a female had left; and (2) the distension of the female's abdomen obviously decreased between entry into and exit from a burrow (Fig. 2.1). Larval hatching was assumed to have occurred when a male fish stopped guarding his burrow and started to dig a new burrow or repair the burrow that he had guarded.

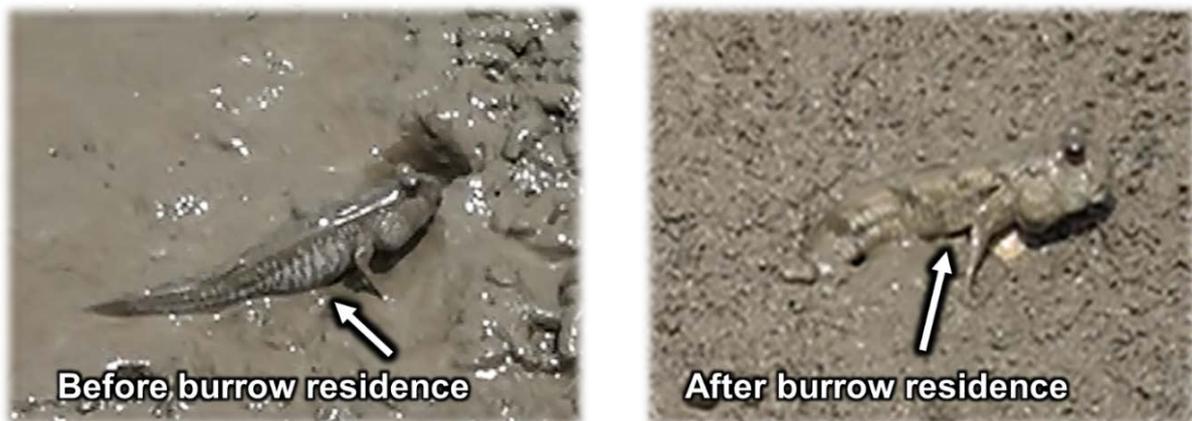


Fig. 2.1. Comparison of the belly distension (arrows) before and after burrow residence of a female *Periophthalmus modestus*, suggesting that spawning took place. (A) the image was taken prior to the burrow entrance of the female. (B) the image was taken at the emergence of the female after spending 285 minutes inside the burrow. Video-recorded on June 7, 2016.

2.3 Results

When a gravid female approached a male showing a courtship display near his burrow, the male fish escorted the female to an opening of the burrow. Then, the male usually entered the burrow first and the female followed within 2 min, except in one pair in which the female entered a burrow first (Fig 2.2 and Table 2.1). On the way to the burrow opening, the female often stopped and stared at the male's courtship display. In some other cases, the male bit the female's operculum, which caused the female to follow the male again. In still some other cases, the

male fish came out from the burrow he had entered and escorted the female fish again while she hesitated to enter. In many cases, a female followed a male at some distance from him and ran away for food or another male.

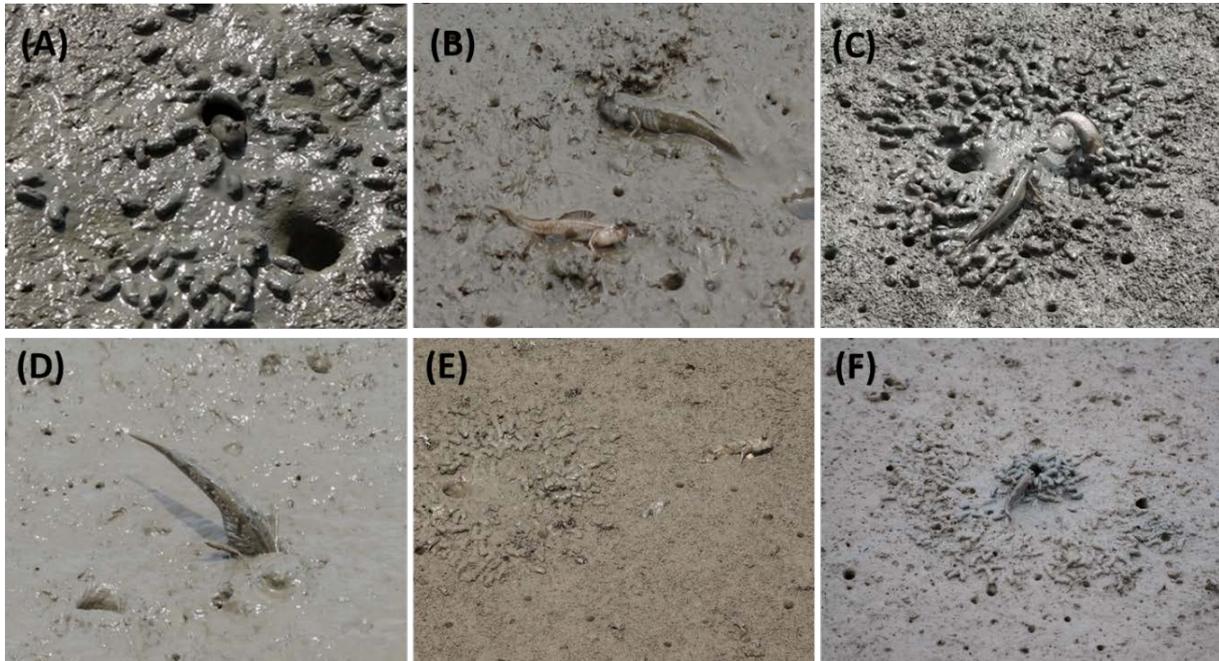


Fig. 2.2. Composite photographs of the on-surface behavior of a male and a female *Periophthalmus modestus* during presumed spawning inside a burrow. (A) After completing the excavation of a burrow, the male protrudes the head, searching for a female; (B) the male turning to a nuptial color (front) is trying to attract the female (back) with distended abdomen; (C) the male is entering the burrow (the female about to enter the burrow); (D) the female is entering the burrow (after male). Note that the size of the female's belly is nearly as large as or larger than the size of the burrow opening; (E) The female came out from the burrow and is leaving it; (F) The male is restoring the burrow for the next spawning round (pair 3).

Table 2.1. Burrow residence of male and female *Periophthalmus modestus* in relation to the tide.

Pair	Date	Lunar age	Emersion of the burrow opening (A)	Male entry	Female entry	Female departure (B)	Inundation of the burrow opening (C)	(A) to (C) (min)	(B) to (C) (min)	Spawning
1	25/5	18.7	13:14	13:16	13:16	18:37	22:18	544	221	Yes
2	29/5	19.7	15:21	15:19	15:19	18:09	2:32 (30/5)	671	503	No
3	7/6	2.4	12:34	13:22	13:22	18:07	20:33	479	146	Yes
4	8/6	3.4	13:10	13:44	13:45	19:30	19:32	382	2	Yes
5	15/6	10.4	6:19	10:16	10:17	14:17	16:48	629	151	Yes

Four pairs were successfully recorded from the entry of the pairs until the female's departure (Pairs 1 and 3-5, Fig. 2.3). Spawning was presumed to have occurred in these four cases. In these cases, females stayed continuously inside the burrow for 245 ± 38 min (SD) (Fig 2.3). In contrast, the male came out onto the mudflat surface and returned to the burrow after less than a minute or as long as 10 min in some cases (Fig. 2.3). When a male was outside the burrow, he remained around openings and protected his burrow from conspecifics and crabs. The male occasionally fed. The frequency of male's emersion largely varied between individuals (Table 2.2). The males of Pairs 3 and 4 showed prolonged sojourn in the burrows toward the later phase of the female's burrow residence, while no such pattern was observed for the males of Pairs 1 and 5. Fish clogged a (or more than one) burrow opening during the female fish confinement (Fig. 2.4). Recording of one pair (Pair 2.2, Fig. 2.3) was terminated when another male entered the burrow 169 min after the female entered the burrow. The female deserted the burrow in one min (i.e., 170 min after her entry).

Table 2.2. Duration of burrow confinement (min) and number of emersions of male and female *Periophthalmus modestus*.

Pair	Male		Female	
	Confinement*	Emersions*	Confinement	Emersions
1	178	52	309	0
2	119	13	170	0
3	239	10	285	0
4	265	35	345	0
5	178	15	240	0

*During the female's residence in a burrow.

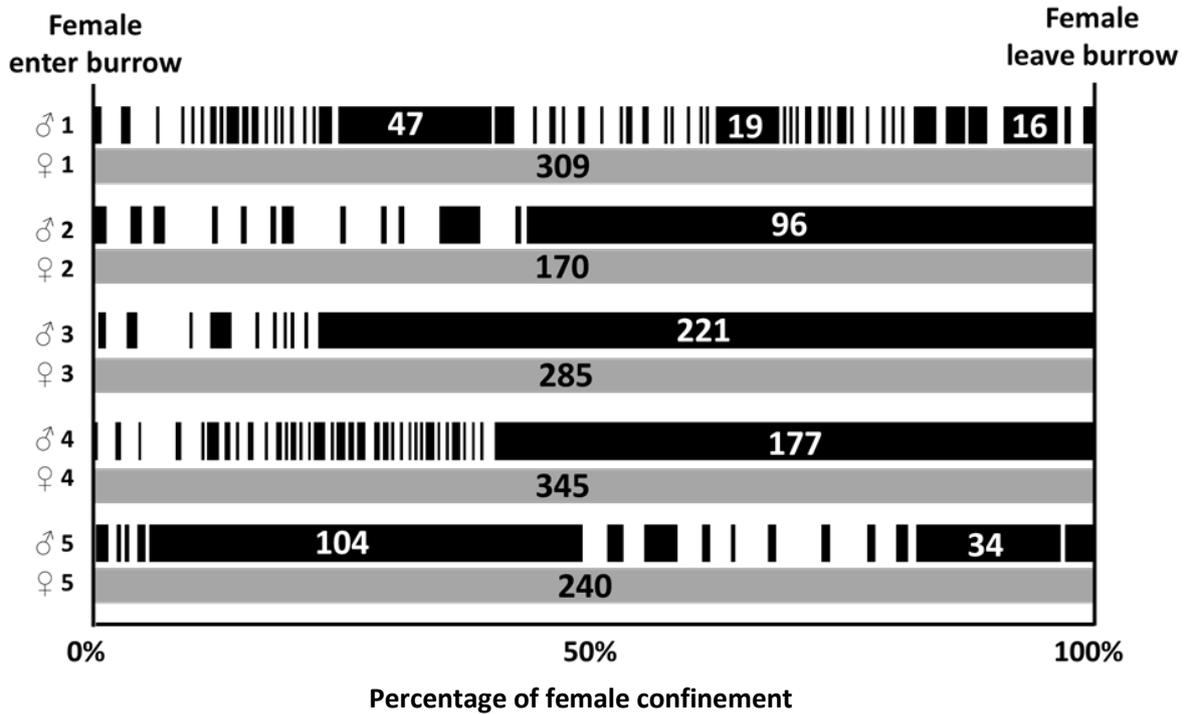


Fig. 2.3. Burrow residence of male and female *Periophthalmus modestus* from female's entry into the burrow (on the left) to the female's departure from the burrow (on the right). Five pairs were recorded. The colored bars represent time when a male and a female remained inside a burrow. The numbers in the bars indicate the duration of long confinement (min). No spawning occurred in the pair 2.



Fig. 2.4. *Periophthalmus modestus* clogged a burrow opening from inside (red arrows). (A) fish started to clog the opening; (B) the burrow opening was partly clogged; (C) the burrow opening was fully clogged.

After presumed spawning in Pairs 1 and 3-5, the males of Pairs 1, 4 and 5 mostly remained inside the burrow until the observation was terminated due to sunset (Male 1 and 4) or rising tide (Male 5), but the male of Pair 3 repeatedly emerged (Fig. 2.5). Thereafter, all the males guarded the burrow for 7-9 days (Table 2.3). Upon termination of the burrow guarding, the males either dug a new burrow or repaired the burrow that he had guarded. Male 2 remained inside the burrow until sunset despite that spawning did not happen.

Table 2.3. Period of egg incubation in *Periophthalmus modestus*.

