

國立臺灣大學生命科學院生態學暨演化生物學研究所

碩士論文

Institute of Ecology and Evolutionary Biology

College of Life Science

National Taiwan University

Master Thesis



日本樹蛙鳴叫特徵和外部形態特徵的地理變異
Geographic Variation of Acoustic and Morphometric
Traits in Ryukyu Kajika frog (*Buergeria japonica*)

王盈涵

Ying-Han Wang

指導教授：林雨德 博士

指導教授：林思民 博士

Advisor: Yu-Teh Kirk Lin, Ph.D.

Advisor: Si-Min Lin, Ph.D.

中華民國 105 年 6 月

June 2016



致謝

歷經三年，嘗試過許多不同方向的題目，最終還是以蛙鳴當作我的碩士論文主題。這個題目動員了許多各地的親朋好友們一同幫忙，不論是在心情上，還是實驗操作上，如果沒有你們，我也無法順利的完成我的研究。今天我想在這張僅有的頁面寫下我滿滿的感謝。

感謝林雨德老師，總是不斷的督促我，並且讓我有很大空間去嘗試不同的問題和解決的方法。一開始和您討論實驗時，常因為問題達不出來，而覺得很氣餒甚至有點自暴自棄。但後來開始做實驗時，我漸漸的開始會不由自主的模擬，如果是您，您會問甚麼問題，我該怎麼解決？在幫助我釐清事情的思考過程中幫助頗大。也感謝您，明知道我的英文很爛，還是不厭其煩，仔細的幫我修改研討會海報、講稿和論文，每回收到您改回來的 word 檔，我似乎都看到您扶著額頭嘆氣，祝福老師在美國一切順心，有一天我們會組團去你們家吵鬧的。

感謝林思民老師，從大學時期就一直忍受我的吵鬧和幼稚到現在，那隻被我帶去營隊玩耍的小鵬都已經國小畢業了，時間的流逝想想都覺得很可怕。謝謝您在我無腦的時候點醒我；在我心情低落的時候，陪我玩拋接食物的遊戲；在我火燒屁股的時候，提水滅火；在我不想做事的時候，讓我可以去魚缸餵魚發呆…。草魚家有太多的回憶，就像我第二個家！謝謝您這幾年的照顧！

感謝田鼠家的大家！全能辣媽淑蕙學姐不論是在生活上還是在我去宜蘭出差時，都給予我很大的幫助。燒燙燙的新科區長柏翰帥哥，陪我討論和說垃圾話。永遠的女神貝珊，感謝細心的妳畢業了都還幫我處理報帳的事。恥力無上限的哲豪，和你一起百無禁忌的聊天頗紓壓。不說話的阿猴，感謝你總是聽我碎念。感謝威森在忙碌作畫之餘，還要接我動物房的求救電話。感謝林杰和懿尹幫我一起處理實驗室的雜物。感謝遠在美國的譚博，幫我修改論文、聊天打屁，以及讓我不在對出國念書有期望，哈。還有雨珊，和我分享你的野外照片和聊天，讓我繼續有動力。還有凌軒，不但幫我完成宜蘭的採樣，還幫我畫了一張好上相的日本樹蛙圖，讓我任何公開發表都可以拿出來炫耀一番。你們雖然不在我身邊，但實際上卻一直都在！

感謝草魚家的各位！無聊大師展博士，你根本是我的救援投手，大事小事找你就對。謝謝老闆娘，每次跟你聊天都很有收穫，而且有種被療癒的感覺，覺得可以一直一直講下去。做人處事第一名的俊文大叔，不論是在處事還是實驗設計上，都給很多幫助。感謝芳神的後備支援，讓我能順利出差。奮青李昱冒著大雨出去幫我錄音，幫我帶回珍貴的資料。小雨蛙大師李閣桓的火線支援，有你幫忙實驗錄音總是相當順暢。感謝 A4，在我出差時幫我照顧我的小寵物們。金黃水果甩呀，雖然平常是塊肉，但其實你是人形英文翻譯機吧！謝謝雨神嘉偉，日本樹蛙錄音最速傳說非你莫屬，祝你趕快學會游泳！接棒的阿薇，雖然很常欺負你，但我還是要說，實驗室有個女孩真好！

感謝蝴蝶家的捧油們！大仔、師兄、阿賢、玉米、黃黎、Bibo、睿睿、中庸、大大、宣安、國偉…感謝你們都會拍打餵食我，讓我免於飢餓。龍博小油龍總會與我分享他在國外或離島的各種經驗，也會帶著我認識許多不同領域的朋友，拓展我的視野。育琦和小灰在我遇到困難時，就算再忙碌也會放下手邊的工作幫助我解決問題。謝謝你們，我會努力變成一隻美麗的蝴蝶！

感謝我的大小天使們，莊銘豐學長總是提醒著我聲學研究該注意的事項。台中場的 823，你的躺躺式錄音法堪稱經典。南投場彥博大鞋長的收留，讓我有冷氣可以吹到爽，一路睡到中午。嘉義場的張嘉豪、李昱緯、何勇霖和 Lara 學姐陪我一起驅逐狗群，尋找錄音點。台南場的惠瓊玲、劉子毓，收留我還帶我去吃好吃的刈包。高屏場的陳惇聿學長和光頭提供我需許多日本樹蛙的樣區。台東的山豬大哥提供我許多資訊，給我東部地區日本樹蛙活動的第一手消息。花東場的神奇保羅，睡覺之餘還很會記路找點！台北場的宇德和 Fish，陪我涉水走到攤地等了整晚都不叫的小日本。琉球群島場的彥博、芳神、貝珊、毅倫、阿平、柔潔、苑佐，去渡假還要分心支援沒加油的兩個笨蛋。這個實驗因為有你們而變得完整！

最後，感謝漢漢和阿鄧，兩位親愛的鄰居是我在台北的家人，不只要幫助賴床阿蟲不要上課遲到，還要當我的小秘書，讓我詢問許多學校相關規定和事宜，如果沒有你們，我應該真的畢不了業吧！還有我親愛的老爸老媽和老姐，你們的支持與關愛是我繼續努力下去的強大動力。

