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Doctoral Dissertation

**Population divergence by local adaptation  
to acoustic environment in  
the Ruddy Kingfisher (*Halcyon coromanda*)**  
(アカショウビンにおける音響環境への局所適応が  
もたらす集団の分化に関する研究)

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# Abstract

Differing quality and quantity of available resources among habitats, causes differentiation in mating signals. The acoustic environment is one such resource, and it can readily be divided by frequency (Hz). Among avian species that learn their calls, there are many examples showing that the characteristics of calls diverge in relation to the gradient or variance in the acoustic environment, such as the amplitude of environmental noise and sound attenuation, even among adjacent regions. In this study, we targeted a species that does not learn its calls, but inherits them. In such a species, adapting to the local acoustic environment may cause isolation among populations and eventually lead to speciation. Here we show that the differences between the acoustic environments of adjacent islands may cause pre-mating isolation in the Ruddy Kingfisher. We investigated differences in the characteristics of this migratory kingfisher's innate calls among populations in the Ryukyu Archipelago. Their innate calls parallel their genetic structure. The kingfisher seems to avoid the loud environmental noise caused by cicadas. In addition, a playback experiment revealed that assortative mating might be occurring. Our results show that even nearby populations of birds that have flight capability may speciate due to acoustical adaptation.



# General Introduction

Among many taxa, acoustic signals have been shown to be important in mate attraction, territory defense, and species recognition (Boughman 1998, Herman. 2016). Acoustic environment is a resource type that can be described by frequency (Endler 1992). Biotic and abiotic factors affect the acoustic environment. A signaler benefits from having its signal detected by a potential receiver. As both sound transmission and masking play a crucial role for the signal-to-noise ratio at the position of a potential receiver, signalers shift the frequency of their acoustic signals according to the environment property.

High-frequency noise is generally more common in tropical forest than temperate forest. This is due to the presence of high frequency noise made by insects or frogs (Morton 1975; Padgham 2004; Boncoraglio and Saino 2007). As a result, birds in tropical forest are selected to sing at lower frequency range than birds in temperate forest (Weir et al. 2012). On the contrary, the song frequency shift upward in urban area than rural area due to large low-frequency noise emitted by artificial activities such as cars and factories (Slabbekoorn and Peet 2003).

Dense vegetation attributes the signal attenuation. Attenuation rate is generally higher in forest environment than more open habitats, including grassland, marshland, and scrub. Song attenuation is particularly strong at high frequency because high frequency sound has a short wavelength, which makes it difficult to diffract obstacles in space (Bradbury and Vehrencamp 2011). To minimize the effect of attenuation on their song, birds that live in dense habitat such as tropical forest sing at lower frequency (Morton 1975; Padgham 2004; Tobias et al. 2010). The difference in signal attenuation rate among habitats affects the song frequency of the birds inhabiting there. For example, Amazonian birds that live in bamboo forest sing at lower frequency than birds live in terra firm forest reflecting the high attenuation rate of high frequency signal in bamboo forest (Tobias et al. 2010).

In this way, signalers shift their frequency to compensate the maximum signal-to-noise ratio avoiding the frequency range with noisy environmental noise and/or low attenuation ratio. The characteristics of acoustic environment vary according to biotic and abiotic factors. Even among conspecific populations, acoustic signals can vary if they inhabit in habitat with acoustically different characteristics.

The diversification of mating signal leads to pre-mating isolation among conspecific populations (Boughman 2001). When acoustic signals are responsible in mate choice and diverse by local acoustic adaptation among populations, the difference causes pre-mating isolation (Schluter 2001). Species-specific acoustic signal is important in intra-specific recognition and act as behavioral barrier to reproduction (Becker, 1982; Grant & Grant, 1998). Territory defense and mate attraction becomes less functional among populations that have different song types (Slater & Catchpole 1990; Irwin et al. 2001; Brambilla et al. 2008). Bird populations often have population specific song characteristics even among conspecific passerine populations due to their adaptation to

different acoustic environments in their habitats (Mundinger, 1982; Kroodsma, 2004). Intra-specific song variations prevent gene flow among conspecific populations at the initial stage of differentiation (Dingle et al. 2008). In this way, geographical difference of bird song causes pre-mating isolation among populations that are potentially accessible.

Differences in acoustic environment can cause population diversification by applying different selective pressure to each population. In general, birds are relatively unaffected by geographical barriers owing to their capability over a wide range (Price 2008). However, when local adaptation to the acoustic environment of each habitat is subject to strong selection pressures, it is thought that migration is restricted even among adjacent habitats. There are plenty of studies on the variation of bird song among populations in response to the acoustic environment of their habitats (Soha et al. 2004, Ruegg et al. 2006; Leader et al. 2008; Potvin et al. 2013; Branch & Pravosudov 2015; Ortiz-Ramirez 2016). However, previous studies have not revealed the parapatric diversification caused by song divergences that are shaped by local acoustic adaptation. This is probably because the objects of such previous studies have been birds that learn their songs. Song learning has been reported from *Passeriformes*, *Psittaciformes*, and *Trochilidae* (Kroodsma 2004, Rios-Chelen et al. 2012). It has been inferred that these birds are able to learn adaptive new calls even when in locations different from their natal areas, where the characteristics of the acoustic environment are different. In such avian species, differences among acoustic environments do not prevent dispersion across populations. In this thesis, I investigated geographical variation of Ruddy Kingfisher's (*Halcyon coromanda*) song as a cause of pre-mating isolation. Ruddy Kingfisher is classified as Coraciiformes, which is thought to have innate call characteristics (Kroodsma 1982). They breed in Ryukyu Islands as a summer visitor and thought to winter in Philippines and Talaud Islands (The Ornithological Society of Japan 2012, Woodall 2017). However, no Kingfisher that was ringed in Japan has ever been observed or recaptured abroad (Biodiversity Center of Japan 2019). Their migration route and wintering sites are remained unknown. We describe the migration and wintering site of Ruddy Kingfisher in the first study of its kind (Chapter I). *H. c. Bangsi* breeds throughout the Ryukyu Archipelago. There are many subspecies in the Ryukyu Archipelago that are classified into different subspecies or whose genetic structure is known to be different even between adjacent islands (Seki et al. 2007; Hamao et al. 2016). I compared the morphological traits and song characteristics among populations of the migratory Ruddy Kingfisher. I found geographical variations in song characteristics but no morphological differences (Chapter II). The difference in song indicates the parallel difference in their genetic structure because they are thought to have innate song characteristics. By comparing the acoustic environment of the study Islands, I investigated the factors that affect the song characteristics (Chapter II). Furthermore, I conducted playback experiment to examine whether pre-mating isolation are induced among populations by song differences (Chapter II). In order to investigate the correspondence of genetic structure among

populations with the geographical difference of the songs and the results of playback experiments, I constructed a haplotype network of COI sequences from a database (Chapter II).

Chapter I

**Site fidelity and  
migratory behavior  
of Ruddy Kingfisher  
on Miyakojima**

## INTRODUCTION

The majority of kingfisher *Alcedinidae* species have pan-tropical distributions, and most (104 out of 114 species) are resident (Woodall 2017; Gill & Donsker 2020). The migratory strategies of the few migratory species of *Alcedinidae* are poorly understood; however, a phylogeny of kingfishers by Andersen et al. (2017) indicates that their migratory strategies may have evolved independently.

Ruddy Kingfisher *Halcyon coromanda* occurs in South-East Asia to Nepal, south China, north east China, Korea and Japan. Ten subspecies of the Ruddy Kingfisher *Halcyon coromanda* have been recognised, three of which are migratory (Woodall 2017; Gill & Donsker 2020). In Japan, 2 subspecies (*H. c. major* and *H. c. bangsi*) are recorded and are exclusively summer visitors (The Ornithological Society of Japan 2012). *H. c. bangsi* breeds in the Tokara Islands, Amami Islands, and Ryukyu Islands. Although they were thought to winter in Philippines and Talaud Islands (Woodall 2017), none of the birds ringed in Japan have been recorded or recaptured there (Biodiversity Center of Japan 2019), and their migration routes and wintering sites have remained unknown. Revealing the migratory movement of a migratory subspecies of the Ruddy Kingfisher may help our understanding of the evolution of migration in kingfishers.

In this chapter, I describe the first use of Global Positioning System (GPS) data loggers to track the autumn migration and wintering locations of Ruddy Kingfisher from a breeding population on Miyakojima, Japan. This kingfisher is particularly suitable for migration research using GPS data loggers because of their body mass and high recapture rate in the study area (Uemura unpublished data). I also describe their site fidelity in this population.