Pair	Spawning date	Hatching date	Incubation days
1	May 25, 2016	June 3, 2016	9
3	June 7, 2016	June 15, 2016	8
4	June 8, 2016	June 15, 2016	7
5	June 15, 2016	June 22, 2016	7

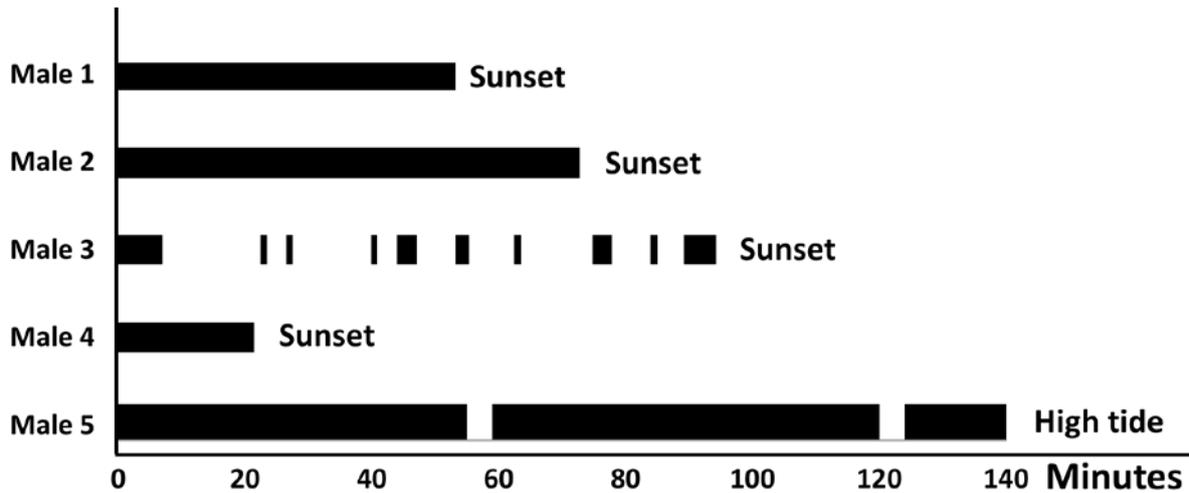


Fig. 2.5. Burrow residence of male *Periophthalmus modestus* after a female left his burrow (min). The observations were terminated at the sunset (pair 1 to pair 4) or at mudflat inundation (pair 5).

Discussion

The most noticeable difference in burrow residence between males and females is that females remained inside a burrow once they entered it, whereas males repeatedly came out and stayed on the mudflat surface for a variable, but usually short, period of time before returning into the burrow. In two cases (Pairs 3 and 4), the male's burrow residence became prolonged and continuous in the later phase of female's residence (Fig. 2.3) and extended beyond the time of female's deserting the burrow (Fig. 2.5). The pattern is somewhat different in Pair 1, in which the male repeated short sojourn inside his burrow interspersed with three occasions of longer (16 to 47 min) residence. The male of Pair 5 showed a long (ca. 100 min) burrow residence in the early phase, followed by an irregular pattern of emersion and confinement. This male remained confined more than 50 min after the female's departure. Since direct evidence is currently lacking about the reproductive behavior of any mudskipper within a burrow, we propose a hypothesized scenario for the reproduction of *P. modestus* at the end of this section

for the future study, on the basis of available information on goby reproduction and fish reproduction in general.

Gobies typically attach adhesive eggs on the underside of rocks, depressions, or on the ceiling of enclosed spaces including burrows (Miller, 1984). These spawning sites are usually prepared by a male prior to his nuptial display. Following male-female interactions, a couple will enter the nest or the burrow and remain inside for variable periods of time before spawning (Table 2.4). It is not always clear whether either of the couples would make short excursions outside after they enter into the nest/burrow. The actual time of spawning in the nest/burrow lasted for 1 to 9.5 h, during which the female extruded 3-30 eggs from the urogenital papilla at each spawning bout. The tip of the urogenital papilla presumably has a tactile sensory function, because it has been repeatedly observed that the female avoided spawning over the already laid eggs and oviposited only on the bare surface (Tavolga, 1954; Matsuo and Takahama, 2001). When the spawning was completed, the female left the nest/burrow while the male continued to stay in it for parental care.

The testis of gobies is accompanied by a unique accessory gland called the sperm duct gland or seminal vesicles (Chowdhury and Joy, 2007; Mazzoldi et al., 2011). The sperm duct gland has been reported for 8 species of mudskippers by Fishelson (1991) and is also confirmed for *Ps. modestus* (Tran Xuan Loi, Graduate School of Fisheries and Environmental Sciences, Nagasaki University, personal communication). The physiological roles of the sperm duct gland have not been fully elucidated (Chowdhury and Joy, 2007), but one probable function is to prolong sperm availability for egg fertilization by slowly releasing sperm from within a viscous secretion from the gland, which is glued onto the nest/burrow surface. Male gobies were observed to rub the urogenital papilla before, during, and after oviposition by a female fish (Tavolga, 1954; Marconato et al., 1996; Ota et al., 1996). Hence, the male goby does not need

to accompany the female throughout oviposition that may last for several hours (Table 2.4) but can guard the nest or burrow from intruders.

Prior to spawning, oocytes must go through the process of final oocyte maturation, accumulating yolk materials and increasing the size by water uptake, and are shed from the ovarian follicles (ovulation, Kagawa 2013). Gonadal development in fish is regulated by environmental factors such as temperature, light, lunar cycle, and social interaction, which generate endocrine changes that drive the gonad maturation (Pankhurst and Porter, 2003). The process of final oocyte maturation to ovulation takes 1.5 to 2 days in marine fishes (Alvariño et al., 1992; Ni Lar Shein et al., 2004; Shiraishi et al., 2008). In addition, the process of ovulation itself takes several hours up to 24 h (Alvariño et al., 1992; Goetz and Garczynski, 1997; Soyano et al., 2008b). Ovulated eggs are retained within the female's body but must be released and fertilized within a certain period of time. Otherwise, they degenerate and are progressively resorbed (Samarin et al., 2015). The duration of egg fertility inside female's body varies largely among species and temperature, ranging from 30 min to 12 h for most tropical and temperate species but it can be as long as 30 to 40 days in cold water species (Samarin et al., 2015). Larval hatching was almost completely inhibited when ovulated eggs were fertilized 12 h after ovulation in the seven-band grouper (Soyano et al., 2008b).

We hypothesize the following scenario for the reproduction of *P. modestus* within its burrow. (1) Gravid females have nearly or completely finished ovulation before burrow entry. We presume this because the total duration needed for final oocyte maturation and ovulation is much longer than the observed period of female's residence in a burrow (240 to 350 min, Table 2.2). Therefore, final oocyte maturation must have been terminated and ovulation may have been partly or fully completed before female's entry into a burrow. (2) We assume that the spawning chamber is filled with air prior to

spawning for the following three reasons. First, a female presumably cannot maintain metabolism during spawning that may last for several hours if the burrow including the spawning chamber is filled with hypoxic water (water P_{O_2} of *P. modestus* burrow is 1.5 kPa, only 7% of air saturation, Ishimatsu et al., 2007). Yet, we cannot preclude the possibility that the female will swim up to the air-water interface of the burrow and gulp air. Second, fish embryos are generally sensitive to hypoxic conditions (Hassel et al., 2008; Elshout et al., 2013; DePasquale et al., 2015). Rombough (1988) summarized early data on metabolic intensity during early developmental stages and showed that mass-specific oxygen consumption can be high during cleavage, suggesting high sensitivity to hypoxia. On the other hand, how hypoxia affects sperm motility and fertilization success is scarcely known for fishes (Dzyuba and Cosson, 2014). Third, burrow water is thought to contain high concentrations of ammonia (Ip et al., 2004), which disrupts embryogenesis in fishes (Bardon-Albaret and Saillant, 2016). (3) The time between female's entry to spawning is estimated to be at least 170 min. This is based on the observation of Pair 2, in which the female left the burrow without spawning due to the entry of an intruder male (Fig. 2.3). This would allow the time for spawning to range from 70 to 180 min, i.e. the difference between female's burrow residence time except the Pair 2 (240 to 350 min, Table 2.2) and the presumed time before spawning (170 min). The fecundity of *P. modestus* was reported as 5200 ± 765 eggs/burrow (Ishimatsu et al. 2007). This means 29 to 74 eggs spawned per min. (4) A male fertilizes the eggs during, after, and even before spawning as in other gobies (Ota et al., 1996). A male *Andamia tetradactyla*, the only fish for which spawning and fertilization in the air is known, rubs the urogenital papilla on the eggs after a female finishes spawning

(Shimizu et al., 2006). The sperm duct gland was found in some blennies (Chowdhury and Joy, 2007), yet it remains unknown if *A. tetradactyla* has it or not. The unpublished observation that *P. variabilis* added air only after spawning in an aquarium needs confirmation (Rupp, unpublished).

Clogging of burrow openings after female's entry for reproduction was reported for gobies, for example, *Leucopsarion petersii* (Akiyama and Ogasawara, 1994) and *Valenciennesia longipinnis* (Takegaki and Nakazono, 1999). Hong et al. (2007) stated that those burrows of the mudskipper, *Boleophthalmus pectinirostris*, always contained a spawning chamber and the eggs therein when the openings were clogged by mud.

Table 2.4. Duration of nest residence and spawning in gobies observed under captivity.

Species	Nest residence before spawning	Spawning (h)	Nest type	Number of eggs per extrusion	Fecundity	Ref.
Gobiidae						
<i>Bathygobius soporator</i>	>0.5 h	3-9	On surface	10-30	15000, 17500, 18000	(1)
<i>Glossogobius olivaceus</i>	10 h to 6 d	3.5-9.5	On surface	No data	60800	(2)
<i>Leucopsarion petesii</i>	14-22 d	1-2.5	Burrow	3-6	No data	(3)
<i>Pterogobius elapoides</i>	1-2 d	1	On surface	No data	1800	(4)
Eleotridae						
<i>Eleotris oxycephala</i>	No data	2-3.75	On surface	3-10	37800, 178000	(5)
Odontobutidae						
<i>Odontobutis obscura</i>	12 h	6-12	On surface	5-10	960	(6)

(1) Tavalga 1954; (2) Senta and Wada 1970; (3) Akiyama and Ogasawara 1994; (4) Dôtu and Tsutsumi 1959; (5) Matsuo and Takahama 2001;

(6) Mashiko 1976.

Chapter 3

Reproductive ecology of *Periophthalmodon septemradiatus* in the Mekong Delta, Vietnam

3.1 Introduction

The emergence of vertebrates from water to land, which is thought to have occurred in the mid to late Devonian Period (approximately 390 to 360 million years ago), represents one of the most pivotal events in the history of life on Earth (Clack et al., 2012). Discoveries of new fossils of transitional animals, e.g. elpistostegalian fishes (the fishes most closely related to tetrapods) and early tetrapods, during the last few decades, have refined our knowledge on the process considerably, especially on how body structure changed accompanying the habitat transition (Daeschler et al., 2006; Boisvert et al., 2008; Pierce et al., 2012; Porro et al., 2015). These palaeontological studies have also given important clues to unveiling the environmental settings and ecosystem structures of the sites where the transition might have occurred (Schoch, 2014). The idea that vertebrates abandoned drying-up freshwater bodies in drought to seek larger remaining ponds (Romer et al., 1958) had been widely accepted in the 20th century, but has become questioned by more recent researchers (Laurin et al., 2010), particularly after the finding of trace fossils, which dated older than the first known body fossils of vertebrates, and was initially thought to indicate the coastal transition to land (Niedźwiedzki et al., 2010). In addition, the new analysis of stable isotopes of the bones of early tetrapods has lent support for the euryhalinity of these animals, which supposedly helped rapid global distribution and colonization of different landmasses (Goedert et al., 2018). Even though these palaeontological studies provide the most direct evidence for the process of land invasion by early vertebrates, the fossil records are inherently fragmentary, and some aspects of their ecology remain difficult to reconstitute from these materials. Some of these knowledge gaps may, therefore, be

complemented by studying extant animals that are showing such a transition today (Gordon and Olson, 1995).