摘要



日本樹蛙 (*Buergeria japonica*) 廣泛分布在琉球群島和台灣，為橫跨不同島系之間的兩棲動物，模式標本產於琉球群島北部的奄美大島。近年研究指出，日本樹蛙族群在島間具有高度的分化，且台灣島內也並非單系群，顯示日本樹蛙族群的分類地位有重新檢視的必要。聲音及外部形態是蛙類繁殖與物種辨識重要的依據，因此本研究利用這兩組特徵進行日本樹蛙族群間地理變異的檢測。總共分析來自琉球群島和台灣，共 17 個族群的宣示叫聲以及 13 個族群的外部形態特徵。使用卡方檢測和廣義線性混合模型 (GLMM) 檢測鳴叫聲在地區間的差異，並使用主成份分析濃縮 19 個外部形態特徵，接著根據過去利用遺傳訊息建立的親緣關係樹進行判別分析，尋找最佳的分群方式，並根據分群結果利用 GLMM 進行不同單系群間的差異性檢測。結果顯示，日本樹蛙的叫聲由短鳴叫和長鳴叫所組成。台灣東部和西南地區具有一種其他地區未曾記錄到的長鳴叫類型；而這些族群短鳴叫的鳴叫時間和鳴叫分貝上升時間也比其他地區短促。另外，台灣西北地區的主頻率則比其他地區來的高。利用形態特徵進行判別分析的結果也顯示，分群為台灣東部和西南地區 (台灣群)，和琉球群島及台灣西北地區 (日本群) 兩個單系群時具有最佳的判別正確率，且台灣群在頭部特徵上顯著的大於日本群。綜合以上結果和前人的遺傳訊息，皆顯示日本樹蛙有一個形態不易辨識，但叫聲明顯分化的隱蔽種存在。

關鍵字：日本樹蛙、外部形態、宣示叫聲、族群分化、隱蔽種

Abstract



Delimitating species is a central concern in evolutionary biology, taxonomy, and biological conservation. Acoustic and morphological traits play key roles in frogs, especially in species recognition and reproduction. *Buergeria japonica* is widely distributed from Ryukyu Archipelago to Taiwan. Recent studies indicated the extremely high genetic differentiation with non-monophyletic relationship among populations in Taiwan, which revealed the necessity to reevaluate the taxonomic status of this species across their distributional range. In this study, we sampled advertisement calls from 17 localities and morphology from 13 localities across Ryukyu and Taiwan to examine the variation among populations. By analyzing their advertisement calls, we found an exclusively unique type of long call occurring in the southwestern and eastern populations in Taiwan. Moreover, these populations also represent faster call duration and shorter call rise time compared to those from the other regions. On the other hand, the dominant frequency in the northwestern populations in Taiwan was higher than the others. Morphological measurements with discriminant analysis indicated that the species should be separated into two forms, which correspond to Taiwanese clade (the southwestern and eastern populations in Taiwan) and Japanese clade (Ryukyu and the northwestern populations in Taiwan) defined by mitochondrial sequences. Principal

component analysis showed that traits on the head from Taiwanese clade are significantly higher than the other. These results indicated that there is a cryptic species in Taiwan, which represents different acoustic signal but with similar morphology from *B. japonica sensu stricto*.

Keywords: advertisement call, *Buergeria japonica*, cryptic species, morphology, population differentiation



Content

致謝	i
摘要	ii
Abstract.....	iii
Introduction	1
Materials and methods.....	6
Acoustic signal data.....	6
Acoustic signal data collection	6
Advertisement call analyses	6
Morphological data.....	8
Morphological data collection	8
Morphological analyses.....	9
Results	10
Acoustic data	10
Morphological data.....	10
Discussion.....	12
Reference	17
Content of Tables & Figures.....	26
Appendix	44

Introduction



Establishing species status is one of the major tasks in evolutionary biology. It is not only essential to taxonomy, but also crucial for biodiversity conservation (Bickford *et al.*, 2007; Warwick *et al.*, 2015). Cryptic species are often misclassified or unidentified in the past (Dobzhansky, 1940). The rapid development of molecular techniques has improved our ability to delimitate cryptic species. For example, molecular evidence has revealed the forest cicada, *Cicadetta Montana*, to be a complex of several closely related species distinguishable by calls (Wade *et al.*, 2015). However, delimitating species limits based solely on molecular evidence may lead to other problems in taxonomy. For instance, only using DNA identification in Diptera would lead to misidentifications because of wide overlapping of intra- and interspecific genetic variability (Meier *et al.*, 2006). Furthermore, phylogenetic tree was reconstructed by using unsuitable molecular marker which may not provide sufficient information. The insufficient diagnosis of divergence sometimes presented inconsistent results with actual species tree due to incomplete lineage sorting, gene flow, recombination, stochastic coalescence events and sex-biased dispersal rates (Carstens and Knowles, 2007; Irwin, 2002; Knowles and Carstens, 2007; Maddison and Knowles, 2006; Petit and Excoffier, 2009).

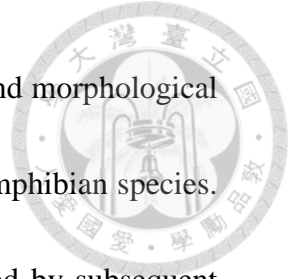
Several criteria should be employed to provide consistent and coherent set of

evidence (Helbig *et al.*, 2002). For example, the Bahama Woodstar, *Calliphlox lryra*, was described as a distinct species based on morphology until Peter (1931) considered it as a geographic variation and revised its status as a subspecies, *C. evelynae lryra*.

Recently, additional supports from morphology, call, song, mechanical sounds, and genetics re-elevated the Bahama Woodstar back to the species level (Feo *et al.*, 2014).

Similarly, Alström (2008) used six lines of evidence to clarify the species status of the spotted bush warbler (*Bradypterus thoracicus*) complex. These studies showed the importance of using multiple criteria while examining species status.

Acoustic signals and body morphometric features are factors that should be included as diagnostic criteria for amphibians. Acoustic signals have been shown to be vital to mate choice and resource defense (Ritchie, 2007) in anurans (Gerhardt and Huber, 2002), as in many other taxa such as insects (Cocroft and Rodríguez, 2005; Gray and Cade, 2000), fishes (Maruska *et al.*, 2012), birds (Price, 1998; Thorpe, 1961), and mammals (McComb, 1987; Voigt *et al.*, 2008). Frog calls have the potential of acting as a diagnostic signal that supports intraspecific recognition or reproductive isolation (Panhuis *et al.*, 2001; Ritchie, 2007; West-Eberhard, 1983). On the other hand, body size and shape are complex traits resulted from many selective pressures in nature (McKinney, 1990; Perrin, 1998; Peters, 1986; Schmidt-Nielsen, 1984). They reflect the multifaceted ecological context where an amphibian species evolves (Barbault, 1988;



Miller *et al.*, 1993; Nauwelaerts *et al.*, 2007). Both acoustic signal and morphological traits have been used to complement genetic data for delimitating amphibian species.