## MATERIALS AND METHODS

### Study area and species

The study was conducted in Ohno Sanrin Forest, on Miyakojima in the Ryukyu Archipelago, Okinawa, Japan (24°48'30.7" N, 125°18'57.1" E). This secondary evergreen forest is flat and dominated by *Pinus luchuensis* and *Bischofia javanica*. Ruddy Kingfishers arrive in the study area during early April, excavate their nest cavities in tree snags, and commence egg laying in May and June (Uemura unpublished data). They leave Miyakojima until late September (Wild Bird Society of Miyako (2014)). The recapture rate is 32% in our field (Uemura unpublished data).

### Capture, marking and tagging

I captured three pairs of Ruddy Kingfishers using mist-nets, in front of their nests from 19 to 23 July 2016. Each captured individual was weighed to the nearest 1 g using a 300 g spring scale (40300, Pesola AG); their body masses ranged from 94 g to 119 g (mean±SD 103.7±7.7g n=6). Individuals were ringed with numbered aluminium and coloured plastic rings (Japanese Ministry of the

Environment) for identification in the field, and blood samples were taken to confirm their sex (Fridolfsson and Ellegren 1999). GPS data loggers (PinPoint-10, Loteck Wireless Inc.) were attached to the back of each kingfisher using Rappole-Tipton style harnesses made of 2 mm tubular Teflon ribbon (Rappole and Tipton 1991) (Fig. I-1). The GPS loggers and the harness were weighed to the nearest 0.1g using a digital scale (KP-104, Tanita Corporation). The GPS loggers, including the harness, weighed approximately 2.4g, amounting to 2.0 % to 2.6 % of each individual's body mass.

In addition to these individuals for the GPS tagging, 154 adult birds from 2013 to 2017 and 110 nestlings from 2015 to 2017 were ringed with numbered aluminium and coloured plastic rings for identification in the field.

### **Recapture and reconfirmation**

In order to recapture the GPS tagged individuals, I used mist-nets to recapture the tagged kingfishers in the Ohno Sanrin Forest in the breeding season (April to June) 2017. I tried to recapture them almost everyday as far as the weather permitted. When a focal kingfisher was recaptured, I retrieved GPS data logger and re-weighed them. Recapture of ringed individuals other than tagged with GPS and reconfirmation of them by visual observation was counted and calculated the return rate.

### **GPS locations and analysis**

Non-breeding season was divided into three-time intervals, August to October, November to February and March to April, and GPS data collection was individually programmed to each interval. For the first and the third period, the loggers were set every three days to track their migration while it was set every nine days for the second period to save battery of the tag. All fixes were made at 2130 JST. As the location data were downloaded from the GPS data loggers as geographic coordinates no further processing was necessary to determine locations. I eliminated fixes with dilution of precision values lower than 10. This thresholding is sufficient for a reliable point indication. Locations were mapped using Google Earth (7.1.2.2041, Google, US). The onset of migration was defined as the date after which all subsequent locations occurred away from the breeding area. A wintering site was defined as the last site a bird remained for more than a month.

## **RESULTS**

Three of the six marked kingfishers were recaptured on Miyakojima in 2017: male A on 20 April, male B on 10 June, and female C on 11 June. On the dates of recapture male A weighed 96 g (4 g lighter than at initial capture), male B weighed 104g (2 g lighter), and female C weighed 112g (18 g heavier). Although each of the kingfishers showed some feather wear (from their harnesses) on their

backs and knees, none exhibited plumage or skeletal disorders. I could not observe or recapture the other marked kingfishers.

When I tagged focal individuals in 2016, pair A was rearing 3 nestlings, pair B was incubating 2 eggs, and pair C was rearing 3 nestlings. After I tagged them, all nestlings raised by pair A and C successfully fledged, and one nestling from pair B fledged while one egg did not hatch (Table I-1). The body mass of the 12 day-old nestlings ranged from 97.7 to 116.2 (mean±SD 107.2±6.0g n=7) while it was 57.6 to 123.1 (mean±SD 105.3±11.4g n=49) in all 12 day-old nestlings in 2016. Male A occupied the same territory in 2017 as in 2016, mated with female C in 2017 and successfully fledged four nestlings. In 2016, female C had mated with male B.

I was able to download data from males A and B, but not from female C. Female C's GPS data logger antenna had disappeared and further missing parts prevented any data from being downloaded (Vandentillaart personal communication).

Male A remained in its breeding territory on Miyakojima until 24 September 2016 (Fig. I-2A and I-2B). Two locations were obtained during male A's migration, the first, on 30 September, was off Okinawa Island, about 220 km northeast of Miyakojima at an altitude of 3,945 m, and the second, on 6 October, was off Polillo Island at an altitude of 2,353 m. Male A continued its migration until at least 9 October, but the battery ran out after the logger failed to record its locations on 12 and 15 October. Male A may have landed on Tablas Island on 9 October because its GPS data logger recorded an altitude of 98.2 m at a point where the elevation is known to be 44 m above sea level. The altitude data obtained during migration were based on more than four satellites, thus it was thought to be credible. The distance between Miyakojima and Tablas Island is 1,429 km, whereas that between Polillo Island and Tablas Island is 300 km. The total migration distance including the point off Okinawa Island and off Polillo Island was 1815km.

Male B was last recorded in its breeding territory on Miyakojima on 24 September 2016; by 3 October it had reached The Philippines. No data were obtained during migration because the GPS data logger failed to record locations on 27 and 30 September. Male B spent the period from 3 October to at least 17 December around the Dangla River, Polillo Island (Fig. 2C), after which the data logger battery failed. The GPS data logger recorded altitude of 65m to 117m at points (mean±SD 90±15m n=10) where the elevation is known to be about 40 m above sea level. The distance between Miyakojima and Polillo Island is 1,153 km. Although the GPS data loggers had been programmed to fix data until the birds returned to their breeding grounds in spring 2017, their batteries ran out after just a few months.

I ringed 131 adults and 110 nestlings in this population. 46 adults and 9 nestlings were recaptured or observed on Miyakojima after the following year. the return rate was 35.1% for adults and 8.1% for nestlings.



## DISCUSSION

The weights of males A and B, and female C did not change much between tagging in 2016 and recapture in 2017. After I deployed tags on them in 2016, all pairs reared chicks of normal body mass. Furthermore, male A and female C successfully fledged four nestlings (naturally occurring fledging of four in this population (Hamachi et al. unpublished data)) after their GPS data loggers had been removed. It appears that the GPS data loggers did not constitute a deleterious burden for the birds.

The GPS data showed that both male (A and B) Ruddy Kingfishers remained within their breeding territories from their capture in July 2016 until late September when they began their migration. The possible height bird reached in the area was approximately 40 to 60 meter high. The altitude data showed an error range of -40 to +60 m. This confirms that the GPS data provided by these tags were reliable enough even when the birds were in the flat forest.

After its migration, male B remained around the Dangla River on Polillo Island for more than 45 days, indicating that it wintered in the area, which is dominated by mangrove forest (Hampson et al. 2003). Male B's GPS data logger recorded its altitude as 65.3 to 117.2 m. The area is known to be about 40 m above sea level, and given that tropical rain forest trees can reach heights of more than 70 m and mangrove trees may reach up to 40 m, the altitude data recorded appear to correctly assess the bird's position in forest.

Male A commenced its migration by 30 September and initially moved northeast before turning south. Seven days later, male A arrived off Polillo Island. The two data points obtained were both over the sea and indicated that male A migrated at high altitude. Above 2,000 m, stable winds blow in what is called the free atmosphere; there, the wind is scarcely affected by either geographical features or convection currents (Roland 1988). Flight above this altitude may be especially efficient for long distance migration (Kerlinger and Moore 1989). An initial flight of male A to the northwestward may indicate that he got caught in a typhoon wind. The 17th Typhoon in 2016 approached Miyakojima during 26 and 27 September. Typhoon wind and clouds reaches to the altitude he was flying. Male A landed on Tablas Island on 9 October, but the lack of subsequent data prevented us from judging whether it wintered there or continued its migration further south.

Due to the limited data I obtained, I could not reveal their migration and wintering sites fully. However, the wintering site inferred for male B and A was consistent with the wintering range of *H. c. bangsi* described by Woodall (2017). *H. c. bangsi* is thought to overwinter in Philippines and Talaud Islands (Woodall 2017).

Although males A and B bred in neighboring territories on Miyakojima, Japan, their wintering sites in The Philippines were far apart. The link among breeding and overwintering locations may be weak in Ruddy Kingfishers.

In this study, only two locations during migration between Miyako and Tablas islands were obtained from male A. In the future, improvement of loggers will make it possible to obtain more data during migration between breeding site and wintering site by setting data loggers to record locations more frequently (e.g. twice a day). As the data logger batteries lasted less than six months it should be possible to save power by commencing data recording from late September onwards when Ruddy Kingfishers begin migrating.