Mudskippers are amphibious gobies belonging to the subfamily Oxudercinae (Jaafar and Murdy, 2017). These fishes usually inhabit intertidal mudflats of tropical and subtropical coasts and estuaries and show various degrees of behavioral, morphological, and physiological adaptations to the terrestrial environment. During low tide, mudskippers emerge from the water for various activities such as feeding and defending territories, while during high tide some species remain out of the water but others retreat into burrows (Takita et al., 2011). Courtship occurs only during low tide, and the mating pair enters a burrow while the mudflat is exposed. Eggs are laid on the wall of a spawning chamber within the burrow. Parental care comprises the continuous addition of fresh air into the spawning chamber during low tides but terminates with submersion of hatch-competent embryos during high tide (Martin and Ishimatsu, 2017). Although goby lineages are estimated to have appeared in the latter half of the Eocene, approximately 48.7–36.2 million years ago (Thacker, 2015) (see also Reichenbacher et al., 2018 for the oldest goby fossil dating 19.1–20.4 million years ago) and thus are far more modern than the transitional animals of the Paleozoic, mudskippers could still provide insights into how the land invasion would alter body structure and other life-history traits. Despite the fact that most mudskippers are highly euryhaline (Clayton, 1993; Ishimatsu and Gonzales, 2011), their occurrence in low salinity waters has only been sporadically reported (see Discussion). Through my field survey in the Mekong Delta during the last four years, I found that one species of mudskippers, *Periophthalmodon septemradiatus*, inhabits over a 150 km stretch of the riparian zones along the Mekong River in Vietnam. The Mekong River flows as two main channels, the Hau River and the Tien River, through the Mekong Delta, and the Tien River further subdivides into the Co Chien River and a few other channels before reaching the coast (Fig. 3.1).

In this chapter, I report on the reproductive ecology of *Pn. septemradiatus* inhabiting from brackish to freshwater reaches of the Mekong River, discuss how studying this species will benefit the understanding of the vertebrate transition to land from waters of different salinities, and propose a possible scenario for the expansion of the distribution range of this species into freshwater reaches of the Mekong River.

3.2. Methods

Study sites. The field study of *Periophthalmodon septemradiatus* was carried out mainly along one main channel of the Vietnamese part of the Mekong River, the Hau River, for the period of December 2015 through June 2018. In addition, I also conducted a field survey along another main channel, the Tien River–Co Chien River, in five trips to obtain complementary data. In total, 10 field trips were made (see Table 3.1).

Table 3.1. Summary of field trips in the lower Mekong River in Vietnam

Year	Date	Lunar age	River surveyed
2015	Dec. 14–15	3.1–4.1	TC
2016	June 6–8	1.4–3.4	H, TC
	Sept. 29–30	28.1–29.1	H, TC
	Nov. 27–Dec.8	28.8–9.0	H
2017	April 10–16	13.4–19.4	H
	June 25–July 6	1.4–12.4	H
	Sept. 19–Oct. 10	28.7–20.3	H
	Dec. 12–27	24.0–9.2	H, TC
2018	Jan. 30	13.4	TC
	June 10–22	26.0–8.7	H

Environmental measurements. Water depth, temperature, and salinity of river water were monitored using data loggers; HOBO U20L for water depth and temperature and HOBO U24-001 and -002 for water conductivity (Onset Computer Corp. Massachusetts, USA) at three sites including the lower and upper limits of *Pn. septemradiatus* distribution (E1 and E2, see Fig. 3.1 and Table 3.3), and further up where no specimens of *Pn. septemradiatus* were found (E3, see Fig. 3.1 and Table 3.1). Water conductivity was determined also with a probe (Horiba 9382, Tokyo, Japan) and a portable conductivity meter (Horiba ES-51, Tokyo, Japan), and used for correcting the data obtained by the conductivity loggers. Water conductivity was converted to salinity using PSS-197849 (Hill et al., 1986). Dissolved oxygen (DO) concentration of river water was determined with a probe (Horiba 9520) and a meter (Horiba D-55), which was calibrated every day with a sodium nitrite solution and humidified air. The obtained DO concentration was converted to oxygen partial pressure using measured water temperature, salinity, and oxygen solubility coefficient (Dejours, 1981). At each site, the data loggers were set vertically, guarded in a perforated PVC pipe (diameter 9 cm, length 90 cm) which was capped on each side. The pipe was tied to a stick pushed into the bottom sediment to position the data loggers 20–30 cm above the river bottom. Another depth data logger was kept in air for compensation for changes in barometric pressure. Sampling frequency was set at 5 minutes and sampling duration was 5 days or longer. The data were extracted to Excel files according to the instruction manual. During burrow density determination (see Reproductive ecology), river and burrow water samples were collected, immediately filtered (0.45 μm), acidified to pH 2 by the addition of concentrated nitric acid, and stored at room temperature until analysis. Na, K, Ca, and Mg concentrations of the river and burrow water were analyzed with an ICP-AES (ULTIMA2, Horiba, Tokyo). Sr concentration was determined with ICP-QQQ (Aglient 8800, Aglient Technologies, Tokyo).

Table 3.2. The locations of environmental monitoring along the Hau River, Mekong Delta, Vietnam (Environmental monitoring was conducted in April, September, and December 2017 and in June 2018).

Site ID	District, Province	Latitude	Longitude	Distance from the river mouth (km)
E1	Cu Lao Dung, Soc Trang	9°30'35.22"N	106°13'43.02"E	8
E2	Chau Thanh, An Giang	10°26'57.78"N	105°22'20.76"E	150
E3	Chau Phu, An Giang	10°34'41.46"N	105°14'26.28"E	172

Observations of fish occurrence. The fish occurrence was surveyed during low tide along the mud banks of the main channel and tributaries of the Hau River and the main channel of the Tien River-Co Chien River. I used binoculars, digital cameras, and video recorders to identify species, and recorded fish behaviors. I took special care before concluding the absence of *Pn. septemradiatus* in a specific site, by observing some hundred meters of nearby river banks.

I used the keys by Takita et al. (1999) for field identification of mudskippers. Accordingly, *Pn. septemradiatus* had a brown body, lighter on the dorsal surface, with a dark horizontal line, running dorsolaterally from the eye and posteriorly becoming a row of dark spots. Numerous small dark speckles occurred on the snout, opercles, and the flanks. The young had white speckles (instead of dark ones) laterally on the trunk, and the dorsolateral row of dark spots was obscured posteriorly. In addition, the eyes of *Pn. septemradiatus* often appeared blue-green in color, which was used as an additional characteristic to confirm species identification in the field. For collected samples, I further confirmed the occurrence of two rows of teeth in the upper

jaw, a key characteristic for distinguishing *Periophthalmodon* from *Periophthalmus* (Murdy, 1989).

Fish sampling and measurements. In this study, a total of 284 individuals were collected. 111 fish were collected on the mud bank in December 2017 using a baited hook and line for a comparison of the relationships of body mass and standard length between sites (see Table 3.3). These fish were anesthetized by immersion in a solution of FA-100 (DS Pharma Animal Health, Osaka, Japan). After determination of body mass and standard length, the fish were allowed to recover in river water and released, except 10 individuals from each site, which were euthanized in a concentrated FA100 solution, preserved in 10% neutralized formalin, and used for the determinations of morphometric and meristic parameters. In addition, 4 fish from F3 (the upper limit of fish distribution in the Hau River) were collected for the analysis. I measured 16 morphometric and 10 meristic parameters reported in Jaafar et al. (2016). 88 fish were used for DNA analysis, of which 20 were also used for otolith Sr:Ca analysis, together with 5 additional fish from F5. Fish for DNA analysis were collected by dip nets in 2016 and 2017 as shown in Table 3.3. After collection, they were sacrificed by immersion in ice water, measured for total and standard lengths, and preserved in 96% ethanol. These fish were preserved in the laboratory for further morphological analyses.

Table 3.3. Summary of fish sampling during this study along the Hau River, Mekong Delta, Vietnam

Sampling date	Site ID	District, Province	Latitude	Longitude	Nd (No)	Ns (Nm)	Distance from the river mouth (km)
Sept. 29, 2016	F1-a	Cu Lao Dung, Soc Trang	9°38'26.28"N	106°09'9.16"E	10 (3)		27
Sept. 30, 2016	F2	Cao Lanh, Dong Thap	10°24'39.96"N	105°43'10.62"E	5 (5)		127
Dec. 1, 2016	F3	Chau Thanh, An Giang	10°26'31.80"N	105°23'26.10"E	5 (5)		148
Dec. 5, 2016	F4	Thot Not, Can Tho	10°13'0.54"N	105°33'7.32"E	5 (5)		119
Dec. 8, 2016	F1-b	Cu Lao Dung, Soc Trang	9°39'31.58"N	106°9'38.20"E	4 (2)		27
Apr. 11, 2017	F3	Chau Thanh, An Giang	10°26'31.80"N	105°23'26.10"E	10 (0)		148
Apr. 12, 2017	F5	Binh Thuy, Can Tho	10° 2'58.62"N	105°43'25.50"E	13 (0)		96
Apr. 14, 2017	F1-c	Cu Lao Dung, Soc Trang	9°33'0.44"N	106°15'21.54"E	10 (0)		12
June 18, 2017	F3	Chau Thanh, An Giang	10°26'31.80"N	105°23'26.10"E	26 (0)		148
Oct. 6, 2017	F5	Binh Thuy, Can Tho	10°2'58.85"N	105°43'25.95"E	0 (5)		96
Dec. 15, 2017	F1-c	Cu Lao Dung, Soc Trang	9°33'0.44"N	106°15'21.54"E		45 (10)	12

Dec. 20, 2017	F3	Chau Thanh, An Giang	10°26'31.80"N	105°23'26.10"E	0 (4)	148
Dec. 21, 2017	F4	Thot Not, Can Tho	10°13'0.54"N	105°33'7.32"E	32 (10)	119
Dec. 23, 2017	F5	Binh Thuy, Can Tho	10° 2'58.62"N	105°43'25.50"E	34 (10)	96

Nd, the number of fish used for DNA analysis. No, the number of fish used for otolith analysis. All fish used for otolith analysis was also used for DNA analysis, except on Oct. 6, 2017, when the fish were used for otolith analysis only. Ns, the number of fish used for body mass, and standard length determinations. They were released after the determinations, except for those used for morphometric analysis (Nm). On Dec. 20, 2017, fish were collected exclusively for morphometric analysis.

Reproductive ecology. The density and physicochemical conditions of *Pn. septemradiatus* burrows were studied along the bank of the Hau River and its tributaries in June 2018 (see Table 3.4). Transects of 15–30 m were set parallel to a stream (ca. 3 m from the water's edge) and the locations of burrows along the transects were recorded. Burrows were initially located by the shape and size of the openings. They were then emptied of water and excavated to confirm its identity on the basis of configuration (two sloped shafts that led to a dome-shaped spawning chamber at the bottom, often with the upper surface of the spawning chamber a bright brownish color, Quang Minh Dinh et al. in preparation). For several burrows at each transect, burrow water was analyzed for DO concentration with a probe (Horiba 9520) and a meter (Horiba D-55). The DO probe was inserted directly through a burrow shaft into the standing water inside. Redox potential of the upper surface of the spawning chamber and surrounding mud was determined with a probe (Horiba 9300) and a meter (Horiba D-55), immediately after excavation. After the measurement of redox potential, the horizontal cross-section of the chambers was photographed and measured for their dimensions. Burrow air was sampled by the method reported earlier (Ishimatsu et al., 2007). When eggs were found, they were transferred onto a piece of sponge soaked in water and brought back to the laboratory. Eggs were photographed with a stereoscope and a camera for determination of egg and clutch size. Several egg clutches were used for preliminary trials of incubation in different salinities. Courtship behavior was photographed on several trips. To elucidate the migration history of larvae and juveniles, I determined Sr:Ca ratio of the sagittal otolith (Table 3.3). Fish were preserved in 96% ethanol and brought back to Can Tho University. The otoliths were excised under a dissecting microscope and packed for bringing them to Japan. The otoliths were processed with the method reported earlier (Yokouchi et al., 2018), and analyzed for Sr and Ca concentrations in a line along the longest axis from the core to the edge by a wavelength-dispersive X-ray stereoscopy using electron microprobe analyzer (JXA-8230, JEOL) housed at

the Atmosphere and Ocean Research Institute, The University of Tokyo. Microscopic observation of the otolith samples confirmed that the otolith core was properly exposed and that Sr and Ca analysis started from the core. CaSiO_3 and SrTiO_3 were used as quantitative standards. All Sr:Ca ratios were expressed as weight ratios. Fish from F1-a, F1-, and F1-c were lumped as one group because of their close vicinity to each other.