For example, the application of genetic data as a first step followed by subsequent confirmation from acoustic signal or morphology has contributed to a steep increase in amphibian species diversity in Madagascar (Köhler *et al.*, 2005; Vieites *et al.*, 2009).

In Taiwan, two newly described frog species, *Kurixalus berylliniris* and *K. wangi* were established by combining molecular phylogenetic, morphological, and acoustic evidences (Wu *et al.*, 2016).


The old world tree frog family Rhacophoridae has more than 380 species from 18 genera (Delorme *et al.*, 2005; Frost, 2014; Frost *et al.*, 2006). The most basal group of Rhacophoridae, genus *Buergeria*, consists of four species (Biju *et al.*, 2016; Wilkinson and Drewes, 2000). One of the species, the Ryukyu Kajika frog, *Buergeria japonica* (Hallowell, 1861), distributes widely on the East Asian Arc from Ryukyu Archipelago to Taiwan. Studies of several terrestrial species found restricted distribution and high endemism on the East Asian Arc (Ota, 1998), such as grass lizards (Lin *et al.*, 2002), newt (Tominaga *et al.*, 2010), geckos (Honda *et al.*, 2014), and fireflies (Osozawa *et al.*, 2015). Multiple historical events of land connections and fragmentations due to repetitive glacial cycles may have contributed to the diversification and distribution of many species that are isolated by oceanic barrier (Ho, 1986; Kimura, 2000; Lin *et al.*,

2002).



Mountain ranges and river systems are species barriers as well. The island of Taiwan is formed through the collision of the Philippine Sea Plate and the Eurasian Plate, which results in several mountain ranges and the development of numerous river systems (Teng, 1990). The intricate network of mountain ranges and river systems creates the opportunity for species differentiation within small geographic scale. For example, the Pallas's squirrel, *Callosciurus erythraeus*, has very high genetic differentiation among populations on different mountain tops (Oshida *et al.*, 2006). The geographic boundary of two sister grass lizards, *Takydromus viridipunctatus* and *T. luyeanus*, is a narrow stream (Tseng *et al.*, 2015). These topographic features have similar effects on amphibians in Taiwan. Without an effective osmoregulatory mechanism, phylogeographic patterns of amphibians may fit best to the topography. A genetic landscape model of the Brown tree frog (*Buergeria robusta*) showed that its phylogeographic pattern matched the topography of Taiwan splendidly (Lin *et al.*, 2012). Similar findings have been reported for several other amphibian species, such as *Sylvirana latouchii* (Jang-Liaw *et al.*, 2008) and *Rana sauteri* (Jang-Liaw and Lee, 2009). These topographic features may have had similar effects on *Buergeria japonica*.

A recent study (Tominaga *et al.*, 2015) revealed that *B. japonica* has extremely high level of genetic differentiation among the East Asian Arc islands groups. Another



study (Lin *et al.* 2013) investigated the genetic differentiation of *B. japonica* among populations from different river systems in Taiwan. The results placed the populations in northwestern Taiwan and those in southern Ryukyu in the same clade, while assigned populations from the eastern and southwestern Taiwan to their own clade. The maximum genetic distance among populations exceeded 17%. Both studies indicated the necessity to re-evaluate the taxonomic status of *B. japonica* across different regions. It is highly plausible that cryptic species exist in Taiwan and should be separated from *B. japonica* whose type locality came from Amami Oshima (Hallowell, 1861). In this thesis, I evaluated the taxonomic status of the Ryukyu Kajika frog, *B. japonica*, with two additional criteria: calls and morphology. I examined: (1) the acoustic differentiation among regions; and (2) the morphometric differentiation among regions across the geographic distribution range of the species along the East Asian Arc, including Taiwan.



Materials and methods

Acoustic signal data

Acoustic signal data collection

During the breeding season of *Buergeria japonica* from April to September 2015, I recorded the calls of 281 individuals sampled from 17 localities in Taiwan and Ryukyu archipelago (Table 1, Fig. 1 and Fig. 2). We recorded the advertisement calls using a Sony ECM-CG50 directional microphone attached to a digital recorder (Sony PCM-M10) at a 44.1 kHz, 16 bit resolution. The microphone was held toward the calling male as close as possible during recording. At least one minute and 10 calls were recorded for each male. The callers were subsequently captured to prevent from repeat sampling. Frogs were released at their original localities immediately after all recording works at the same day. Since ambient temperature and moisture may influence call characteristics (Oseen and Wassersug, 2002), we also recorded air temperature and humidity using a thermohygrometer (Lutron, Taipei, Taiwan).

Advertisement call analyses

Records were digitized and characterized as acoustic traits by Raven Pro v1.4 (Cornell Lab of Ornithology, Ithaca, NY, USA). The call types of *B. japonica* could be classified into short calls and long calls by the number of pluses after the first rising peak

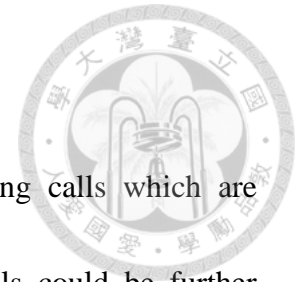


on the waveform. Calls with less than three pluses after the first rising peak on the waveform were defined as short calls. In contrast, calls with more than three pluses after the first peak were identified as long calls (Fig. 3).

Five spectral and temporal properties of each short call were extracted, which have been proposed to associate with female choices (Bee *et al.*, 2010; Gerhardt, 1991; Gerhardt and Huber, 2002). They are (1) dominant frequency (DF, in kHz), which was the highest peak reached by a call on the power spectrum (FFT = 1024 points, Hanning window); (2) the interquartile range (IQR), which was calculated by subtracting the 3rd quartile value from the 1st quartile value of frequency bandwidth of a call; (3) call duration (DT), which was the time from the beginning to the end of a call; (4) call rise time (RT), which was the time from the beginning to the highest peak on the waveform of a call; and (5) call fall time (FT), which was the time from the highest peak on the waveform to the end of call (Fig. 4). Call parameters of an individual were obtained by averaging the values of 10 calls from the same caller.