Chapter II

**Geographical variation in  
bird calls as a cause of  
population divergence.**

## INTRODUCTION

The gene flow of most terrestrial vertebrates is significantly interrupted by geographical barriers (Burnell and Hedges 1990, Beheregaray et al. 2004). Birds are relatively unaffected by such barriers owing to their capacity for flight over a wide range of distances (Price 2008). However, many avian species have diverged, even among adjacent regions (Mayr and Diamond 2001; Seki et al. 2007). Such divergence and the resulting localized distributions are thought to have resulted from ecological speciation. Ecological speciation is driven by local adaptation to different types, and/or different abundance levels, of resources among habitats.

Acoustic space is a resource type that can be divided by frequency range. Vocal behavior related to breeding is known in many taxonomic groups including insects, amphibians, birds, and mammals (Winn and Winn 1978; Czarnowsky & Forester 1985; Aspi and Hoikkala 1985). Bird song is one of the best-known sexual traits that function both as territorial defense and mate attraction (Kroodsma and Miller 1996). Sexual selection should favor individuals whose sexual traits are efficiently recognized by potential mates or competitors in natural habitats. Different habitat favors different sexual traits (Morton 1975). Although there has been great support for this sensory drive hypothesis shaping visual tuning, there is also increasing support for auditory tuning (Cummings and Endler. 2018). The signaler can obtain or defend a territory and a mate by broadcasting their signals efficiently. Conversely, signals that are not suited to the acoustic environment are maladaptive. The divergence in auditory traits occurs to avoid loud environmental noise. Using the same frequency in calls as occurs in a loud environment is not adaptive, because loud environmental noise masks calls (Boncoraglio and Saino 2007). To readily distinguish deliberate signals from environmental noise, the signal-to-noise ratio should be high (Fay 1988; Forrest 1994).

Weir et al. (2012) compared many passerine species and showed that species breeding in the tropics are evolutionarily selected to sing at lower frequencies than species breeding in the temperate region so as to avoid high frequency loud environmental noise in tropical forests. Many studies have revealed that characteristics of calls differ even among conspecific passerine populations due to their adaptation to different acoustic environments in their habitats (Hunter and Krebs 1979, *Parus major*; Anderson and Conner 1985, *Cardinalis cardinalis*; Slabbekoorn and Smith 2002, *Andropadus virens*; Slabbekoorn and Peet 2003, *Parus major*; Kirschel et al. 2009, *Hylia prasina*).

Individuals that sing differently in certain areas are often reproductively isolated because inter- and intra-sexual recognition becomes less functional (Slater and Catchpole 1990; Irwin et al. 2001; Brambilla et al. 2008; Ripmeester et al. 2010). In this way, the local adaptation of calls leads to reproductive isolation preventing gene flow between populations, and eventually leading to ecological speciation.

Previous studies have revealed that the geographical song divergence have been shaped by local adaptation to acoustic environment that causes premating isolation among adjacent populations

(Dingle et al. 2008, Caro et al. 2013, Halfwerk et al. 2016). However, many previous studies on call dialects have not necessarily shown linkage between the characteristics of calls and genetic structures. (Soha et al. 2004; Ruegg et al. 2006; Leader et al. 2008; Potvin et al. 2013; Branch and Pravosudov 2015; Ortiz-Ramirez 2016). This is probably because the objects of such previous studies have been birds (such as songbirds and parrots) that learn their calls (Rios-Chelen et al. 2012). It has been inferred that these birds are able to learn adaptive new calls even when in locations different from their natal areas, where the characteristics of the acoustic environment are different. In avian species that learn their songs, differences among songs do not prevent dispersal between populations and will not lead to reproductive isolation. Conversely, among birds with innate calls, dispersal across populations that have different call dialects, due to different acoustic environments, will be restricted.

Vocal learning has been found in three taxonomic groups of birds: Passeriformes (Kroodsmas 1982), Psittaciformes (Gramza 1970; Nottebohm 1970; Rowley and Chapman 1986), and Trochilidae in Apodiformes (Baptista and Schuchmann 1990). In order to investigate the relationship between call characteristics and genetic structures, it is necessary to study bird species from groups other than these three. However, little research has been done on the link between call characteristics and genetic structures in birds with innate calls (cf. Bertelli and Tubaro 2002; Guo et al. 2016).

In this study, I investigated whether or not the Ruddy Kingfisher *Halcyon coromanda* has identifiably different calls among populations and whether or not any such differences parallel genetic differences. Calls are thought to reflect genetic structure. At first, I compared the maximum frequency of calls among three island populations in the Ryukyu Archipelago, southern Japan. Subsequently, to examine whether or not kingfishers assortatively respond in mating behavior, based on geographic call variation, I conducted a playback experiment on Miyako Island (Miyakojima). Playback experiments are generally used to examine whether different populations view each other as potential competitors or mates by examining male response intensity (Irwin et al. 2001; Irwin et al. 2010). As the broadcast calls were recorded on various islands, I expected that the intensity of responses by kingfishers would differ among calls. There are cases where call differences are a feature adapted to the acoustic environment of the local habitat and cases where it is a feature associated with a change in body size resulting from other selection pressures (Wallschläger 1980; Podos 2001; Mahler and Tubaro 2001). As parameters of acoustic features that affect call frequency, I measured frequency dependent environmental noise and attenuation. Kingfisher should use the frequency range with small environment noise and/or small attenuation rate if their calls fits the acoustic adaptation hypothesis. Then I compared these measurements with call frequency. In addition, I measured and compared the body size of different Ruddy Kingfisher populations. Finally, I performed genetic analyses to determine whether genetic differences between populations, as suggested by vocal differences and playback experiments, are supported by their genetic structure.

## METHODS

### Study species

The Ruddy Kingfisher is a summer visitor to the Ryukyu Archipelago (Ornithological Society of Japan 2012). It winters in the Philippines (Uemura et al. 2019). After arrival on its breeding grounds, it commences breeding in April, migrating back to the Philippines during late September and October. Both sexes exhibit strong site fidelity in their breeding sites; males especially return to the territories they occupied in the previous year (Hamachi and Uemura unpublished data). Some individuals, marked as chicks in their nest have been confirmed to return to our research site on Miyakojima. The Ruddy Kingfisher's call has a monotonous falling tone that fades away. The fundamental tone is distributed in the range of approximately 1–2.4 kHz. Occasionally, calls with harmonic tones have also been noted. The kingfishers continue vocalizing, giving advertising calls, through their breeding season, especially at dawn and dusk.

### Call recordings and analysis

I recorded Ruddy Kingfisher calls on three islands in the Ryukyu Archipelago: Amami-Oshima, Iheyajima, and Miyakojima (Figure II-1). Recordings of calls were made from April to June in 2015 and 2016. I did not make recordings in bad weather such as heavy rain or typhoon conditions. At least 10 calls per individual were recorded. Recordings were made in wave format files with a sampling rate of 44.10 kHz using a Fuji Planning LiSN LS370 parabolic microphone connected to a SONY PCM-D50 digital recorder. Recording locations were obtained by GPS (GARMIN eTrex 20xJ).

Calls were analyzed with *Raven Pro* version 1.5 (The Cornell Lab of Ornithology, Ithaca, New York). Spectrograms were made using a Hanning window with a window size of 2514, resulting in a fine frequency resolution of 4 Hz, with the monochrome palette setting (Charif et al. 2010).

I measured the maximum frequency of a call visually on a spectrogram, and obtained at least 10 measurements of maximum frequency per individual. The maximum frequency occurred within approximately 0.1 s from the onset of each call. Ruddy Kingfisher calls fade away, obscuring the end of each call, making it difficult to make other measurements such as call duration or minimum frequency. Calls with harmonic tones were eliminated from the analysis.

### Environmental noise sampling

To assess whether environmental noise differs between the islands, I placed OLYMPUS DS-750 digital recorders on each island and recorded ambient environmental noise during May and June when Ruddy Kingfishers settle in their territories and breed. Recordings were made automatically twice a week using the timer function of the recorder set to start at 40 min before the earliest sunrise

time during the period and end 2 hours and 30 min after the latest sunrise time during the same period. I obtained 3 hours and 10 minutes of environmental noise samples per day. Recordings were made in wave format files with a sampling rate of 44.10 kHz. Recordings were made at sites where I had recorded Ruddy Kingfisher calls in the previous year. The recorder was covered with a waterproof box exposing the microphone under the box, and placed at the height of about 2.5 m from the ground (Figure II-2). Recordings were conducted in three locations on each of the three islands: Amami-Oshima, Iheyajima, and Miyakojima. However, recording at one location on Iheyajima failed because of poor contact between the recorder and the battery.