DNA analysis. Total DNA was extracted from approximately 30 mg of tissue (muscle or fin) using a standard DNA lysis solution containing Proteinase K. A 940-base pair (bp) segment of the mitochondrial cytochrome c oxidase subunit II (COII) and a 934-bp segment of the mitochondrial D-loop (DL) was PCR-amplified with the newly developed primers, Pn.sept_CoII_F1 (5'- ACACATTTGAAGAGCCTGC-3') and Pn.sept_CoII_R1 (5'- AGCTTAAAAGGCTGACGC-3') for COII, and Pn.sept_DL_F1 (5'- TAGCTCCCAAAGCTAGCATTC-3') and Pn.sept_DL_R1 (5'- TCAGGACCAAGCTTTTGTGC-3') for DL. The PCR amplification was carried out in a reaction volume of 25 μl containing 1X EmeraldAmp MAX PCR Master Mix (TaKaRa Bio Inc.), 400 nM of forward, and reverse primer, 1 μl of 10-fold diluted DNA, and the remaining volume made up by nuclease-free water, at the following conditions: initial denaturation at 94°C for 1 min 30 sec, 30 cycles of amplification with each cycle containing 94°C for 30 sec, 50°C (COII) or 60°C (DL) for 30 sec, 72°C for 1 min 30 sec (COII) or 2 min (DL), and a final extension at 72 °C for 5 min. The amplicons were purified using ExoSAP-IT (Thermo Fisher Scientific Inc.) and sequenced by outsourcing (FASMAC Co., Ltd.).

Table 3.4. The locations of burrow density determination

Site ID	District, Province	Date	Tributary/ Main channel	Latitude	Longitude
B1	Cu Lao Dung, Soc Trang	June 12, 2018	T	9°39'33.6"N – 9°39'41.0"N	106°09'21.5" E– 106°09'53.9"E
			M	9°38'42.2"N – 9°38'28.4"N	106°08'23.3" E– 106°08'34.8"E
B2	Binh Thuy, Can Tho	June 14–16, 2018	T	10°02'50.0"N – 10°02'50.8"N	105°43'22.2"E – 105°43'23.8"E
			M	10°02'51.9"N – 10°04'25.4"N	105°45'23.6"E – 105°47'31.9"E
B3	Thot Not, Can Tho	June 17–18, 2018	T	10°09'26.7"N – 10°13'00.1"N	105°33'07.0"E – 105°38'39.0"E
			M	10°09'45.3"N – 10°09'49.3"N	105°38'30.6"E – 105°38'34.4"E
B4	Chau Thanh, An Giang	June 19–20, 2018	T	10°26'46.9"N – 10°26'57.1"N	105°22'17.6" E– 105°22'21.1"E
			M	10°27'1.8"N – 10°27'28.4"N	105°20'43.9" E– 105°22'5.2"E

Data analysis. To compare body mass–standard length relationships of fish from the three sampling sites, the residuals from the regression of the pooled data were analyzed by one-way ANOVA. Sr:Ca data along the longest axis of each otolith were binned into “Peak” segment where Sr:Ca ratios were > 10 , “Core” segment central to “Peak” with Sr:Ca ratio < 10 (but in some fish there was no “Core” area), and “Periphery” segment peripheral to “Peak” with Sr:Ca ratio < 10 . Mean values were obtained for each segment of each fish, and grand mean values were calculated for each site. One-way ANOVA was applied to detect a statistical difference between sites. One-way ANOVA was also used for the comparison of morphometric and meristic data and for the comparison of the river and burrow water ion concentrations between sites. When the normality test failed, a non-parametric test was used (Kruskal-Wallis one way of ANOVA on ranks), followed by Tukey or Mann-Whitney test for pairwise multiple comparisons.

DNA sequences were aligned, edited, and trimmed to a common length using MAFFT version 755 and GENETYX 12.0.4 (GENETYX Corp.). The number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π) was calculated using DnaSP 5.1056. I used Contrib 1.0257 to obtain haplotype richness after rarefaction (rarefied allelic richness) by taking into account differences in sample size. Arlequin 3.5.2.258 was used to calculate population pairwise ϕ_{st} (an analog of F_{st} that includes sequence divergence) with 10,000 permutations. Isolation by distance was assessed by plotting pairwise ϕ_{st} against geographical distance (km) and using a Mantel Test with 10,000 permutations. To infer relationships between populations and sampling sites, a haplotype network for each gene was created using Haplotype Viewer (<http://www.cibiv.at/~greg/haploviewer>) with a Maximum-likelihood (T92 model for COII, T92+G+I model for DL) tree reconstructed by MEGA 7.0.1860.

Values are expressed as means \pm 1 standard deviation where possible.

3.3 Results

Fish distribution. *Periophthalmodon septemradiatus* was found up to 148 km from the river mouth of the Hau River in the An Giang Province and 155 km from the river mouth of the Co Chien River in the Dong Thap Province (Fig. 3.1). I found no individual of *Pn. septemradiatus* in the reaches further upstream from these spots to the border with Cambodia. The fish were abundant in tributaries, but were far fewer in the main channels; only small individuals were occasionally found around mangrove bushes in the banks of the main channel of the Hau River (no detailed survey was done along the Tien River–Co Chien River). In the lowermost sites (F1-a,b,c), *P. septemradiatus* occurred sympatrically with other mudskippers (*Pn. schlosseri*, *Periophthalmus chrysospilos*, *Ps. gracilis*, *Ps. variabilis*, and *Boleophthalmus boddarti*), but it was the only mudskipper species in the more upstream sites.

Habitat environment. The semidiurnal fluctuation of water level was recorded in all the monitoring sites (including E3 where *Pn. septemradiatus* was not observed, 172 km from the river mouth, see Fig. 3.1 and Table 3.2). The tidal range during spring tides at E1 (8 km from the river mouth) reached three meters while it was less than one meter at E2 and E3, which agrees with the data reported earlier¹⁹. Salinity remained zero at E2 and E3 irrespective of the season or time of the day, while it fluctuated with the tide at E1, ranging from zero to approximately 10 at high spring tides. Water temperature showed occasional daily fluctuations of < 5°C (25–30°C) at E1, but it remained at about 30°C at E2 and E3. The PO₂ of river water ranged from 8.1 to 23.4 kPa (mean ± SD, 15.0 ± 6.5, N = 6). The habitats in tributaries were shaded by thick vegetation of Nipa palm and other plants, unlike the open mudflats in coastal areas where other species of mudskippers are abundant.

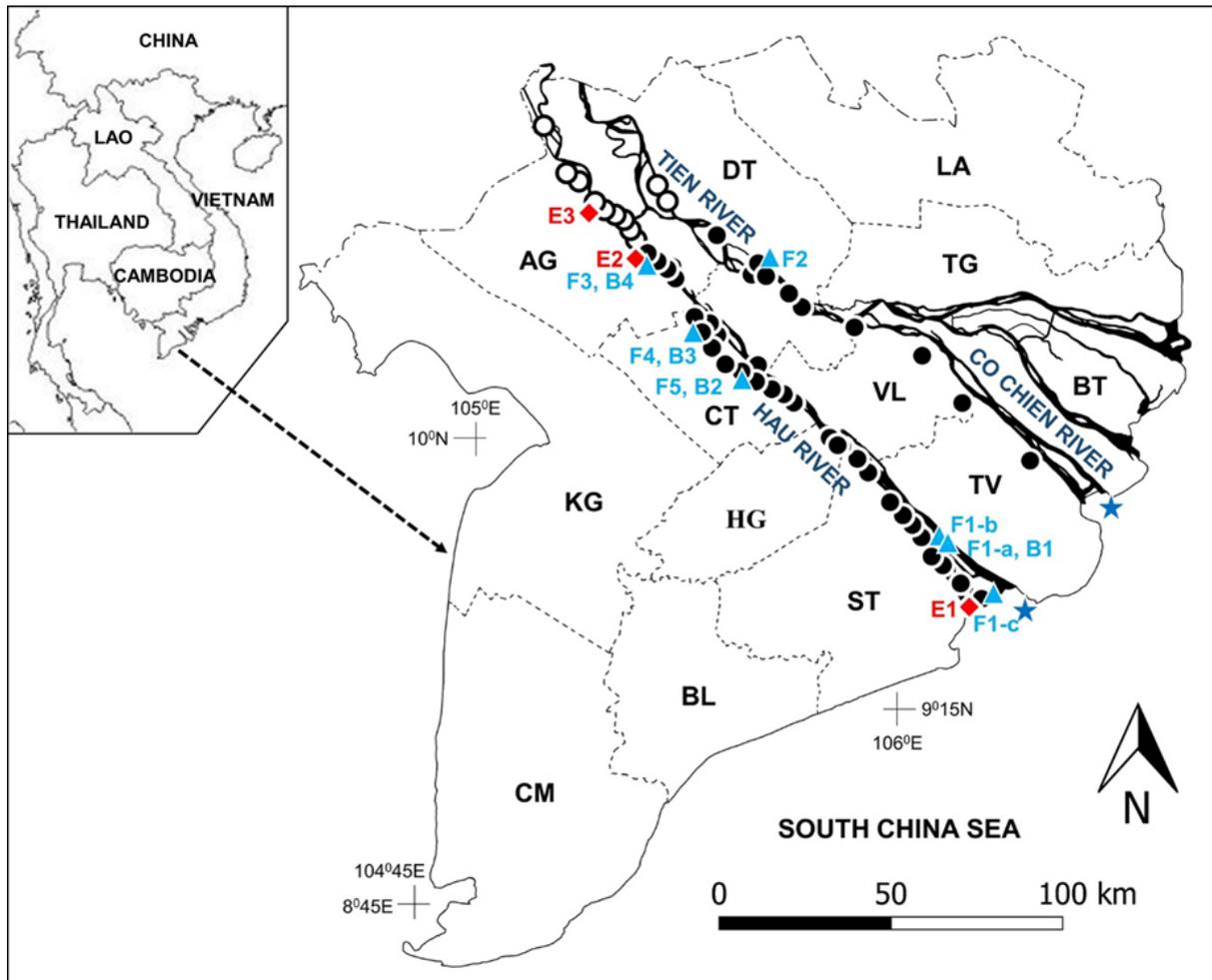


Fig. 3.1. The distribution of *Periophthalmodon septemradiatus* along the two major channels of the Mekong River, the Hau River, and the Tien River–Co Chien River. Filled circles represent sites where the mudskipper was observed, while open circles are where the mudskipper was not observed. The red squares labeled as E1 to E3 are the sites of environmental monitoring, and the blue triangles labeled as F-1 to F5 the sites of fish sampling. Burrow density was determined in close vicinity to four fish sampling sites (B1 to B4). Two stars represent the locations used to calculate the distances of each site from the river mouth. The abbreviations in the map represent the names of provinces or a city (CT). AG, An Giang; BL Bac Lieu; BT, Ben Tre; CM, Ca Mau; CT, Can Tho; DT, Dong Thap; HG, Hau Giang; KG, Kien Giang; LA, Long An; ST, Soc Trang; TG, Tien Giang; TV, Tra Vinh; VL, Vinh Long. The map was created with Qgis 3.4 (http://qgis.org/downloads/QGIS-OSGeo4W-3.4.7-1-Setup-x86_64.exe) (QGIS Development Team (2018) and Microsoft Powerpoint - version 1904 (<https://products.office.com/en-ie/powerpoint>) for labels and icons.

Body size, morphometry, and meristic characters. There was no significant difference in the relationship of standard length (SL, cm) and body mass (BM, g) between fish from the three sampling sites (F1-c, F4 and F5; Table 3.3) as shown by a comparison of residuals from the regression line for the pooled data ($\log \text{BM} = -1.876 + 3.077 \times \log \text{SL}$, $r_2 = 0.962$, $H_2 = 5.442$, $P = 0.066$, Kruskal-Wallis one-way ANOVA, Figure 3.2).

Morphometric analysis revealed statistically significant differences in the first dorsal fin base length ($H_2 = 9.694$, $P = 0.008$), the second dorsal fin base length ($F_{2,27} = 3.781$, $P = 0.036$, one-way ANOVA), the caudal fin length ($F_{2,27} = 8.181$, $P = 0.002$), the pectoral fin length ($F_{2,27} = 6.899$, $P = 0.004$), the pectoral fin height ($H_2 = 8.871$, $P = 0.012$), body width at the anus ($H = 8.739$, $P = 0.013$), head width ($F_{2,27} = 10.860$, $P = 0.001$), and eye diameter ($F_{2,27} = 20.259$, $P = 0.001$) between fish from F1-c, F4 and F5 (Table 3.5). The number of fin rays was significantly different in the second dorsal (male only, $H_2 = 8.700$, $P = 0.013$) and caudal fins ($F_{2,27} = 4.870$, $P = 0.016$) (Table 3.6).