I summarized temperature and humidity by principal component analysis (PCA), then chose PC1 as the “ambient factor” (Appendix 1). To test differences in the call properties among regions, we used general linear mixed model (GLMM) in which regions were treated as a fixed factor and sample localities were nested in the regions as a random factor. We included “ambient factor” as covariate. The GLMM was performed using SAS

9.4 statistic software (SAS Institute Inc., Cary, NC).



In addition to the short calls, *B. japonica* also produce long calls which are comprised of a series of complicated pulses. This kind of signals could be further identified as type 1 and type 2. Type 1 was identified as calls with more than four consecutive and similar amplitude pulses following the first rising peak on the waveform (Fig. 3a, 3b). In contrast, type 2 are calls with two peaks presented at two terminal of one call (Fig. 3c, 3d). We recorded the type of long call from each frog and calculated the number of individual of each call type from each region. To assess the association between long call type and regions, we used χ^2 test and computed adjusted standardized residuals for each cell of contingency table to assess significant deviations in frequency of a particular region in call type.

Morphological data

Morphological data collection

In 2015, I captured a total of 133 adult male frogs from 13 populations (Table 1; Fig. 1 and Fig. 2). They were euthanized by providing lethal dose of benzocaine, fixed in 10% formalin immediately, and transferred to 70% ethanol for permanent storage. I measured SVL as well as 18 other morphometric measurements as shown and defined in Table 2 and Fig. 5. The choice and definition of measurements follow those of previous studies

(Matsui, 1984, 1994, 2011; Matsui et al., 2013; Poyarkov Jr et al., 2014). All measurements were taken by myself with a digital caliper and recorded to the nearest 0.1 mm (Mitutoyo, Kanagawa, Japan).



Morphological analyses

First, I examined the association between SVL and regions. The 18 morphometric traits were divided by SVL in order to standardize those measurements and were used in statistical analyses. To reduce the number of variables, I input all parameters into a principle component analysis (PCA), and used principal components with eigenvalues greater than 1 as new variables in the subsequent analyses, including discriminant analysis (DA) and general linear mixed model (GLMM). I identified five individuals as outliers (based on conditioned non-Gaussian distribution). They were excluded from analyses (excluded individuals were listed on Appendix 2). We used discriminant analysis to discriminate among populations to establish groups, and evaluate different ways of grouping based on phylogenetic tree and different divergence time (Lin *et al.*, 2013; Tominaga *et al.*, 2015). We chose the best grouping way and use GLMM to test the difference of principal component among groups.



Results

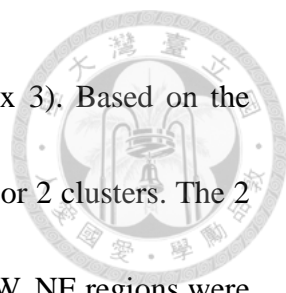
Acoustic data

We recorded short calls from a total of 251 individuals from 17 populations, 7 regions. They were included in short call analyses, except the population from Okinawa region because of its small sample size ($n=2$). The call duration (GLMM, $F_{5,9}=5.25$, $p=0.016$), call rise time ($F_{5,9}=5.23$, $p=0.016$), and dominant frequency ($F_{5,9}=0.019$, $p=0.019$) of short calls were significantly different among regions. Both call duration and call rise time in the SE, SW, NE regions of Taiwan were shorter, while dominant frequency in the NW region was higher than those of the other regions. Call fall time ($F_{5,9}=0.29$, $p=0.906$) and the interquartile range ($F_{5,9}=0.11$, $p=0.988$) were not different among regions. Ambient factor had no effect on any short call properties (Table 3; Fig. 6).

We recorded long calls from a total of 281 individuals from 17 populations, 7 regions. The types of long call were significant association with regions ($\chi^2 = 185.5$, d.f.=6; $p<0.0001$). There was a statistically higher frequency of type 2 long call than random expectation in the SE, SW, NE regions of Taiwan (Table 4; Fig. 7).

Morphological data

Based on the results of PCA, I included six principal components (from PC1 to PC6)



as new variables and used them in discriminant analysis (Appendix 3). Based on the similarity among regions, I tried to discriminate the regions by 5, 3, or 2 clusters. The 2 cluster approach yielded to the best discrimination results: the SE, SW, NE regions were placed in one and the remaining regions in the other cluster (Fig. 8a). Such a cluster division correctly assigned 89.84 % of individual to a cluster. The 3-cluster approach yielded 86.72 % correct assignment (Fig. 8b); the 5-cluster approach yielded 81.25 % correct assignment (Fig. 8c). The individuals in the two clusters differed significantly in PC2, which was associated mainly with head dimensions ($F_{1,14}=15.85, p=0.001$; Table 5 ; Fig. 9).


Discussion




The taxonomic status of Ryukyu Kajika frog, *Buergeria japonica*, has been proposed to be questionable by two recent studies based on molecular evidence (Lin *et al.*, 2013; Tominaga *et al.*, 2015). In this study, we used two additional criteria: acoustic signal and morphology, to provide further analyses on their species status. The results from both criteria are consistent with molecular evidence. All together, they support the splitting of *B. japonica* on the East Asian Arc from Ryukyu Archipelago to Taiwan into two clades: a clade that includes populations in the Ryukyu Archipelago and northeast Taiwan (hereafter defined as Japanese clade), and the other that includes other populations from eastern and southwestern Taiwan (Taiwanese clade).

Both short and long calls are significantly different between the two clades. Taiwanese clade has shorter call duration and call rise time than that in Japanese clade, whereas the type 2 long call is found exclusively in Taiwanese clade. The two clades can also be separated in morphology specifically by PC2. PC2 is contributed mainly by head dimensions, such as head length and width. This result showed that the head dimensions of the Taiwanese clade is relatively larger than those of Japanese clade. Referring to the type locality in Amami Island, the *B. japonica sensu stricto* should be strictly assigned to populations distributed from Ryukyu Archipelago to the northwestern region of Taiwan, whereas populations on the eastern and southwestern regions of Taiwan should be a new

species.