I assessed the spectral characteristics of the local environmental noise from the noise recordings. I extracted the first 10 seconds of every 10 minutes from the noise recordings and combined them into a 3 minutes and 10 seconds audio file. Since I used five recordings (once a week) to measure environmental noise, the total recordings used for each recording site was 15 minutes and 50 seconds. The Power spectra were obtained in Raven Pro 1.5 from the recording (total 10 min for each recording). In order to eliminate the effect of white noise mainly caused by wind from environmental noise, a spectrum representing the relative sound volume was drawn based on the sound volume at 2 kHz at each recording location (2 kHz is the typical maximum frequency of a Ruddy Kingfisher's call).

### **Transmission experiments**

To assess whether sound transmission features differ between islands, I conducted sound transmission experiments on each island. I played back artificial sounds and recorded them, after transmission through vegetation, at the locations where environmental noise was recorded (cf. Slabbekoorn et al. 2002). One tone was 1863 Hz and the second was 2327 Hz, generated using Audacity® 1.3.13-beta (1999-2019 Audacity Team). Both tones were constant frequency tones of 1 sec duration and of equal amplitude throughout the tones, except for 1s ramps for smooth onset and offset of the tones. Volume levels for playback and recording of stimuli were standardized throughout all experiments. The sounds were recorded at 4.0 m and 16.0 m from the speaker. Recordings were made in wave format files with a sampling rate of 44.10 kHz, using OLYMPUS LS-7 digital recorder. Central Mic ON, Mic Sense Middle, Rec Mode PCM 44.1kHz/16bit, Rec Level Manual Limit/Comp OFF; Zoom Mic +6zoom Low Cut Filter, Off; VCVA, Off; V-Sync Rec, Off; Timer Rec, Off; Rec Scene, Off; Rec Monitor, Off; Plug-in Power, Off; Pre-Recording, Off. Speaker and microphone heights were 2.0 m. The transmission characteristics of the habitats were compared by examination of the amplitude at 16.0 m as a proportion of the amplitude at 4.0 m at each location. I conducted this experiment at 14 locations on Amami-Oshima, six locations on Iheyajima, and five locations on Miyakojima.

### **Playback experiment**

I carried out playback experiments on Miyakojima Island between 30 minutes after sunrise and 8:00 AM, when birds were most active, from the 6th of June until 27th of July 2016. I chose calls from recordings with a high signal-to-noise ratio as stimulus calls. These calls were recorded on Amami-Oshima, Iheyajima, and Miyakojima in 2015. Call stimuli of Miyakojima were recorded at a location more than 6.5 km away from the experimental site, in order to use calls that were unfamiliar to the experimentally targeted individuals. This distance is considered to be sufficient to meet requirements because the Ruddy Kingfisher's home range is within an approximately 140 m radius (Hamachi and Uemura unpublished data). Each playback stimulus consisted of one-minute playback phases repeated three times with a two-minute period of silence between the phases. A three-minute silent phase was added at the beginning. A total playback file consisted of 12 minutes: during the first three minutes I began playback and left the site, the next nine minutes (playback phase) assess the response to the playback call. Eleven calls were repeated in the one-minute playback phase because Ruddy Kingfishers call 11 times per minute on average. Files were played from a TOSHIBA BK-701 speaker at the same 100dB volume used by Ruddy Kingfishers. Response calls made by birds nearby were recorded with an OLYMPUS LS-7 digital recorder placed within one meter of the loudspeaker. Experiments were made once a day in one location. The location I chose for the experiment was at least 300m from the previous day's location. In total, I conducted 74 playback trials, and used 66 trials in the analysis: Miyakojima's calls were used in 34 trials, Iheyajima's calls were used in 18 trials, and Amami-Oshima's calls were used in 14 trials. Eight trials failed due to incorrect recorder set up, battery failure, or speaker failure. The order of the three playback stimuli was randomized within each series.

I scored the number of calls during each playback phase by listening to the playback at a certain volume and counting all the calls. No other species on these islands give calls similar to those of the Ruddy Kingfisher, thus there is no danger of mistaken identity. Calls were not distinguished whether it was aggressive response or not and were not identified the individual.

### **Body size measurement**

I compared the morphometry of Ruddy Kingfisher populations using study skins stored at the Yamashina Institute for Ornithology and the National Museum of Nature and Science, and birds I captured on Miyakojima. I measured total head length and the length of the bill from the nostril to the tip. It was not possible to obtain samples from all of the islands on which I conducted vocalization research, so I used body size data from other islands, where I did not conduct research. It was also not possible to use body weight as a parameter of body size because I used mostly study skins, except for the birds captured on Miyakojima.



### ***Statistical analysis***

I compared the maximum call frequencies among populations using multiple comparisons following the Tukey-Kramer method after conducting Bartlett's test. I used environmental noise amplitude and sound attenuation rate as indicators of the acoustic environment. I compared the reaction intensities to playback stimuli using a multiple comparison test using Bonferroni's method. It was not possible to compare body size measurements statistically, because there was too great a difference in the number of birds measured among populations. All statistical analyses were conducted in R version 3.2.2 (R Core Team 2017).

### **Haplotype Network**

The cytochrome oxidase subunit 1 (COI) sequences of Ruddy Kingfishers were downloaded from GenBank and BOLD. I used the sequences obtained from adult birds that were caught on Amami and Okinawa and the Sakishima Islands during April to September. Amami-Oshima belongs to the Amami Islands; Iheyajima belongs to the Okinawa Islands; and Miyakojima belongs to the Sakishima Islands. I used seven individuals from the Amami Islands, seven from the Okinawa Islands, and three from the Sakishima Islands to construct the haplotype network. Sequence alignments were performed using MEGA version 7.0 (Kumar et al. 2016). I used the 544-bp region of the COI gene to construct the haplotype network. The haplotype network was constructed in PopART ver. 1.7 (Leigh and Bryant 2015) using the minimum-spanning algorithm to visualize the relationship between haplotypes and their distribution. Detailed information about the haplotypes is presented in Supplementary Data Table S1.

## **RESULTS**

### **Call**

I analyzed calls of 59 individual Ruddy Kingfishers (20 on Amami-Oshima; 16 on Iheyajima; 23 on Miyakojima). The maximum frequencies of calls differed among populations although there were large overlap. Kingfishers on Miyakojima produced calls with a lower maximum frequency than those on Amami-Oshima and Iheyajima (Tukey-Kramer's multiple comparison test: Miyakojima-Amami-Oshima,  $P=0.031$ ; Miyakojima-Iheyajima,  $P=0.020$ ) (Figure II-3).

### **Habitat-dependent environmental noise**

The environmental noise characteristics differed between islands (Figure II-4). In general, environment noise level was highest in the low frequency range resulting from rain, rustle of leaves, and anthropogenic noise such as cars and airplane. Recordings on Miyakojima were characterized by a distinctive band ranging from 5 to 8 kHz. Recordings on Amami-Oshima and Iheyajima were

characterized by relatively low amplitude levels in the range, and amplitude levels decreasing from low to high frequencies.

### **Transmission experiment**

I compared the amplitude of artificial tones, after they were transmitted through the vegetation on each island, at 16.0 m as a proportion of the amplitude at 4.0 m. There was no correlation between average yearly temperature and attenuation rate (1863Hz,  $P=0.43$ ; 2327Hz,  $P=0.78$ ; Figure II-5).

### **Playback experiment**

The playback experiment showed that the response intensity to the playback stimulus differed depending on playback call type. Playback calls recorded on Miyakojima elicited stronger responses than other calls (Bonferroni's multiple comparison test: Miyakojima-Okinawajima,  $P=0.028$ ; Miyakojima-Amami-Oshima,  $P=0.017$ ) (Figure II-6).

### **Body size measurement**

I measured the total head length from individuals obtained from each island, but did not conduct any statistical analysis due to the large difference in bird numbers measured among islands. When the difference of the ratio between groups is too large, there is no proper method for multiple comparisons. However, there was no apparent tendency in the measurements among populations (Figure II-7).

### **Haplotype Network**

I found six haplotypes based on 554 bp of COI gene sequences among 17 birds, which clustered into two main groups in the haplotype network (Figure II-8). Haplotype 1 was the most common haplotype. All 11 birds in haplotype 1 were from Okinawa and Amami Islands, except one from Sakishima Islands. The second major haplotype was haplotype 2, which comprised all samples from the Sakishima Islands. These two haplotypes were separated by one base pair, with a p-distance of 0.002. There were three additional haplotypes from the Amami Islands that was separated by two to five base pairs from haplotype 1 and one additional haplotype from the Sakishima Islands that was separated by one base pair from haplotype 2.