The smallest fish collected in F1-c (12 km from the river mouth), F5 (96 km), and F3 (148 km) were juveniles and already amphibious (total length 27, 24 and 25 mm for F1-c, F5 and F3, respectively). No larvae have yet been collected.

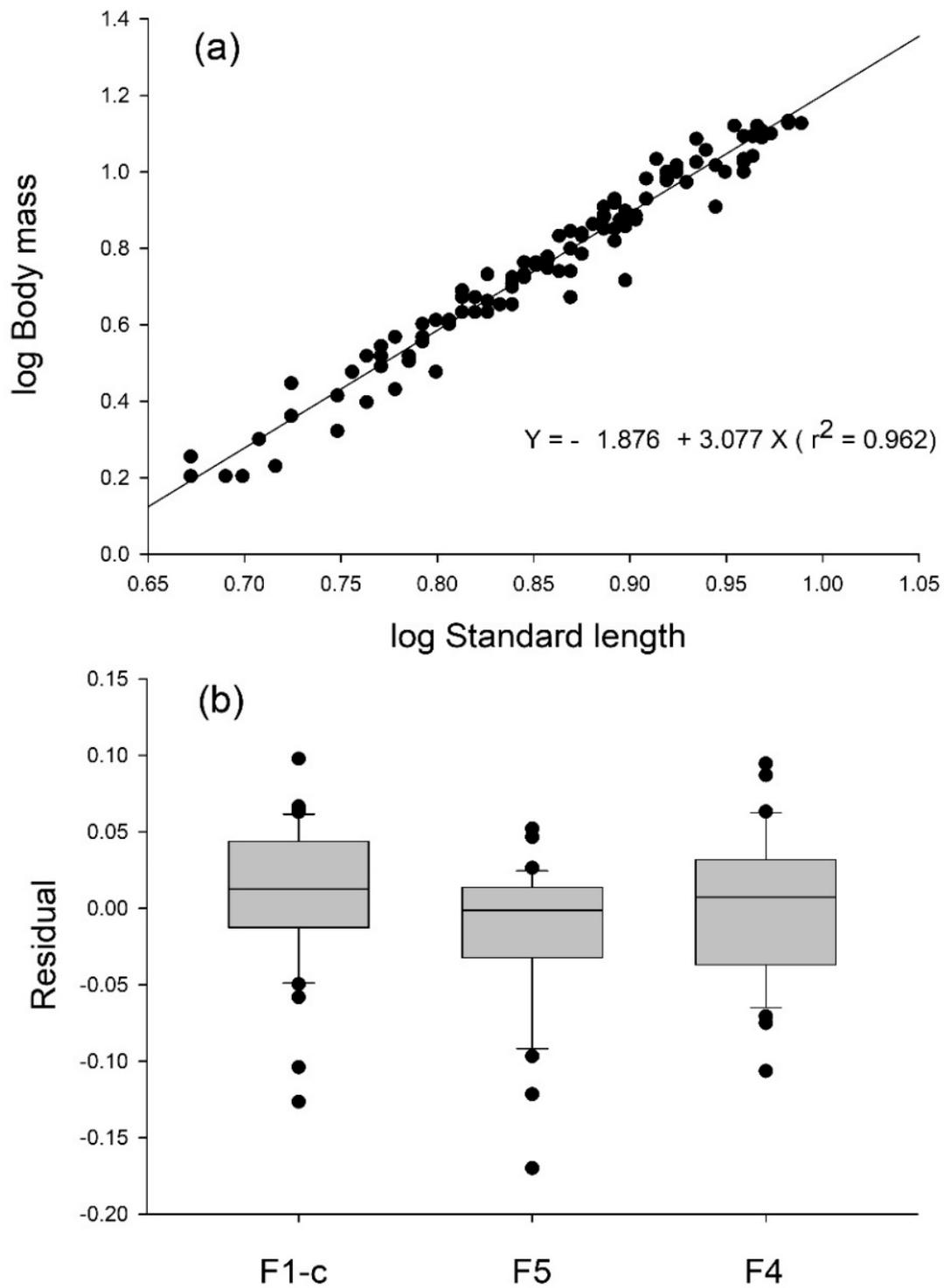


Fig. 3.2. (a) Relationship between body mass (g) and standard length (cm) of *Periophthalmodon septemradiatus* collected at F1-c, F4, and F5. (b) A box plot of the residuals from the regression shown in (a) for samples collected at F1-c, F4, and F5. The lines within the boxes mark the medians. The whiskers above and below the boxes indicate the 90th and 10th percentiles.

Table 3.5. Summary of morphometric analysis of *Periophthalmodon septemradiatus* collected from four sites along the Hau River

	F1-c	F5	F4	F3
% of Standard length				
Head length	29.6 ± 0.55	29.5 ± 0.89	30.2 ± 1.68	30.1 ± 0.54
Predorsal length	38.2 ± 1.28	38.1 ± 1.17	38.0 ± 1.56	38.6 ± 0.26
First dorsal fin base length	16.2 ± 0.65 ^a (3.6,4.5,5.7)	15.4 ± 1.77 ^{ab} (7.7)	13.4 ± 2.53 ^b	14.4,16.0 (4.7,5.2)
Second dorsal fin base length	24.9 ± 1.21 ^a	23.0 ± 1.65 ^b	23.9 ± 1.74 ^{ab}	24.6 ± 1.95
Anal fin length	17.7 ± 0.99	18.0 ± 1.22	17.3 ± 1.19	17.2 ± 0.62
Pelvic fin base length	6.3 ± 0.56	6.4 ± 0.37	6.2 ± 0.43	6.4 ± 0.32
Caudal fin length	25.7 ± 0.85 ^a	26.0 ± 1.20 ^a	24.3 ± 0.95 ^b	27.0 ± 1.90
Pectoral fin length	19.8 ± 0.94 ^{ab}	20.7 ± 1.20 ^a	18.8 ± 1.20 ^b	21.5 ± 0.64
Pectoral fin height	8.3 ± 0.28 ^a	8.1 ± 0.68 ^{ab}	7.7 ± 0.42 ^b	7.8 ± 0.39

Body depth at the anus	17.6 ± 0.70	16.8 ± 0.83	16.7 ± 0.82	16.7 ± 0.44
Body width at the anus	13.3 ± 0.59 ^a	12.0 ± 0.87 ^b	12.6 ± 2.54 ^{ab}	12.0 ± 0.54
	F1-c	F5	F4	F3
% of Head length				
Head width	61.3 ± 2.57 ^a	64.5 ± 2.56 ^b	68.2 ± 4.44 ^c	62.4 ± 5.30
Head depth	68.8 ± 1.79	71.0 ± 2.40	68.9 ± 2.93	66.5 ± 2.71
Snout length	37.0 ± 2.71	35.7 ± 2.25	36.5 ± 1.04	37.1 ± 3.16
Eye diameter	20.2 ± 1.17 ^a	21.9 ± 1.42 ^b	18.5 ± 0.81 ^c	20.6 ± 1.98
Jaw length	31.7 ± 2.16	33.4 ± 2.04	31.5 ± 2.81	31.9 ± 1.33

The number of fish used is 10 for each site except F3 where only 4 fish were used. Statistical comparisons were applied only to three sites because of the low number of fish in F3. Data in the same row with different alphabetical letters are significantly different (see text). The data for the first dorsal fin were compared only for males because of the sexual dimorphism. The values in parentheses are for females. There were no females in the sample from F4. Mean ± SD.

Table 3.6. Summary of meristic analysis (Fin ray number) of *Periophthalmodon septemradiatus* collected from four sites along the Hau River

	F1-c	F5	F4	F3
First dorsal fin	14.4 ± 0.53 (5,5,6)	15.0 ± 0.87 (12)	13.6 ± 1.77	13,13 (5,8)
Second dorsal fin	12.2 ± 0.42 ^{ab}	12.9 ± 0.57 ^a	12.1 ± 0.88 ^b	12.0 ± 0.00
Anal fin	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
Pelvic fin	5.0 ± 0.00	5.0 ± 0.00	5.0 ± 0.00	5.0 ± 0.00
Left pectoral fin	14.3 ± 0.82	15.0 ± 0.82	14.6 ± 0.97	14.0 ± 0.00
Caudal fin	19.5 ± 0.85 ^a	18.7 ± 0.67 ^{ab}	18.3 ± 1.06 ^b	19.5 ± 0.29

The number of fish used is 10 for each site except F3 where only 4 fish were used. Statistical comparisons were applied only to three sites because of the low number of fish in F3. Data in the same row with different alphabets were significantly different (see text). The data for the first dorsal fin were compared only for males because of the sexual dimorphism. The values in parentheses are for females. There were no females in the sample from F4. Mean ± SD.

Table 3.7. Summary of meristic analysis (Scale number) of *Periophthalmodon septemradiatus* collected from four sites along the Hau River

	F1-c	F5	F4	F3
Longitudinal series	47.3 ± 0.82	47.1 ± 0.57	46.7 ± 0.48	47.8 ± 0.50
Transverse backwards	7.1 ± 0.32	6.8 ± 0.42	7.4 ± 0.84	7.3 ± 0.50
Transverse forwards	5.0 ± 0.00	5.1 ± 0.32	5.5 ± 0.53	5.0 ± 0.00
Predorsal midline	18.8 ± 1.14	18.2 ± 0.42	18.7 ± 0.48	18.0 ± 0.00

The number of fish used is 10 for each site except F3 where only 4 fish were used. Statistical comparisons were applied only to three sites because of the low number of fish in F3. Data in the same row with different alphabets were significantly different (see text). The data for the first dorsal fin were compared only for males because of the sexual dimorphism. The values in parentheses are for females. There were no females in the sample from F4. Mean ± SD.

Reproductive ecology. Courtship behavior was observed throughout the distribution range. The reproductive behavior seemed to be more frequent in the early rainy season (June) than in the other months, but I did not quantify it. Upon courtship, the male changed its body color from a subdued brownish one to vivid metallic deep blue (Fig. 3.2a). Based on field observation, the body color appears to be able to revert to the non-nuptial one within minutes. The female did not change body color and maintained a muted tannish color even during courtship. A successful male then escorted the female to his burrow (Fig. 3.2b). The courtship was more frequent once the tide started to ebb at spring tide.

The burrow density was high along muddy banks of tributaries, but scarce along the main channel of the Hau River (Table 3.1, see also Fig. 3.1 and Table 3.4) except in B1, where the burrow density was similar between the main channel and tributaries. The burrow density in the uppermost site (B4) was low even in tributaries. Eggs were collected at B1 (4 clutches), B2 (4), and B3 (2). Eggs were laid on the upper wall of the spawning chamber in a single layer. Mean (\pm SD) clutch size was 6600 ± 1600 (2 egg clutches each from the three sites), and there appears to be no trend for clutch size to vary with sampling sites. Eggs were elliptical, measuring 0.7–0.8 mm in long-axis and 0.5 mm in short-axis lengths with a clump of adhesive filaments at one end of the long axis. There was a small significant difference in the long-axis length and short-axis length between the sites ($<10\%$ of each axis length, one-way ANOVA), but I tentatively regarded these to be biologically insignificant due to the small sample size. Not all egg clutches could be used for clutch size estimation because the complete recovery of eggs was not achieved in some cases.



Fig. 3.2. (a) A male of *Periophthalmodon septemradiatus* developing nuptial coloration. (b) A male *Periophthalmodon septemradiatus* about to enter his burrow followed by a female.