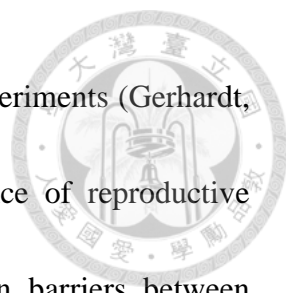
Using morphological traits has been the major criteria for species delimitation. However, my study showed that it may not be sensitive to distinguish between cryptic *Buergeria* species. Although Japanese and Taiwanese clades differ significantly in morphology principle component PC2, it explains only 16.9% of overall morphological variations. In addition, PC2 contains more than head dimensions, and would render the use of morphological traits as a species identification criteria tedious if not inaccurate. Therefore, PC2 is not an ideal diagnostic features between the two clades. This situation is similar to several cases of cryptic species. For example, the greenish warbler, *Phylloscopus trochiloides* subspecies are hard to be distinguished through morphology. However, they display distinct acoustic signals (Irwin *et al.*, 2008; Irwin, 2002). Another case in bird comes from the famous Chinese song bird Hwamei, *Garrulax canorus*. The songs of several morphologically similar geographic populations are significantly different in syllable and syntax. The two sibling species were thus further divided into two valid species, *G. canorus* and *G. taewanus*, by consolidating the results from song variation and gene information (Li *et al.*, 2006; Tu and Severinghaus, 2004). An anuran case comes from *Mircohyala ornata*, an abundant species in South Asia (Kuramoto and Joshy, 2006; Matsui *et al.*, 2005). The species distributes from India to Taiwan to Ryukyu Archipelago and was separated into three species. The populations on Ryukyu



Archipelago and Taiwan are now recognized as distinct species, *M. okinavensis* and *M. fissipes*, based on both genetic data (Matsui *et al.*, 2005) and acoustic evidence (Kuramoto and Joshy, 2006). The three species can be clearly discriminated by temporal acoustic characteristics, especially pulse repetition rate (Kuramoto and Joshy, 2006). My study shows another case of using acoustic signals in anuran cryptic species delimitation. The two morphologically similar clades of *B. japonica* are significantly distinct in acoustic signals.

Acoustic signal plays a key role in pre-mating isolation. Referring to Paterson's Recognition Species Concept (RSC) (Coyne, 1993; Paterson, 1985), the differentiation of advertisement calls gave the primary line of evidence to suggest the biological identity in this candidate species. In addition to RSC, the genetic data (Lin *et al.*, 2013) provided strong evidence to my suggestions with Evolutionary Species Concept (ESC) (Simpson, 1961) because the genetic distance among populations in *B. japonica* could reach 17%. Genetic divergence of these clades exceed to the case of two newly discovered species, *Kurixalus wangi* and *K. berylliniris*. In that case, the maximum sequence divergence among three *Kurixalus* species was 11% (Wu *et al.*, 2016).

The evolutionary processes shaping the isolation between closely related species is a compelling issue in speciation. Acoustic signals could be the key reproductive barrier that maintains Japanese and Taiwanese clades as different uniqueness species. Such a



hypothesis could potentially be tested directly using mate choice experiments (Gerhardt, 1991; Gerhardt and Huber, 2002), or by observing the occurrence of reproductive character displacement (RCD) in nature. Given that reproduction barriers between populations could break down when the two clades get into contact, the reproductive signals, such as calls and songs, may be heightened in the contact zone to avoid interspecific competition for signal space and to prevent the two clades from hybridization. The heightened difference in the reproductive barrier leads to reproductive character displacement, defined from the prediction that the traits would be more distinguished in sympatric than in allopatric zones among closely related species (Albert *et al.*, 2007; Arthur, 1982; Grether *et al.*, 2009). This hypothesis has been observed from African tinkerbird *Pogoniulus bilineatus* and *P. subsulphureus* (Moyle, 2002), and American green tree frog *Hyla cinerea* and *H. gratiosa* (Hobel and Gerhardt, 2003).

In *Buergeria japonica*, the Japanese and Taiwanese clades could get into contact in lowland areas in western and northern Taiwan. The former is likely between ZhouShui Stream and BaZhang Stream, while the latter might be between XinDian Stream and LanYang Stream. A preliminary observation in my study showed that there was indeed a greater difference in type 1 and 2 long call usage ratio between regions at contact zones than the other regions. Future studies could pursue the topic of reproductive character displacement between the two clades which could further get insight into the role of

acoustic signal in species delimitation.



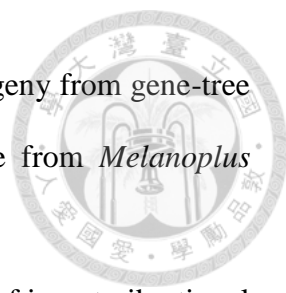
Conclusion


My study demonstrated the geographic variations of acoustic and morphological traits in *Buergeria japonica*. Both the two kinds of advertisement calls in *B. japonica* showed divergence among regions, with one of the two types of long call appeared exclusively in the populations from southern and eastern Taiwan. In addition to acoustic signal, the morphological trait also indicated the subtle difference on integrated head trait, which is bigger in these populations than in the others, although it was hard to distinguish them by macroscopic observation. Based on the type locality, we propose to redefine the taxonomic status of *Buergeria japonica*, and treat the populations in southern and eastern Taiwan as a new species.



Reference

- Albert, A.Y.K., Millar, N.P., and Schluter, D. (2007). Character displacement of male nuptial colour in threespine sticklebacks (*Gasterosteus aculeatus*). *Biological Journal of the Linnean Society* 91, 37-48.
- Alström, P., Rasmussen, P.C., Olsson, U., and Sundberg, P. (2008). Species delimitation based on multiple criteria: the Spotted Bush Warbler *Bradypterus thoracicus* complex (Aves: Megaluridae). *Zoological Journal of the Linnean Society* 154, 291-307.
- Arthur, W. (1982). The evolutionary consequences of interspecific competition. *Advances in Ecological Research* 12, 127-187.
- Barbault, R. (1988). Body size, ecological constraints, and the evolution of life-history strategies. In *Evolutionary biology* (Springer), pp. 261-286.
- Bee, M.A., Cook, J.M., Love, E.K., O'Bryan, L.R., Pettitt, B.A., Schrode, K., and Velez, A. (2010). Assessing acoustic signal variability and the potential for sexual selection and social recognition in boreal chorus frogs (*Pseudacris maculata*). *Ethology* 116, 564-576.
- Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., and Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution* 22, 148-155.
- Biju, S.D., Senevirathne, G., Garg, S., Mahony, S., Kamei, R.G., Thomas, A., Shouche, Y., Raxworthy, C.J., Meegaskumbura, M., and Bocxlaer, I.V. (2016). *Frankixalus*, a new Rhacophorid genus of tree hole breeding frogs with oophagous tadpoles. *PLoS One* 11, e0145727.