## **DISCUSSION**

Geographical variation in the maximum frequency of calls among populations was confirmed in the Ruddy Kingfisher. Although such geographical variation in acoustic signal has been shown in numerous studies on songbirds, the impact of the acoustic environment on their genetic structure was rather low (Slabbekoorn and Smith 2002; Slabbekoorn and Peet 2003). Since Ruddy Kingfishers

have innate call, the differences between calls may reflect intraspecific genetic differences. Several migratory bird species occur widely among the Ryukyu Archipelago and in each case they are classified as a single subspecies. In contrast, many resident species with limited flight capabilities are classified into multiple subspecies (Ornithological Society of Japan 2010). Despite the Ruddy Kingfisher being a migratory species breeding on many islands in the Ryukyu Archipelago, I suggest that differences in genetic structures exist. If the Ruddy Kingfisher's call characteristics are the product of acoustic adaptation to their habitat, their dispersal to acoustically different habitats must be restricted, unlike birds that are capable of call learning. Therefore, differences in calls among populations may act as a behavioral barrier to gene flow.

The results of our playback experiments indicate that Ruddy Kingfishers distinguish the calls of their own population from calls of the other populations. Individual kingfishers on Miyakojima responded more strongly to calls from their own population than to calls recorded on other islands. Several other studies have also given examples of the impact on response for ecologically divergent calls (Irwin 2000; Patten et al. 2004) or calls from different populations (McGregor et al. 1983; Tomback et al. 1983; Searcy et al. 2002). These results, showing that individuals discriminate between divergent calls, were gained from many songbirds that learn their calls. However, such discrimination has rarely been confirmed except among songbirds. Some dove species, despite the fact that they have innate *coo* calls, respond differently to the coos of their own species and other species (de Kort and ten Cate 2001; Secondi et al. 2003; Den Hartog et al. 2007). Indeed, the dove's interspecific *coo* discrimination may promote assortative mating, but it is unclear whether or not the coos have diverged in relation to the acoustic environment. Furthermore, there is no previous information concerning bird species with innate call characteristics discriminating intraspecific call variations. A study on quails showed that they do not discriminate their own species based on calls (Gee 2005). To reveal the impact of local acoustic adaptation on ecological speciation, assortative responses to calls from their own population should be tested on a bird species with an innate call. Our results showed that the Ruddy Kingfisher responded assortatively in relation to intraspecific call characteristic differences. This behavior may promote assortative mating because male response is believed to reflect female preference (Brambilla et al. 2008). Female response to advertisement calls given by unsuitable mates is maladaptive for males. Thus, the kingfisher's assortative response to calls may be the first stage of ecological speciation.

Two possible factors may cause diversification of call features; local acoustic adaptation, and extracted alteration from their body size. Measurements of total head length, and bill length were similar among populations despite the call maximum frequency differing between Miyakojima and other islands. Therefore, it is considered that the difference in call frequency is not caused by a difference in body size. In contrast, environmental noise seems to explain the call maximum frequency. Shifts in call frequency, in relation to noise loudness, are well known among the

Passeriformes (reviewed in Ortega 2012), and our results show that this trend even exists in the Ruddy Kingfisher. However, the relationship between call frequency and sound attenuation rate was unclear.

Environmental noise at 5 kHz to 8 kHz was relatively loud on Miyakojima and was mainly caused by the buzzing of cicadas. Although kingfishers call most actively early in the morning, they also call after cicada's begin buzzing. The Blackish Cicada *Cryptotympana facialis* on Miyakojima appears in early June and makes a loud noise, whereas on Iheyajima they appear in July; they do not occur at all on Amami-Oshima. Although other cicadas also occur on each island, the Blackish Cicada on Miyakojima and Iheyajima is outstandingly noisy. Since Blackish Cicada's buzz at a frequency of 2–10kHz, the presence of this cicada in the kingfisher's breeding season should greatly affect the kingfisher's call frequency.

The haplotype network supported the existence of premating isolation formed by geographical song divergence. The major haplotype on the Sakishima Islands was different from the one on the Amami and Okinawa islands. The difference in haplotypes and call maximum frequency corresponded well. If call differences do not restrict gene flow among populations, then haplotypes will be shared. Acoustic adaptation by sensory drive may actually prevent gene flow among populations of the Ruddy Kingfisher. Unlike many previous studies in which high levels of gene flow have been found even among populations with distinctive song dialects (c.f. Ortiz-Ramirez et al. 2016), our study showed the correspondence of call difference and genetic difference. I sometimes observed *Halcyon coromanda major*, the subspecies of Ruddy Kingfisher breeding in mainland Japan, as a spring migrant in early April on Miyakojima (Uemura unpublished data). Kingfishers that breed on the Okinawa and Amami islands should also pass through and rest on Miyakojima during migration; however, they do not settle on Miyakojima. We should note that there is an alternative hypothesis that the haplotype network suggests the gradual genetic drift across populations (isolation by distance). When migration between populations is difficult because of the large distances between habitats, genetic drift can lead to genetic differentiation. In our study, there were two large haplotype groups from three island populations. Miyakojima and Iheyajima are about 350km apart, and Iheyajima and Amami-Oshima are about 200 km apart. The structure of the haplotype network may not be explained by isolation by distance alone. Although I attempted to analyze the genetic difference on a finer scale, by using microsatellite markers, the analysis has not been successful yet. I aim to investigate the extent of gene flow among populations in a future study. In conclusion, this study has shown that innate call variations among adjacent populations were an assortative response, and contribute to premating isolation. The differences in their calls seem not to be related to differences in body size. This indicates that call divergence, as an adaptation to habitat-related acoustic structures, promotes ecological speciation in the Ruddy Kingfisher. I used male response intensity to the playback experiment as an indicator of premating isolation. Several studies

have shown that song characteristics used by males, to regard focal individuals as potential competitors, are also used by females when choosing potential mates (Patten et al. 2004). Furthermore, females are capable of superior discrimination to conspecific song than are males (Searcy and Brenowitz 1988; Searcy et al. 2002; Danner et al. 2011). However, more empirical data about female response to playback stimuli are needed to verify the isolation effect more directly. I revealed genetic differences among populations that have different call frequencies. I also require more detailed genetic data from the Ruddy Kingfisher to assess the extent of gene flow.

# General Discussion

This dissertation revealed the population divergence could occur in bird species that are capable of flying long distances. Population divergence can be caused due to local adaptation to the habitat resources that cause pre-mating isolation.

At the beginning of this study, I described the migration behavior and site fidelity of the Ruddy Kingfisher in Chapter I. The return rate of the kingfisher was high, and males in particular returned near the territories that he occupied in the previous year. The high return rate suggests that it is important for kingfishers to choose the breeding site according to their breeding traits that are suitable to the habitat. Furthermore, I confirmed that some birds that born in the Miyakojima Island returned to the island as breeders.

Data obtained by GPS data loggers revealed the migration of the kingfishers. Ruddy Kingfishers leave Miyakojima Island and migrate to Philippines to winter there. As previously thought, kingfisher fly long distance to migrate between breeding site and wintering site (Woodall et al. 2017).

In general, birds are less restricted gene flow among populations over geographical barriers than other vertebrate such as mammals and amphibians. As I revealed in the study in Chapter I, Ruddy Kingfisher are able to fly long distance. Species that are capable of flying long distances may be less susceptible to population isolation due to geographic barriers. However, I found geographical difference in song frequency among populations in the Ryukyu Archipelago. Kingfishers in Miyakojima Islands sing at lower frequency range than the other two Islands. On the other hand, there were no difference in their body size among populations. This suggests the difference in song frequency is not the byproduct of the difference of body size. The song frequency was thought to have changed according to the acoustic environments in each habitat.

Acoustic environment is described by the frequency distribution of environmental noise and the transmission characteristics of sound. The environmental noise at high frequency range was larger on Miyakojima Island than the other two islands. The large noise in this range was attribute to cicadas. Environmental noise at this range was not so loud in Iheyajima Island and Amami-oshima Island. Kingfishers may have shifted their song frequency to lower ward to avoid the loud environmental noise at high frequency.

The difference in song characteristics caused by the local adaptation to acoustic environment may lead population divergence. In Chapter II, I conducted the playback experiment to evaluate the formation of pre-mating isolation by difference in song characteristics. The playback calls recorded in Miyakojima elicited stronger responses than other calls. The different reaction intensity to play back song indicated the difficulty in establishing breeding territories on islands where the characteristics of acoustic environment are different. The genetic analysis of COI gene from database supported the existence of isolation among populations. Their identifiably different calls among populations parallel genetic differences. In this study, I examined the mechanism of

parapatric ecological speciation, which is difficult to detect, in terms of local adaptation to the acoustic environment.