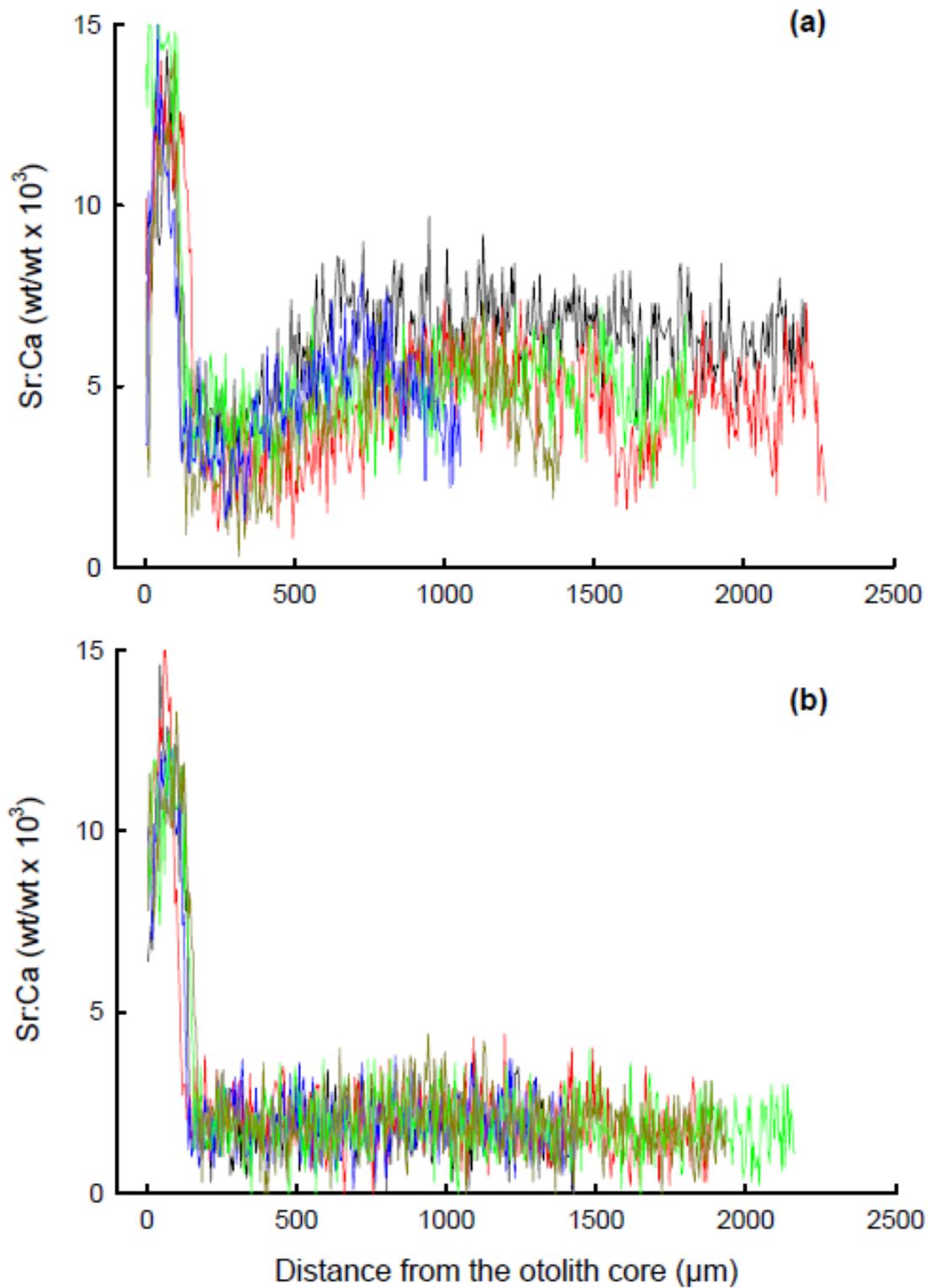


Fig. 3.3 Sr:Ca ratio (weight/weight $\times 10^3$) of the sagittal otolith of *Perioththalmodon septemradiatus*. (a) The specimens were collected at F1-a, a brackish water site (N = 5). (b) The specimens were collected at F4, a freshwater site (N = 5). Different colors indicate different individuals.

The spawning chamber was filled with air (51.0 ± 3.7 ml/burrow, $N = 3$) as in other mudskippers. Oxygen-rich conditions inside the chamber were also supported by the higher redox potential of the upper wall mud of the chamber (144 ± 48 mV, $N = 6$) than the values of the surrounding mud (-144 ± 46 mV, $N = 6$, $P = 0.00004$, $df = 5$, paired t-test). Burrow water showed mean PO₂ of 5.2 ± 1.9 kPa ($N = 11$) and salinity < 0.3 except at B1 where no data were available.

Sr:Ca ratio of the sagittal otolith showed mean values of 6.9–8.8 in the innermost “Core” segment, 11.4–12.2 in the “Peak” segment, and 1.9–4.8 in the outer “Periphery” segment (Table 3.2 and Fig. 3.3). There were significant differences in the Sr:Ca ratio in the “Periphery” segment between sites ($H_4 = 18.116$, $P = 0.001$, Kruskal-Wallis one-way ANOVA), with the values from F1, F2 and F3 significantly higher than from F4 and F5. However, there is no correlation between the “Periphery” values and the distance from the river mouth. No significant difference was found for Sr:Ca ratio of “Peak” segments between five sites ($H_4 = 2.369$, $P = 0.668$, Kruskal-Wallis one-way ANOVA). The Sr:Ca ratio at the innermost measurement point varied from 6.4 to 13.9 with one exceptional fish showing 3.4 (F1). The shift from the “Peak” to the “Periphery” segment always occurred within 200 μm from the otolith core (Fig. 3.3). Burrow water ion concentrations in the upper reaches of the Hau River (B2–4) were significantly higher than in adjacent river water for Na ($H_2 = 14.506$, $P = 0.001$, Kruskal-Wallis one-way ANOVA), K ($H_2 = 21.177$, $P = 0.001$), Ca ($H_2 = 20.710$, $P = 0.001$) and Mg ($H_2 = 20.938$, $P = 0.001$), but no difference was detected for Sr ($H_2 = 3.432$, $P = 0.180$, Table 3.8). No difference was detected between burrow and river water for any ion collected at B1.

Table 3.8. Cation concentrations of the main channel, tributary and burrow water in the upper reaches of the Hau River (B2-4) and in the estuarine island (B1)

	Upper reaches			Estuarine island	
	Main channel (N = 21)	Tributary (N = 15)	Burrow (N = 17)	Tributary (N = 5)	Burrow (N = 5)
Na ⁺	0.45 ± 0.03 ^a	0.51 ± 0.07 ^{ab}	0.64 ± 0.32 ^b	23.0 ± 1.8	22.8 ± 1.9
K ⁺	0.16 ± 0.01 ^a	0.16 ± 0.02 ^a	0.26 ± 0.15 ^b	2.12 ± 0.16	1.94 ± 0.23
Ca ²⁺	0.57 ± 0.03 ^a	0.58 ± 0.03 ^a	0.82 ± 0.37 ^b	1.50 ± 0.12	1.44 ± 0.22
Mg ²⁺	0.25 ± 0.01 ^a	0.26 ± 0.02 ^a	0.42 ± 0.25 ^b	3.47 ± 0.27	3.18 ± 0.39
Sr ²⁺	1.23 ± 0.09	1.23 ± 0.10	1.52 ± 0.58 [*]	7.03 ± 0.04	6.49 ± 0.60

Values are in mmol l⁻¹ except for Sr (μmol l⁻¹). Mean ± SD. The values of the same ion with different superscripts are significantly different (Mann-Whitney test). No statistical test was applied to the data from the estuarine island, Cu Lao Dung (B1 in Fig. 3.1) due to the small sample size. The values for upper reaches are based on combined data from B2, B3 and B4 (see Fig. 3.1. *N = 16)

Population structure. For 679-bp COII, a total of 88 sequences were obtained from *Pn. septemradiatus*, revealing 24 polymorphic sites and 23 haplotypes (Table 3.8). Overall haplotype diversity was $Hd = 0.796$ and nucleotide diversity $\pi = 0.00258$. Likewise, for 828-bp DL, a total of 88 sequences were obtained, revealing 34 polymorphic sites and 47 haplotypes (Table 3.9). Overall haplotype diversity was $Hd = 0.940$ and nucleotide diversity $\pi = 0.00471$. Haplotype richness (Hr), which was calculated with a sample size of 4, was correlated with haplotype diversity values (Spearman correlation coefficient: $r = 0.935$ for COII, $r = 1.000$ for DL, $P < 0.05$ in both cases). There were, however, no clear patterns of genetic diversity across the sampling sites. The most common haplotype of each gene was observed in all sampling sites (Fig. 3.4). Population pairwise ϕ_{st} values showed no genetic differentiation between any pair of the sampling sites using the standard $p < 0.05$ as a significance level (Table 3.8). No isolation by distance was also observed ($r_2 = 0.018$, $p = 0.743$ for COII, $r_2 = 0.053$, $p = 0.088$ for DL). GenBank/EMBL accession numbers are from LC421229 to LC421404.

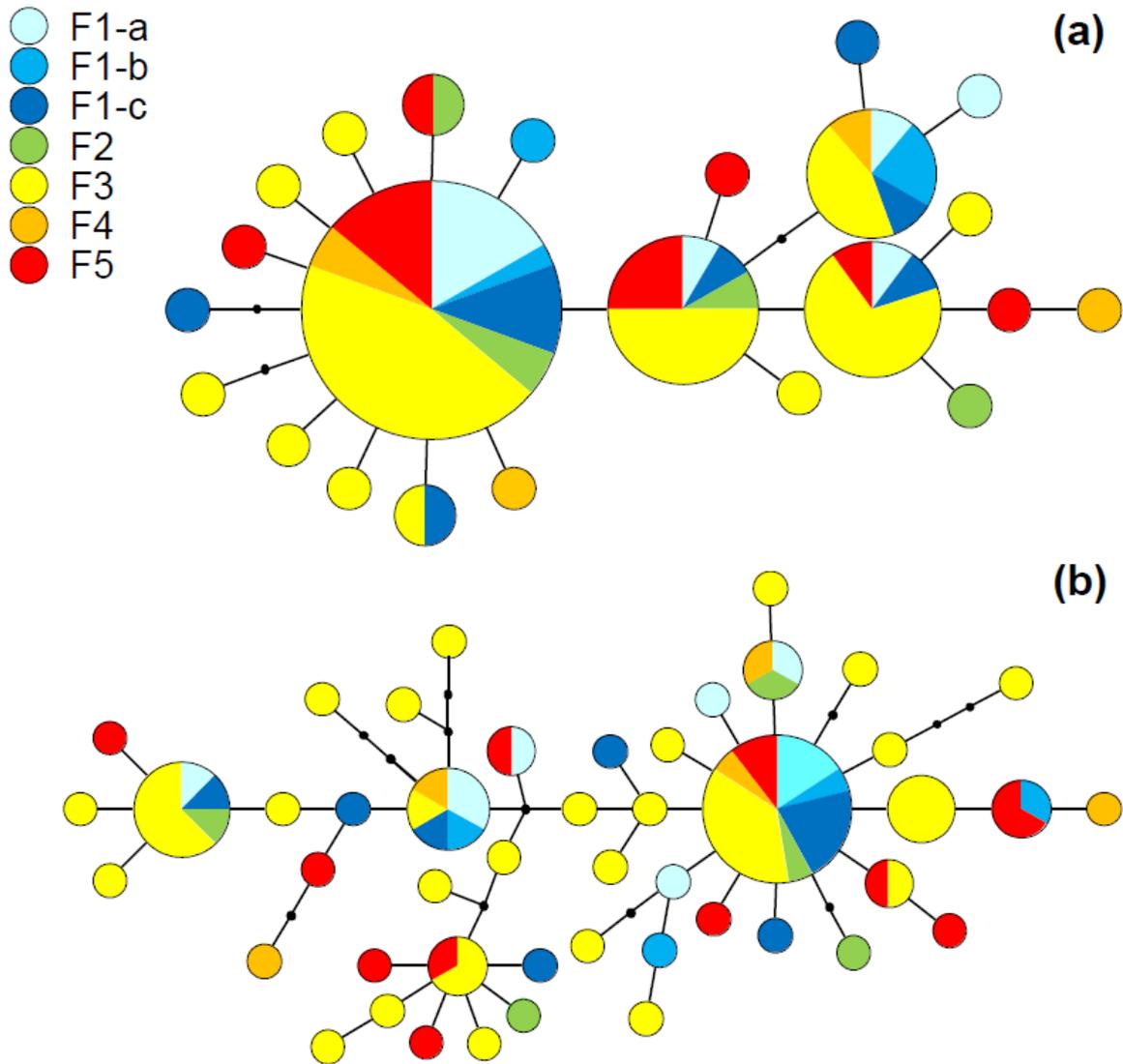


Fig. 3.4 Maximum-likelihood haplotype network for cytochrome c oxidase subunit II (a) and D-loop (b). Each circle represents one haplotype, the size of the circle corresponds to the abundance of individuals and the color indicates the sampling site (see Fig. 3.1). Black dots indicate unsampled mutations (haplotypes).