- 
- Carstens, B.C., and Knowles, L.L. (2007). Estimating species phylogeny from gene-tree probabilities despite incomplete lineage sorting: an example from *Melanoplus* grasshoppers. *Systematic Biology* 56, 400-411.
- Cocroft, R.B., and Rodríguez, R.L. (2005). The behavioral ecology of insect vibrational communication. *Bioscience* 55, 323-334.
- Coyne, J.A. (1993). Evolution and the recognition concept of species - collected writings of paterson, Hugh, E.H. - Mcevey, Sf. *Nature* 364, 298-298.
- Delorme, M., Dubois, A., and Grosjean, S. (2005). Une nouvelle classification générique et subgénérique de la tribu des Philautini. *Bulletin mensuel de la Société linnéenne de Lyon* 74, 165-171.
- Dobzhansky, T. (1940). Speciation as a stage in evolutionary divergence. *American Naturalist* 74, 312-321.
- Feo, T.J., Musser, J.M., Berv, J., and Clark, C.J. (2014). Divergence in morphology, calls, song, mechanical sounds, and genetics supports species status for the Inaguan hummingbird (*Trochilidae: Calliphlox "evelynae" lyrura*). *The Auk* 132, 248-264.
- Frost, D.R. (2014). Amphibian species of the world: an online reference. Version 6.0. New York: American Museum of Natural History.
- Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., De Sa, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., *et al.* (2006). The amphibian tree of life. *Bulletin of the American Museum of Natural History*, 8-370.
- Gerhardt, H.C. (1991). Female mate choice in treefrogs - static and dynamic acoustic criteria. *Animal Behaviour* 42, 615-635.
- Gerhardt, H.C., and Huber, F. (2002). Acoustic communication in insects and anurans: common problems and diverse solutions. (Chicago: University of Chicago Press).
- Gray, D.A., and Cade, W.H. (2000). Sexual selection and speciation in field crickets.

- 
- Proceedings of the National Academy of Sciences of the United States of America
97, 14449-14454.
- Grether, G.F., Losin, N., Anderson, C.N., and Okamoto, K. (2009). The role of interspecific interference competition in character displacement and the evolution of competitor recognition. *Biological reviews of the Cambridge Philosophical Society* 84, 617-635.
- Hallowell, E. (1861). Report upon the reptilia of the north pacific exploring expedition, under command of capt. John Rogers, U. S. N. *Proceedings of the Academy of Natural Sciences of Philadelphia* 1860, 480-501.
- Helbig, A.J., Knox, A.G., Parkin, D.T., Sangster, G., and Collinson, M. (2002). Guidelines for assigning species rank. *Ibis* 144, 518-525.
- Ho, C.S. (1986). A synthesis of the geologic evolution of Taiwan. *Tectonophysics* 125, 1-16.
- Hobel, G., and Gerhardt, H.C. (2003). Reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). *Evolution* 57, 894-904.
- Honda, M., Kurita, T., Toda, M., and Ota, H. (2014). Phylogenetic relationships, genetic divergence, historical biogeography and conservation of an endangered gecko, *Goniurosaurus kuroiwae* (Squamata: Eublepharidae), from the central Ryukyus, Japan. *Zoological science* 30, 309-320.
- Irwin, D., Thimgan, M., and Irwin, J. (2008). Call divergence is correlated with geographic and genetic distance in greenish warblers (*Phylloscopus trochiloides*): a strong role for stochasticity in signal evolution? *Journal of Evolutionary Biology* 21, 435-448.
- Irwin, D.E. (2002). Phylogeographic breaks without geographic barriers to gene flow.

Evolution 56, 2383-2394.

Jang-Liaw, N.-H., Lee, T.-H., and Chou, W.-H. (2008). Phylogeography of *Sylvirana latouchii* (Anura, Ranidae) in Taiwan. *Zoological Science* 25, 68-79.

Jang-Liaw, N.H., and Lee, T.H. (2009). Intraspecific relationships of populations of the brown frog *Rana sauteri* (Ranidae) on Taiwan, inferred from mitochondrial cytochrome *b* sequences. *Zoological Science* 26, 608-616.

Köhler, J., Vieites, D., Bonett, R., Garcia, F.H., Glaw, F., Steinke, D., and Vences, M. (2005). New amphibians and global conservation: a boost in species discoveries in a highly endangered vertebrate group. *BioScience* 55 (8): 693-696 .

Kimura, M. (2000). Paleogeography of the Ryukyu Islands. *Tropics* 10, 5-24.

Knowles, L.L., and Carstens, B.C. (2007). Delimiting species without monophyletic gene trees. *Systematic Biology* 56, 887-895.

Kuramoto, M., and Joshy, S.H. (2006). Morphological and acoustic comparisons of *Microhyla ornata*, *M. fissipes*, and *M. okinavensis* (Anura: Microhylidae). *Current Herpetology* 25, 15-27.

Li, S.H., Li, J.W., Han, L.X., Yao, C.T., Shi, H., Lei, F.M., and Yen, C. (2006). Species delimitation in the Hwamei *Garrulax canorus*. *Ibis* 148, 698-706.

Lin, H.D., Chen, Y.R., and Lin, S.M. (2012). Strict consistency between genetic and topographic landscapes of the brown tree frog (*Buergeria robusta*) in Taiwan. *Molecular Phylogenetics and Evolution* 62, 251-262.

Lin, S.M., Chen, C.A., and Lue, K.Y. (2002). Molecular phylogeny and biogeography of the grass lizards genus *Takydromus* (Reptilia: Lacertidae) of East Asia. *Molecular Phylogenetics and Evolution* 22, 276-288.

Lin, Y.P., Tseng, H.Y., and Lin, S.M. (2013). Inter- and intra-island differentiation: A case from Japanese tree frog (*Buergeria japonica*) in Taiwan and the Ryukyus. In 2013

Congress of Animal Behavior and Ecology (Hualien, Taiwan: Nation Dong Hwa University), pp. 135-136.



Maddison, W.P., and Knowles, L.L. (2006). Inferring phylogeny despite incomplete lineage sorting. *Systematic Biology* 55, 21-30.

Maruska, K.P., Ung, U.S., and Fernald, R.D. (2012). The African cichlid fish *Astatotilapia burtoni* uses acoustic communication for reproduction: sound production, hearing, and behavioral significance. *PLoS One* 7, e37612.