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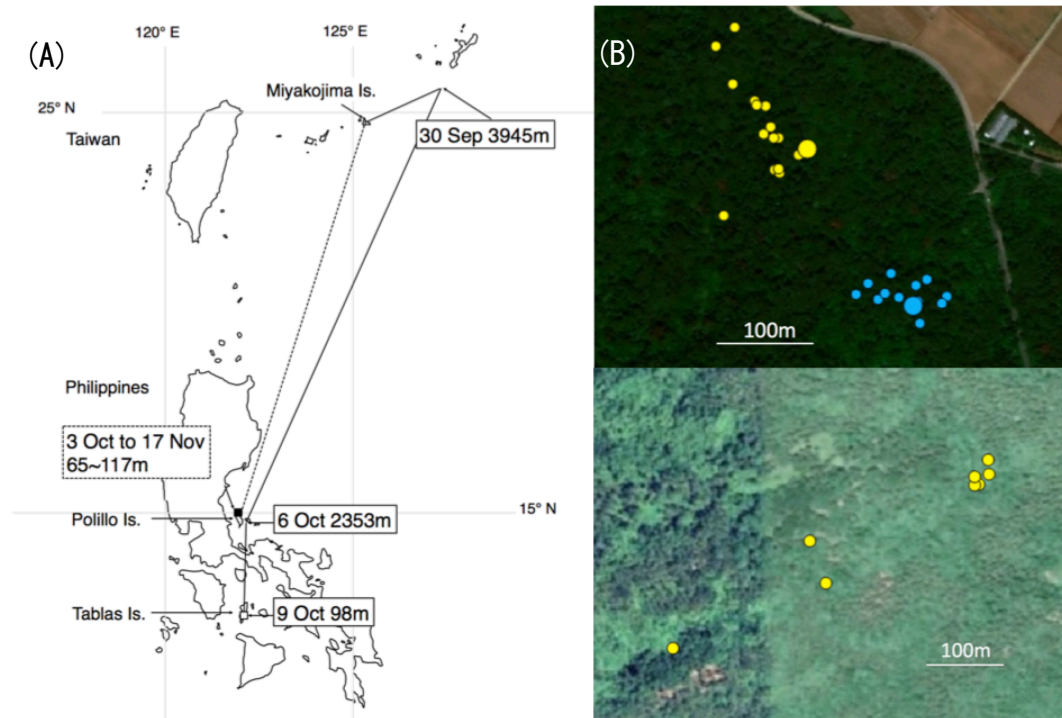
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anniversary issue of the wild bird society of Miyako) Shimoji print, Okinawa (in Japanese)



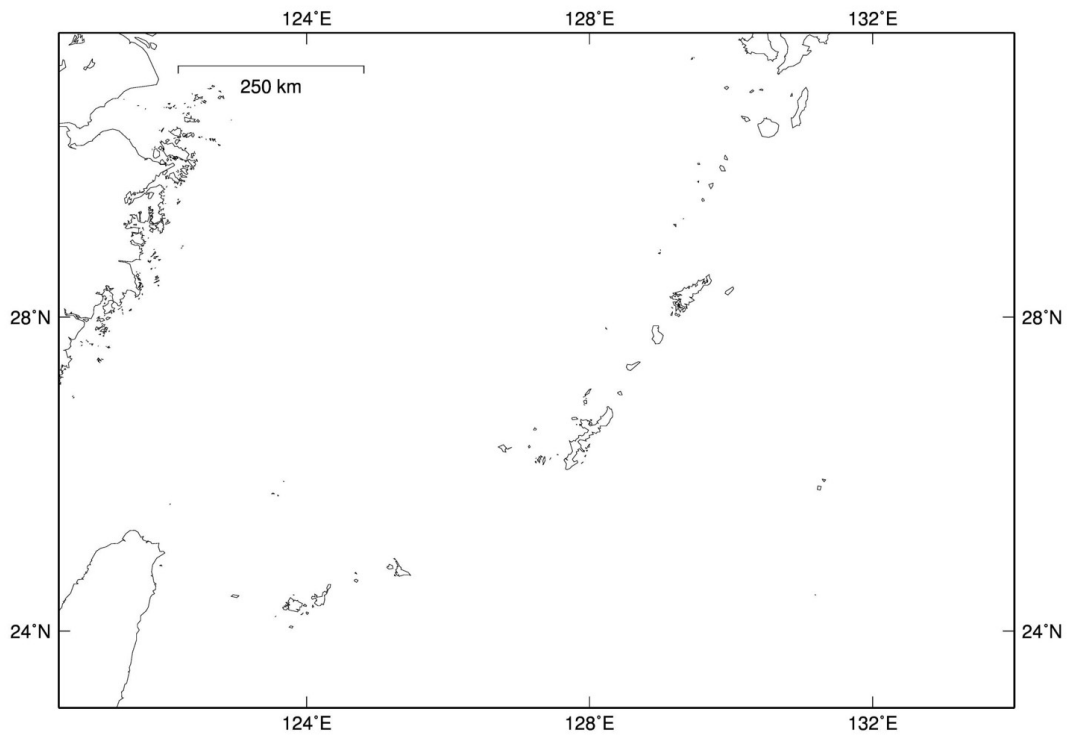
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**Fig. I-1.** Rappole-Tipton style harness attached to a Ruddy Kingfisher.



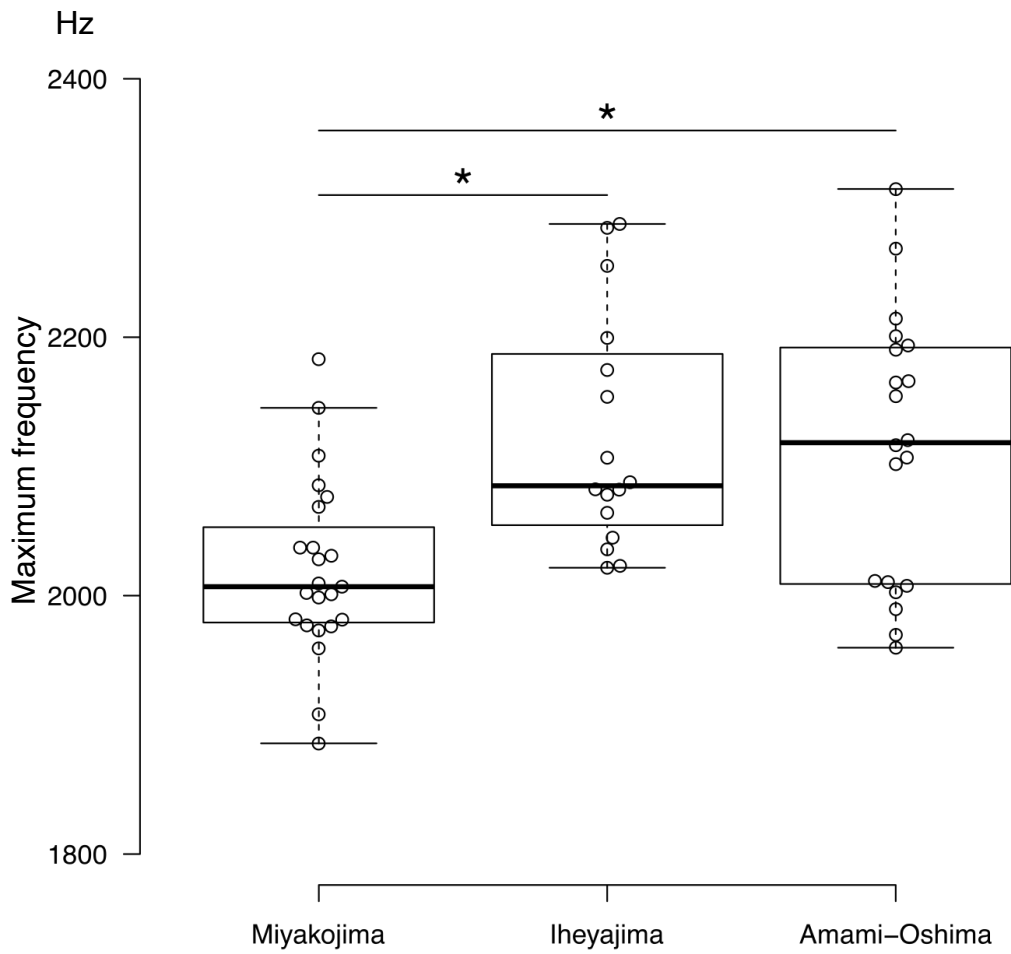
**Fig. I-2.** (A) Autumn migration routes, wintering areas and altitudes of two Ruddy Kingfishers. The solid line represents male A and the dashed line male B. White squares ( $\square$ ) indicate wintering site or stop over site of male A, black squares ( $\blacksquare$ ) indicate wintering area of male B. (B) Location points in Ohno Sanrin Forest, Miyakojima, Japan, between 1 August and 24 September 2016. Blue points represent male A and yellow points represent male B. The large circles represent their nests. (C) Locations of male B on Polillo Island, The Philippines, between 3 October and 17 November 2016.