Table 3.8. Cytochrome *c* oxidase subunit II (COII) and D-loop (DL) molecular diversity indices for *Periophthalmodon septemradiatus*

Site ID	COII n	<i>h</i>	Hd	Hr	π	DL n	<i>h</i>	Hd	Hr	π
F1-a	10	5	0.667 ± 0.163	1.595	0.00242 ± 0.00073	10	7	0.911 ± 0.077	2.500	0.00381 ± 0.00061
F1-b	4	3	0.833 ± 0.222	2.000	0.00368 ± 0.00105	4	4	1.000 ± 0.177	3.000	0.00443 ± 0.00112
F1-c	10	7	0.867 ± 0.107	2.329	0.00331 ± 0.00076	10	7	0.867 ± 0.107	2.329	0.00413 ± 0.00073
F2	5	4	0.900 ± 0.161	2.400	0.00265 ± 0.00084	5	5	1.000 ± 0.126	3.000	0.00507 ± 0.00107
F3	41	12	0.802 ± 0.047	2.028	0.00240 ± 0.00029	41	27	0.954 ± 0.020	2.740	0.00502 ± 0.00038
F4	5	4	0.900 ± 0.161	2.400	0.00412 ± 0.00124	5	5	1.000 ± 0.126	3.000	0.00507 ± 0.00123
F5	13	7	0.833 ± 0.086	2.147	0.00211 ± 0.00044	13	11	0.974 ± 0.039	2.846	0.00551 ± 0.00063
Total	88	23	0.796 ± 0.036		0.00258 ± 0.00023	88	47	0.940 ± 0.017		0.00471 ± 0.00025

Sampling site ID, number of specimens (*n*), haplotype number (*h*), haplotype diversity (Hd), haplotype richness (rarefied allelic richness) (Hr), and nucleotide diversity (π). Hd and π are expressed as mean ± SD.

Table 3.9. Pairwise ϕ_{st} statistics for *Periophthalmodon septemradiatus* cytochrome *c* oxidase subunit II (COII) and D-loop (DL)

	F1-a	F1-b	F1-c	F2	F3	F4	F5
F1-a	-	-0.08527	-0.04575	-0.05719	-0.00246	-0.08156	0.00279
F1-b	0.08232	-	-0.05572	-0.05263	-0.02163	-0.11111	-0.06727
F1-c	-0.05023	-0.00317	-	-0.02967	0.00378	-0.02967	0.01762
F2	-0.04348	0.03481	-0.07547	-	-0.03367	-0.08696	-0.01687
F3	-0.02021	0.04661	-0.04101	-0.03551	-	-0.01236	0.00483
F4	-0.04348	-0.08541	-0.07547	-0.07143	-0.02278	-	-0.01687
F5	-0.01811	0.07801	-0.04206	-0.1006	-0.01767	-0.01937	-

ϕ_{st} values for COII below diagonal, ϕ_{st} values for DL above diagonal. There were no significant differences in any of the values ($p > 0.05$).

2.4 Discussion

This is the first finding of an amphibious fish inhabiting and breeding over a range from a saline environment of an estuarine island to completely freshwater habitats of the same river. *Periophthalmodon septemradiatus* colonizes the riparian zone along the two major channels of the Mekong River (Fig. 3.1), feeding and courting (Fig. 3.2) out of the water on the bank of tributaries, and spawning eggs in burrows excavated in the bank. Although mudskippers are known to be generally euryhaline (Clayton, 1993; Ishimatsu and Gonzales, 2011), there are only two species (*Pn. septemradiatus* and *Periophthalmus weberi*) that have been recorded from freshwater environments. According to the database of the fish distribution in Southeast Asia, *Pn. septemradiatus* was previously collected at 90 and 150 km upstream from the river mouths of the Hau River and the Co Chien River, respectively (Fig. 3.5) (Kano et al., 2013). A brief description of each sampling site is given in the database, but no ecological data are available. In Malaysia, *Pn. septemradiatus* was found in the Selangor River in the Malay Peninsula, about 15 km from the river mouth, where salinity was 0 at low tide and 1–3 during extreme high tides (Khaironizam et al., 2003). *Periophthalmus weberi* was collected at a creek in the Northern Territory, Australia, approximately 60 km upstream from the coast (salinity 0.1) (Larson, 2008). More extremely, *Ps. weberi* was found at about 250 km from the river mouth of the Fly River, Papua New Guinea (Roberts, 1978; Polgar et al., 2010). Unfortunately, the ecology of *Ps. weberi* has not been investigated to date. Other examples of euryhaline, amphibious teleosts include mangrove killifish *Kryptolebias marmoratus*, climbing perch *Anabas testudineus*, and swamp eels *Monopterus* spp. However, the habitat conditions, ecology, and reproductive biology of these fishes are scarcely known (Taylor, 2012). Thus, further investigation of *Pn. septemradiatus* could offer a unique window through which to glimpse how environmental salinity affects the biology and ecology of an animal during the process of niche expansion from the aquatic environment, and would thereby complement our

understanding about where early transitional vertebrates emerged from the water in the Paleozoic.

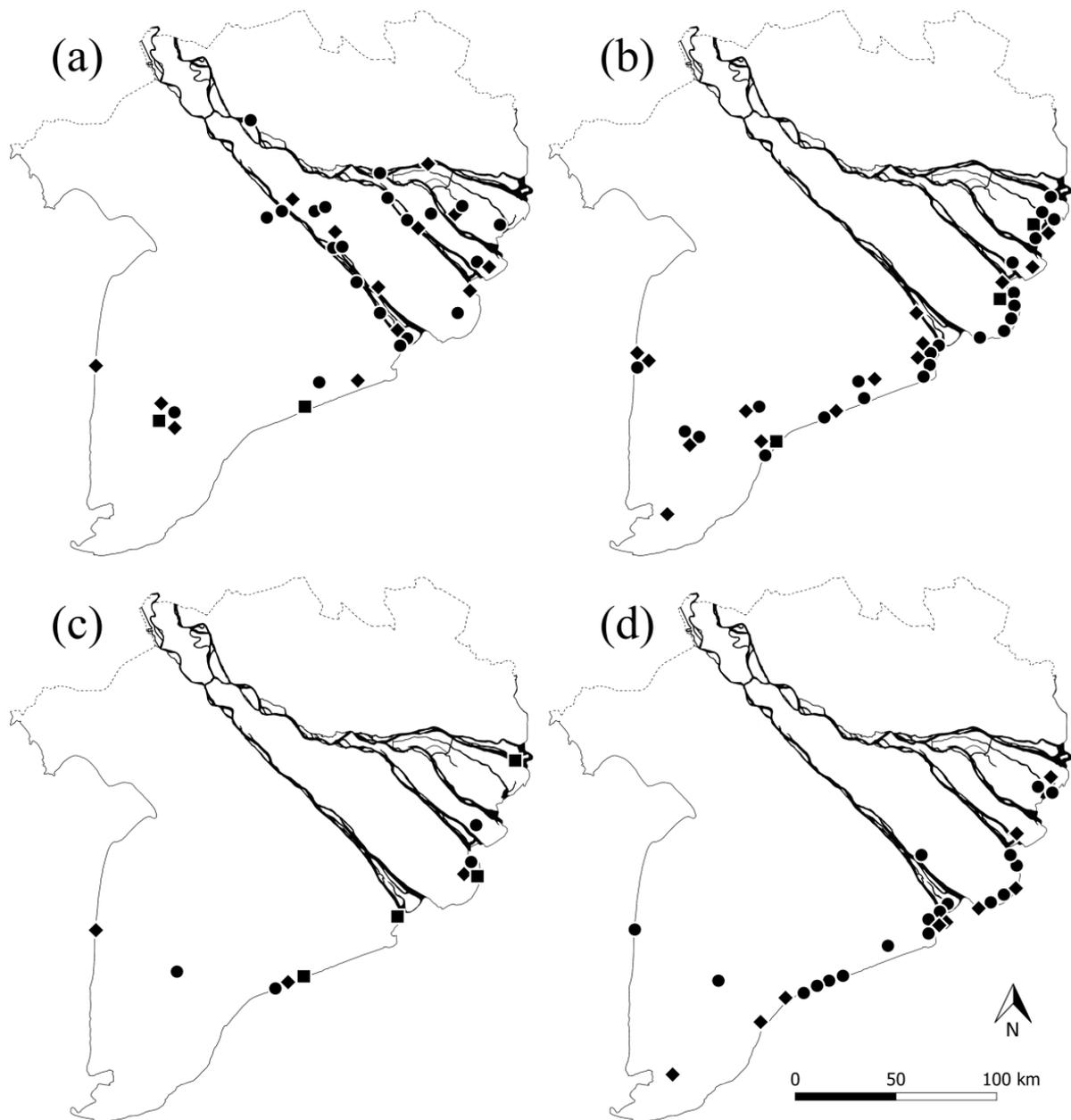


Figure 3.5. The distribution of 11 species of oxudercine gobies in the Mekong Delta. (a) Filled circles, *Perioththalmodon septemradiatus*; filled squares, *Perioththalmodon schlosseri*; filled diamonds, *Parapocryptes serperaster*. (b) Filled circles, *Perioththalmus gracilis*; filled squares, *Perioththalmus variabilis*; filled diamonds, *Perioththalmus chrysospilos*. (c) Filled circles, *Apocryptodon madurensis*; filled squares, *Oxuderces nexipinnis*; filled diamonds, *Scartelaos histophorus*. (d) Filled circles, *Boleophthalmus boddarti*; filled squares, *Pseudapocryptes elongatus*. Data are from Kano et al. (2003).

Periophthalmodon septemradiatus is probably one of the most terrestrial fishes living today. During four years of my field survey in the Mekong Delta, I did not see *Pn. septemradiatus* ventures into the water. The high terrestriality of *Pn. septemradiatus* is further supported by the finding that ants, *Dolichoderus* sp., constituted a substantial portion of the total gut content (> 80% by biovolume analysis) (Dinh et al., 2020). The *Dolichoderus* ants scavenge on the ground floor but also forage for insect secretions on low vegetation and trees (Shattuck et al., 2013). Along the banks where *Pn. septemradiatus* occurs, the ants rarely intrude onto the soft mud substrate of the intertidal zone but occur in areas above it. These observations indicate that *Pn. septemradiatus* feeds largely, if not most, in areas beyond the water's edge. Another example of a highly terrestrial mudskipper is *P. minutus* inhabiting the highest intertidal zone in Australia. The fish is able to withstand continual emersion of more than 20 days during some neap tides by retreating into its burrow (Takeda et al., 2011). *Ps. minutus* also ingests ants in addition to crabs and other animals (Takeda et al., 2011). Carnivory is presumably a crucial factor that allows amphibious fishes to extend habitat usage and exploit available food resources. In contrast, herbivorous amphibious fishes such as *Boleophthalmus* mudskippers (Clayton et al., 2017) and several rock skippers (Blenniidae) (Wilson, 2009) are trophically reliant on the lower intertidal zone because of high moisture conditions necessary for the growth of micro- and macro-algae that these fishes feed on. Early vertebrates are believed to have been all carnivorous (Clack, 2012).

None of the environmental parameters measured in this study seems to be limiting the fish's distribution range. In this regard, it is interesting to note that marine/brackish species of live bivalves were collected to 160 km from the river mouth of the Tien River–Co Chien River (Gugliotta et al., 2017). The most upstream sites of fish occurrence were nearly at the same distance from the river mouth both in the Hau River and in the Tien River, suggesting a common limiting factor for their distribution. The fish's near absence along the main channels is

probably due to the instability of the environment, where mud banks are subject to frequent erosion and deposition. Mangrove bushes probably stabilize local bank conditions, create local food webs and thereby provide a suitable habitat for *Pn. septemradiatus*.