Matsui, M. (1984). Morphometric variation analyses and revision of the Japanese toads (genus *Bufo*, Bufonidae). *Contributions from the Biological Laboratory, Kyoto University* 26, 208-428.

Matsui, M. (1994). A taxonomic study of the *Rana narina* complex, with description of three new species (Amphibia: Ranidae). *Zoological Journal of the Linnean Society* 111, 385-415.

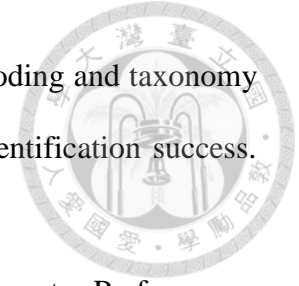
Matsui, M. (2011). Taxonomic revision of one of the Old World's smallest frogs, with description of a new Bornean *Microhyla* (Amphibia, Microhylidae). *Zootaxa* 2814, 33-49.

Matsui, M., Hamidy, A., and Eto, K. (2013). Description of a new species of *Microhyla* from Bali, Indonesia (Amphibia, Anura). *Zootaxa* 3670, 579-590.

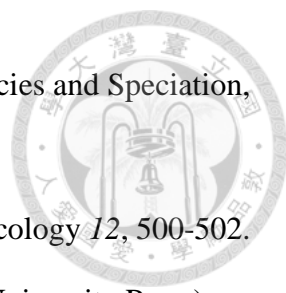
Matsui, M., Ito, H., Shimada, T., Ota, H., Saidapur, S.K., Khonsue, W., Tanaka Ueno, T., and Wu, G.F. (2005). Taxonomic relationships within the Pan-Oriental narrow-mouth toad *Microhyla ornata* as revealed by mtDNA analysis (Amphibia, Anura, Microhylidae). *Zoological Science* 22, 489-495.

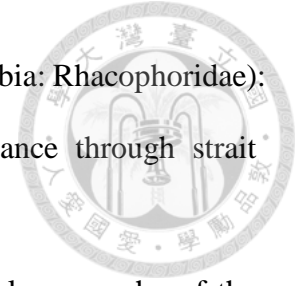
McComb, K. (1987). Roaring by red deer stags advances the date of oestrus in hinds. *Nature* 330, 648-649.

McKinney, M.L. (1990). Trends in body-size evolution. *Evolutionary Trends*, 75-118.



- Meier, R., Shiyang, K., Vaidya, G., and Ng, P.K. (2006). DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Systematic Biology* 55, 715-728.
- Miller, K., Monteforte, P.B., and Landis, L.F. (1993). Scaling of Locomotor Performance and Enzyme-Activity in the Leopard Frog, *Rana Pipiens*. *Herpetologica* 49, 383-392.
- Moyle, R.G. (2002). Molecular systematics of barbets and trogons: Pantropical biogeography, African speciation, and issues in phylogenetic inference. In *Zoology (Biological Sciences)* (University of Rhode Island).
- Nauwelaerts, S., Ramsay, J., and Aerts, P. (2007). Morphological correlates of aquatic and terrestrial locomotion in a semi-aquatic frog, *Rana esculenta*: no evidence for a design conflict. *Journal of Anatomy* 210, 304-317.
- Oseen, K.L., and Wassersug, R.J. (2002). Environmental factors influencing calling in sympatric anurans. *Oecologia* 133, 616-625.
- Oshida, T., Lee, J.-K., Lin, L.-K., and Chen, Y.-J. (2006). Phylogeography of Pallas's squirrel in Taiwan: geographical isolation in an arboreal small mammal. *Journal of Mammalogy* 87, 247-254.
- Osozawa, S., Oba, Y., Kwon, H.Y., and Wakabayashi, J. (2015). Vicariance of *Pyrocoelia* fireflies (Coleoptera: Lampyridae) in the Ryukyu islands, Japan. *Biological Journal of the Linnean Society* 116, 412-422.
- Ota, H. (1998). Geographic patterns of endemism and speciation in amphibians and reptiles of the Ryukyu Archipelago, Japan, with special reference to their paleogeographical implications. *Researches on Population Ecology* 40, 189-204.
- Panhuis, T.M., Butlin, R., Zuk, M., and Tregenza, T. (2001). Sexual selection and speciation. *Trends in Ecology and Evolution* 16, 364-371.

- 
- Paterson, H.E.H. (1985). The Recognition concept of species. In *Species and Speciation*, E. Vrba, ed. (Transvaal Museum Monograph No. 4. Pretoria).
- Perrin, N. (1998). On body size, and energy and fitness. *Functional Ecology* *12*, 500-502.
- Peters, J.L. (1931). Check-list of birds of the world (USA: Harvard University Press).
- Peters, R.H. (1986). The ecological implications of body size, Vol 2 (England: Cambridge University Press).
- Petit, R.J., and Excoffier, L. (2009). Gene flow and species delimitation. *Trends in Ecology and Evolution* *24*, 386-393.
- Poyarkov Jr, N.A., Vassilieva, A.B., Orlov, N.L., Galoyan, E.A., Tran, D., Le, D.T.T., Kretova, V.D., and Geissler, P. (2014). Taxonomy and distribution of narrow-mouth frogs of the genus *Microhyla Tschudi*, 1838 (Anura: Microhylidae) from Vietnam with descriptions of five new species. *Russian Journal of Herpetology* *21*, 89-148.
- Price, T. (1998). Sexual selection and natural selection in bird speciation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* *353*, 251-260.
- Ritchie, M.G. (2007). Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics* *38*, 79-102.
- Schmidt-Nielsen, K. (1984). *Scaling: why is animal size so important?* (England: Cambridge University Press).
- Simpson, G.G. (1961). *Principles of animal taxonomy* (New York: Columbia University Press).
- Teng, L.S. (1990). Geotectonic evolution of late Cenozoic arc-continent collision in Taiwan. *Tectonophysics* *183*, 57-76.
- Thorpe, W.H. (1961). *Bird-song: the biology of vocal communication and expression in birds* (England: Oxford University Press).
- Tominaga, A., Matsui, M., Eto, K., and Ota, H. (2015). Phylogeny and differentiation of



wide-ranging Ryukyu Kajika Frog *Buergeria japonica* (Amphibia: Rhacophoridae): geographic genetic pattern not simply explained by vicariance through strait formation. *Zoological Science* 32, 240-247.