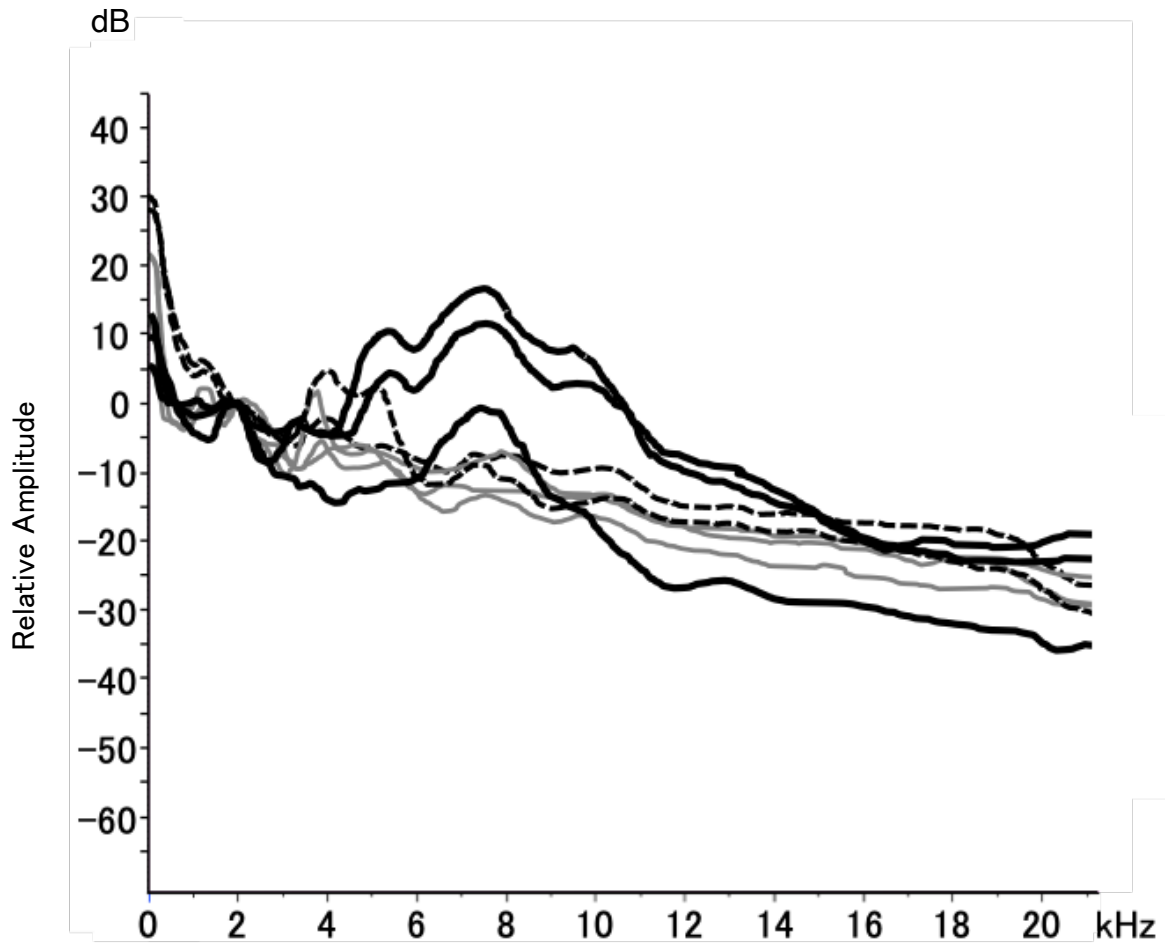
**Fig. II-1.** The three study islands in the Ryukyu Archipelago.



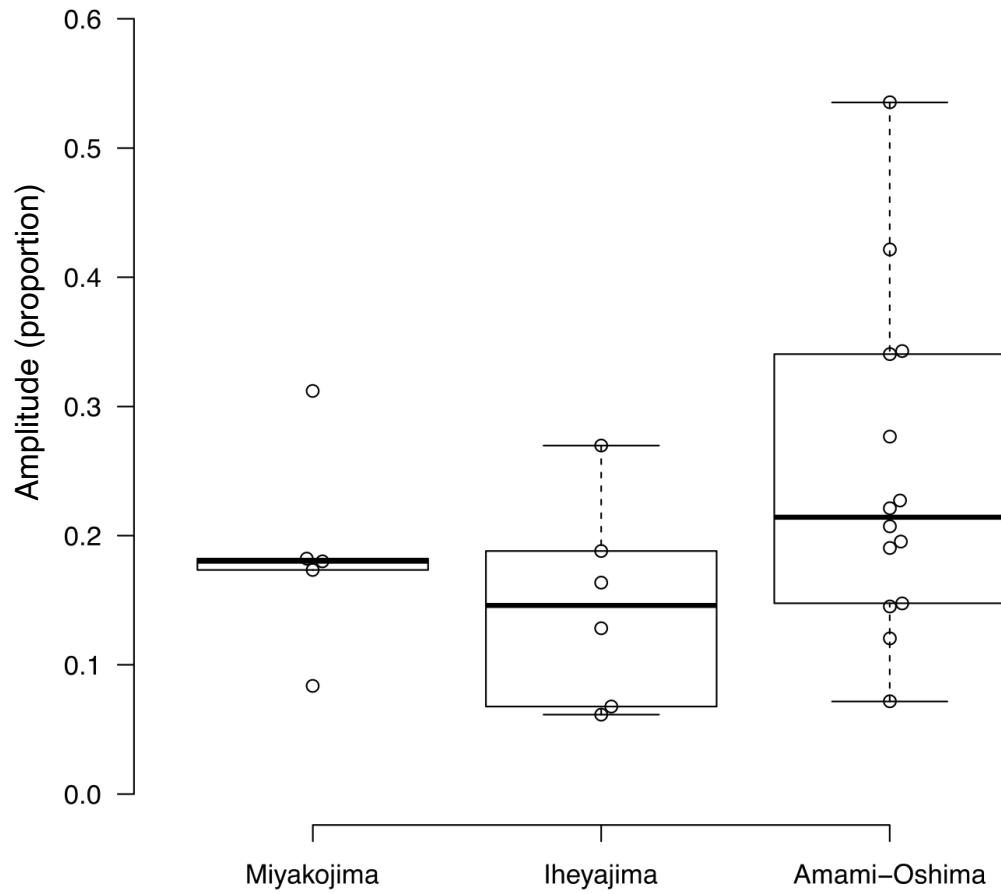
**Fig. II-2.** The IC recorder (OLYMPUS LS-7) *in situ* (Photographs by the author).



**Fig. II-3.** Maximum frequency of Ruddy Kingfisher songs. The boxplots show the median (black line), the interquartile range (box), and full range (whiskers) for the maximum frequency of the call. Each circle represents one individual. Significance levels indicated (\*) refer to  $P < 0.05$  for population comparisons.

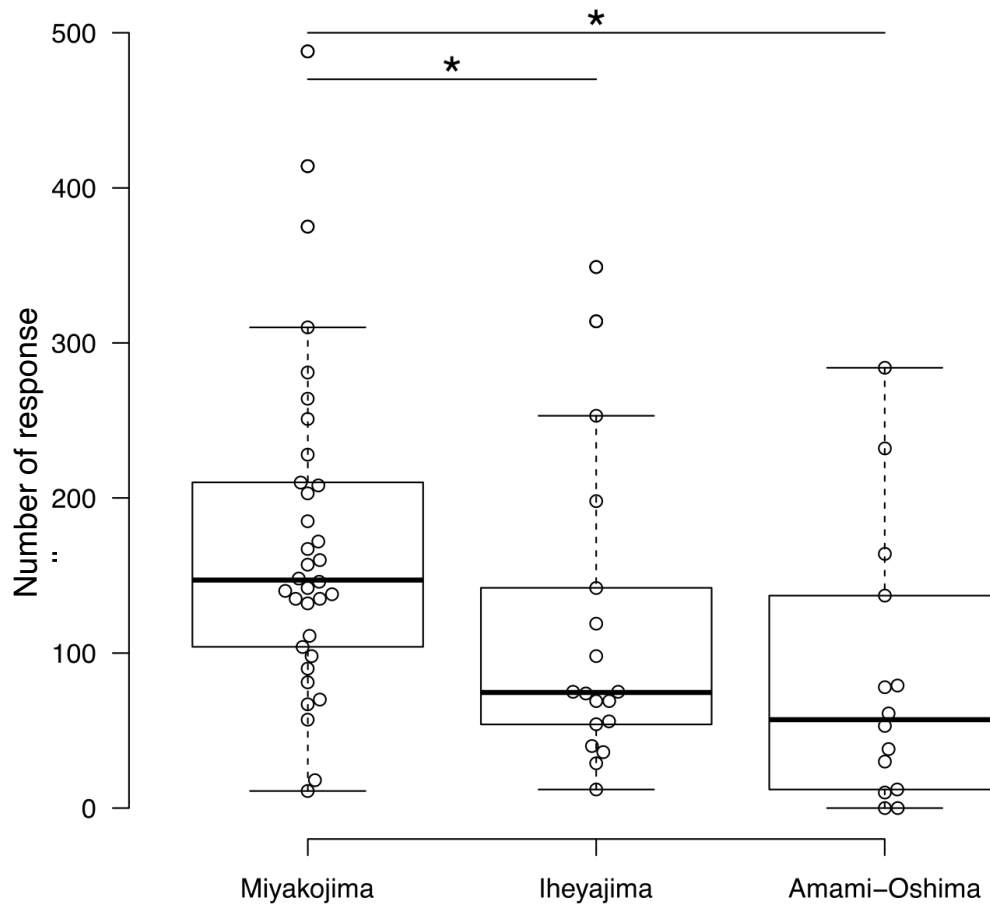


**Fig. II-4.** Spectrum of environmental noise from Miyakojima (black lines), Iheyajima (gray lines), and Amami-Oshima (dashed lines). The lines are drawn relative to the volume at 2kHz.