It should also be noted that *Pn. septemradiatus* does not usually inhabit coastal tidal flats. In contrast, most other oxudercine gobies in the Mekong Delta almost exclusively inhabit coastal zones (Fig. 3.5). There are records of inland collection of oxudercine gobies in the Ca Mau Province (Kano et al., 2013). However, there are extensive canal systems and thick mangrove forests in a large part of the Ca Mau Province, and the salinity in these systems can be 40–45 in the dry season and 8 in the rainy season (Koné and Borges, 2008; Hens et al., 2009). Thus, the inland records of mudskipper occurrence in the Ca Mau Province were likely due to fish migration into those brackish/seawater canal systems. *Parapocryptes serperaster*, a cryptic oxudercine goby, was collected up to 85 km from the river mouth of the Hau River (Kano et al., 2013). The reason(s) for the absence of *Pn. septemradiatus* in the coastal mudflats is currently unknown, but the possibility includes their intolerance to seawater at some life stage(s) or competition with other mudskippers and intertidal animals.

Courtship display was observed throughout the distribution range of *P. septemradiatus*. Moreover, the fertilized eggs were collected from burrows in both fresh (B2 and B3, Fig. 3.1) and brackish water (B1) habitats. These indicate larval hatching in both fresh and brackish water environments. On the contrary, the otolith Sr:Ca data from all five sites indicate larval hatching only in saline conditions, and the subsequent migration to even higher salinity waters before settling in brackish water at F1 or freshwater at the other sites (Table 3.2 and Fig. 3.3). Environmental salinity is usually regarded as the most robust determinant of otolith Sr:Ca ratio (Campana, 1999; Zimmerman, 2005), although the ratio can also be influenced by factors other than salinity, e.g., temperature, ontogeny, and species (Elsdon and Gillanders, 2003). The high

Sr:Ca ratio was not due to lower Ca content in the otolith primordial region, the mass % of CaO in the otolith ranging 50 to 55 throughout the transect from the core to the edge of the otolith.

Two hypotheses may be proposed to elucidate the recruitment of larval *Pn. septemradiatus* to its freshwater habitats. First, larvae hatch in freshwater and migrate downstream to brackish/marine waters and migrate back to the upstream reaches, and the high Sr:Ca ratio in the otolith is due to physiological status or other factors and does not reflect environmental salinity (Correia et al., 2003). Second, larvae that hatch in freshwater are abortive and those that hatch in brackish/marine waters recruit to the entire distribution range and thus constitute the exclusive source of all the populations. The Sr concentration of burrow water was not significantly higher than the adjacent river water (Table 3.7), precluding the possibility of local accumulation of the element in burrows. None of the DNA analyses indicated the segregation of fish populations in my samples, and this is not in conflict with either of the two hypotheses. More field studies should be conducted to reveal larval occurrence in fluvial and coastal waters. In addition, larval rearing needs to be done to examine the relationship between Sr and Ca concentrations in ambient water and otolith Sr:Ca ratio; the growth rate at ambient temperatures in the Mekong River region; and salinity tolerance of larvae and juveniles under controlled conditions. Recently, Dinh et al., (2018) studied monthly changes in the gonadal development of *Pn. septemradiatus* and inferred that the fish was iteroparous.

Irrespective of routes of its recruitment, *Pn. septemradiatus* is most likely emerging from both brackish and freshwater, as suggested by the occurrence of juveniles with a total length of 24 to 27 mm in the three collection sites. The wide salinity range in which *Pn. septemradiatus* emerge provides an opportunity to study whether calcium concentration in environmental water affects the skeletal development of animals in the transition from water to land. Fishes take up calcium, a major constituent of fish bones, mostly from water through the gills (Doherty et al., 2015). However, the gills invariably diminish and atrophy with increasing dependence on aerial

respiration both in extant fishes (Okamoto et al., 2018) and in Devonian transitional animals¹. It seems therefore possible that the difference in calcium concentration in ambient water affects skeletal modification during the mudskipper's metamorphosis from larvae (pelagic) to juveniles (amphibious) (Okamoto et al 2018). My preliminary examination demonstrated a number of subtle, but significant, differences in body morphologies between sites (Tables 3.5 and 3.6), indicating a need for a further detailed study on the skeletal anatomy. A recent study demonstrated that juveniles of *Polypterus senegalus* reared in shallow water (depth 3 mm) for 8 months developed altered anatomy of the pectoral girdles as compared with water-reared control fish, which resembles the ancient anatomical changes in stem tetrapods during the water-to-land transition (Standen et al., 2014).

Nguyen et al., (2000) investigated the coastal evolution of the Mekong Delta. According to their analysis, the coastline ran near the present-day border between Vietnam and into freshwater reaches by remaining where they were left behind by the progression of the coastline, as a consequence of the high degree of freshwater tolerance and the capacity to exploit terrestrial animal food resources. Other mudskippers, both herbivorous and carnivorous, may have been precluded from the area that became increasingly distant from the coast, because of dwindling availability of food resources for them due to environmental instability as discussed earlier. Annual flooding with the peak water levels 3–4 m above usual as measured near the upper distribution limit of *Pn. septemradiatus* (Gugliotta et al., 2017) must have added to the instability of the local riparian ecosystem. Thus, both herbivorous and carnivorous mudskippers would not be able to secure stable, sufficient food resources at the river water's edge, unless they evolved a high degree of freshwater tolerance and terrestriality, enabling them to venture onto land in search for novel food resources. Extrapolating this line of reasoning, it can be speculated that some of euryhaline, transitional vertebrates of the Paleozoic era initially occurred in coastal waters, evolved the capacity of aerial respiration and terrestriality, and

expanded their distribution to freshwater reaches of a large river through progradation of deltaic regions, as we hypothesize for *P. septemradiatus*. A broad range of habitats are advocated as possible sites of the evolution from early sarcopterygian fishes to tetrapods in the Devonian, including estuaries, marginal to fully marine, river channels, flood deposits, tidal pools in a deltaic region, and flooded woodland (Ta et al., 2002; Clack, 2012; Balbus et al., 2014; Retallack, 2011). The hypothesis needs to be tested through more extensive field surveys coupled with statistical biogeography, more detailed genetic analyses including molecular nuclear markers, and laboratory physiological experiments using fishes of different developmental stages.

Chapter 4

General discussion and future direction

Fishes are basically aquatic vertebrates. Almost all of them live in water. But there are small fish groups that show adaptations to the amphibious lifestyle (Graham, 1997). Among them, mudskippers stand out as one of the largest groups that volitionally emerge from the water and spend a significant portion of time on mudflat surface. Studying the reproduction of mudskippers could give useful insights into our understanding of land invasion by ancient vertebrates.

Ishimatsu et al. (1998) found that mudskippers store air in their burrows. This finding was made a big surprise to science. And his research team also found that *P. modestus* brood their eggs in the air but submerge them for hatching (Ishimatsu *et al.*, 2007). But the mechanics of burrow spawning by mudskippers remain unknown like how a male and a female spawn and fertilize the eggs during their cohabitation in a burrow. My study found that, after the male fish

and female fish entered the burrow, the females stayed continuously inside the burrow for 245 ± 38 minutes (SD) (Fig 2.3). In contrast, the male often came out and returned to the burrow (Table 2.2). Three important points during burrow resident time of *P. modestus* include:

(1) Why female fish did not come out from the burrow eggs for extended periods of time? One possibility is that females were not ready for spawning when they entered a burrow. An unpublished study by Takita showed that eggs were not ovulated in females foraging on the mudflat surface (but see page 20).

(2) Why they locked the burrow opening(s) during spawning? This could be to avoid the invasion of other animals living on the mudflat, including conspecifics. I observed that in some cases where another non-burrow guarding male entered a burrow, and it caused the female to leave the burrow.

(3) Do male fish add air after spawning? After the female fish left the burrow, the male fish stayed inside the burrow for a period of time. This may be species-specific because Rupp observed in a laboratory aquarium that a male *P. variabilis* added air only after spawning (unpublished).

There are a few reports on the occurrence of mudskippers in freshwater. However, the earlier reports did not study the ecology of those species or discuss them in the context of vertebrate transition in freshwater and saltwater environments. *Pn. septemradiatus* is highly terrestrial and that the fish inhabits wide reaches from brackish to completely fresh waters renders the species a suitable model for the investigation of how animal's body anatomy, physiology, and ecology are to be modified during emersion to land from waters of different salinities.

What limits the distribution of Pn. septemradiatus? The distribution of *Pn. septemradiatus* is unique not only because of its extended range into the upstream direction but also restricted occurrence along the coastal tidal flats. To gain a clue to understanding factors limiting the

upstream distribution of *Pn. septemradiatus*, I measured the most basic environmental parameters within and beyond the distribution range of the species. None of the measured parameters seems to be responsible for preventing the fish from migrating beyond the observed distribution range. In this respect, it is interesting that Gugliotta et al., (2017) suspected that seawater influence could reach 160 km upstream of the river mouth of the Tien River. It should also be noted that the most upstream sites of fish occurrence were nearly at the same distance from the river mouth between the Hau and Tien Rivers, again suggesting a common limiting force for their distribution.

Where does the fish reproduce, in freshwater, in coastal areas, or both? The courtship display was found from all observation sites from the coast to the upper limit of distribution. These results support that the fish reproduce in both freshwater and coastal areas. On the other hand, the Sr:Ca ratio showed that all the samples (coastal and freshwater samples) were spawned in high salinity conditions because Sr:Ca is much higher than 8.4 (this is presented by de Villiers et al, 1994). This suggests that the fish hatched on the coast and then, migrated and settled on the way from the coast to upstream reaches.

Factors driving mudskipper reproduction using burrows with an air phase may include:

Temperature

The mudskipper burrow may expose to direct sunlight during low tide which may cause desiccation and temperature extremes. By using the spawning chamber with an air phase, mudskipper could avoid these daily rapid changes compare with air or mud surface. The mud surface temperature was higher than the air temperature at the same hour. The deep burrow temperature remained relatively stable (Chen et al., 2007).

Oxygen

The burrow water is extremely hypoxic (Gordon, 1995; Gonzales et al., 2006). Etou et al., (2007) found 100% mortality in embryos of the mudskipper *Periophthalmus modestus* within one hour in water at 10% oxygen saturation, although the level of dissolved oxygen was even lower in burrow water in their natural habitats (Ishimatsu et al., 2007).

Predation

Tidal fluctuation may provide a refuge for embryos in the upper intertidal zone, which aquatic predators cannot reach, although such placement exposes adults and embryos to terrestrial predation (Martin and Raim, 2014). Very few studies have examined the impact of aquatic or terrestrial predation on the survival of terrestrial fish eggs or spawning adults (Martin, 2015).

Future research directions

More field studies should be conducted to reveal larval occurrence in fluvial and coastal waters. In addition, larval rearing needs to be done to examine the relationship between Sr and Ca concentrations in ambient water and otolith Sr:Ca ratio; the growth rate at ambient temperatures in the Mekong River region; and salinity tolerance of larvae and juveniles under controlled conditions. Recently, Dinh et al., (2018) studied monthly changes in gonadal development of *Pn. septemradiatus* and inferred that the fish was iteroparous. Irrespective of routes of its recruitment, *Pn. septemradiatus* is most likely emerging from both brackish and freshwater, as suggested by the occurrence of juveniles with a total length of 24 to 27 mm in the three collection sites. The wide salinity range in which *Pn. septemradiatus* emerge provides an opportunity to study whether calcium concentration in environmental water affects the skeletal development of animals in the transition from water to land.

Comparison *Pn. septemradiatus* with others mudskipper is worthwhile. It is presently unknown why *Pn. septemradiatus* could invade up to 150km from the river mouth in Mekong delta while most other oxudercine gobies in the Mekong Delta almost exclusively inhabit coastal zones. It is also not clear why *Pn. septemradiatus* does not usually inhabit coastal tidal flats. We need to have more surveys to examine the biotic and abiotic factors to answer these questions.

Survival of *Pn. septemradiatus* larvae needs to be studied under different environmental conditions. To clarify the recruitment of larval *P. septemradiatus*, we must conduct laboratory experiments on environmental effects on larval survival, especially focusing on salinity.

Larvae of *Pn. septemradiatus* hatch in a much younger stage and much less developed, compared with other mudskipper species. Ecological implication of this early hatching deserves to pursue.

Reproductive behavior inside the burrow is still unknown. This needs much more sophisticated tactics of installing minimally disturbing observation tools inside a spawning chamber and careful and patient observation in the field. The task seems formidable but it could answer the main question given in the chapter 2, whether spawning and fertilization take place in air or water.

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