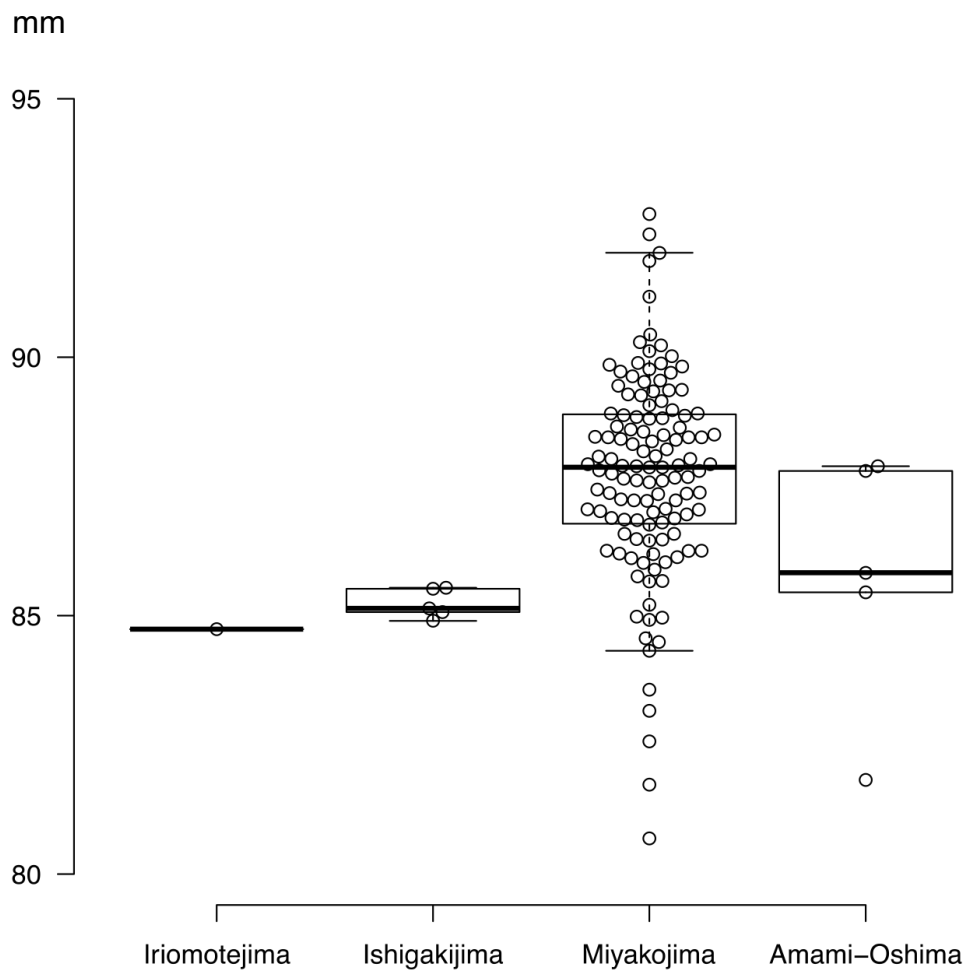


**Fig. II-5.** The sound attenuation ratio on each island. White points show the noise level at 2327 Hz. The number of the experiments was: 5 on Miyakojima, 6 on Iheyajima, and 14 on Amami-Oshima. The boxplots show the median (black line), the interquartile range (box), and full range (whiskers) for the number of calls. Each circle represents one individual.

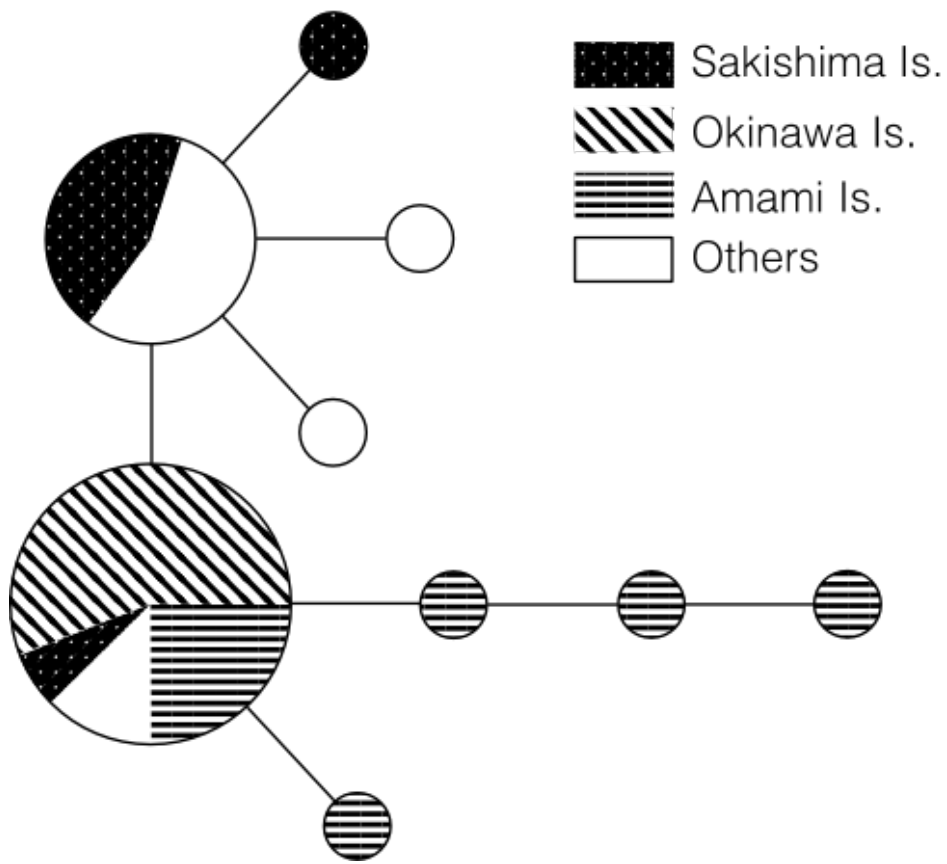




**Fig. II-6.** Number of response calls to playback stimuli. Birds on Miyakojima responded most strongly to playback calls recorded on Miyakojima and less strongly to the calls recorded on other islands. The number of the experiments was: 19 on Miyako, 12 on Okinawa, and 14 on Amami. The boxplots show the median (black line), the interquartile range (box), and full range.



**Fig. II-7.** Total head length of individuals from various islands. The number of individuals was Iriomotejima 1; Ishigakijima 5; Miyakojima 123, and Amami-Oshima 5. The boxplots show the median (black line), the interquartile range (box), and full range (whiskers) for the number of calls. Each circle represents one individual.



**Fig. II-8.** Median-joining haplotype networks of mitochondrial COI sequences. Circle size is proportional to the number of individuals found for each haplotype.

# List of Tables

**Table I-1.** NestID, capture date, recapture date, body weight at capture, body weight at recapture, number of eggs and nestlings at capture, number of fledglings of tagged individuals.

ID	NestID 2016	Capture	Recapture	Weight (g) /Capture	Weight (g) /Recapture	Eggs and nestlings /Capture	Fledglings 2016
male A	A	2016/7/19	2017/4/20	100	96	3 nestlings	3
female A	A	2016/7/19		103		3 nestlings	3
male B	B	2016/7/17	2017/6/10	106	104	2 eggs	1
female B	B	2016/6/15		119		2 eggs	1
male C	C	2016/7/21		100		3 nestlings	3
female C	C	2016/7/26	2017/6/11	94	112	3 nestlings	3