Tominaga, A., Ota, H., and Matsui, M. (2010). Phylogeny and phylogeography of the sword-tailed newt, *Cynops ensicauda* (Amphibia: Caudata), as revealed by nucleotide sequences of mitochondrial DNA. *Molecular Phylogenetics and Evolution* 54, 910-921.

Tseng, S.P., Wang, C.J., Li, S.H., and Lin, S.M. (2015). Within-island speciation with an exceptional case of distinct separation between two sibling lizard species divided by a narrow stream. *Molecular Phylogenetics and Evolution* 90, 164-175.

Tu, H.W., and Severinghaus, L.L. (2004). Geographic variation of the highly complex Hwamei (*Garrulax canorus*) songs. *Zoological Studies* 43, 629-640.

Vieites, D.R., Wollenberg, K.C., Andreone, F., Köhler, J., Glaw, F., and Vences, M. (2009). Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences* 106, 8267-8272.

Voigt, C.C., Behr, O., Caspers, B., von Helversen, O., Knörnschild, M., Mayer, F., and Nagy, M. (2008). Songs, scents, and senses: sexual selection in the greater sac-winged bat, *Saccopteryx bilineata*. *Journal of Mammalogy* 89, 1401-1410.

Wade, E., Hertach, T., Gogala, M., Trilar, T., and Simon, C. (2015). Molecular species delimitation methods recover most song-delimited cicada species in the European *Cicadetta montana* complex. *Journal of Evolutionary Biology* 28, 2318-2336.

Warwick, A.R., Travis, J., and Lemmon, E.M. (2015). Geographic variation in the Pine Barrens Treefrog (*Hyla andersonii*): concordance of genetic, morphometric and acoustic signal data. *Molecular Ecology* 24, 3281-3298.

West-Eberhard, M.J. (1983). Sexual selection, social competition, and speciation.

Quarterly Review of Biology, 155-183.

Wilkinson, J.A., and Drewes, R. (2000). Character assessment, genus level boundaries, and phylogenetic analyses of the family Rhacophoridae: a review and present day status. Contemporary Herpetology 2, 1-24.

Wu, S.P., Huang, C.C., Tsai, C.L., Lin, T.E., Jhang, J.J., and Wu, S.H. (2016). Systematic revision of the Taiwanese genus *Kurixalus* members with a description of two new endemic species (Anura, Rhacophoridae). ZooKeys 557, 121-153.



Content of Tables & Figures

Table

Table 1.	27
Table 2.	28
Table 3.	30
Table 4.	31
Table 5.	32

Figure

Figure 1.	33
Figure 2.	34
Figure 3.	35
Figure 4.	36
Figure 5.	37
Figure 6.	38
Figure 7.	39
Figure 8.	40
Figure 9.	43

Table 1. Region, locality, abbreviation, sample size (acoustic and morphological), and GPS coordination for each *Buergeria japonica* population used in this study.

Region	Sample locality	Abbr.	Acoustic	Morphological	GPS coordination
Amami	Naze Oaza Nesebu	NAZ	5	6	28.379315, 129.438779
	Yuwangama	YUW	10	6	28.363202, 129.412020
	Uken	UKE	0	9	28.276756, 129.302697
Okinawa	North Okinawa	OKI	14	5	26.727186, 128.210264
Yaeyama	Iriomote	IRI	5	4	24.284637, 123.886220
	Ishigaki	ISH	5	9	24.413747, 124.206365
North-Weatern	XinDian Stream	XD	15	9	24.866563, 121.552164
	ZhongGang Stream	ZG	23	8	24.658416, 121.018135
	Wu Stream	WU	19	0	24.065765, 120.737269
	ZhouShui Stream	ZS	15	8	23.784825, 120.778910
North-Eatern	LanYang Stream	LY	19	10	24.618080, 121.635305
	NanAo Stream	NAO	21	8	24.481911, 121.823648
	HePing Stream	HP	24	0	24.346551, 121.769833
South-Western	BaZhang Stream	BZ	18	0	23.381634, 120.530214
	GaoPing Stream	GP	18	0	22.875611, 120.666287
	DongGang Stream	DG	19	11	22.626340, 120.643342
	FongGang Stream	FG	0	10	22.217591, 120.799465
South-Eastern	MeiLun Stream	ML	0	8	24.005365, 121.547298
	MuGua Stream	MG	17	0	23.883503, 121.519595
	XiuGuLuan Stream	XG	19	11	23.502209, 121.329709
	BeiNan Stream	BN	15	0	22.897131, 121.156045
	Baliwan stream	BL	0	11	23.596076, 121.516176
Total			281	133	

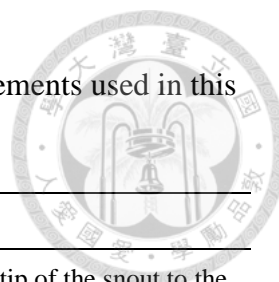


Table 2. Abbreviation and definition of the 19 morphometric measurements used in this study. Adjusted abbreviation was used after standardizing by SVL.

No.	Measurement	Abbr.	Adjusted abbr.	Definition
1	Snout-vent length	SVL		The length between the tip of the snout to the cloaca
2	Head length	HL	aHL	The length between tip of snout to the hind border of jaw angle
3	Head width	HW	aHW	The maximum width of the head on the level of mouth angles in ventral view
4	Eye length	EL	aEL	The length between the anterior and posterior corners of the eye
5	Interorbital distance	IO	aIO	The shortest length between the medial edges of eyeballs in dorsal view
6	Intercanthal distance	IC	aIC	The length between the front edge of the eyes
7	Eye-nose length	END	aEND	The length between the anterior corner of eye and the nostril center
8	Width of upper eyelid	UEW	aUEW	The widest length from the medial edge of eyeball to the lateral edge of the upper eyelid
9	Forelimb length	FLL	aFLL	The length from the third finger to the end of the forelimb armpit
10	Forelimb arm length	FAL	aFAL	The length from the forelimb armpit to the elbow
11	Length of hand	HAL	aHAL	The length from the proximal end of outer metacarpal tubercle to the tip of the third finger
12	Finger 1st length	1FL	a1FL	The length from the first fingertip to the base of the finger
13	Finger 3rd length	3FL	a3FL	The length from the third fingertip to the base of the finger.
14	Hindlimb length	HLL	aHLL	The length of straightened hind limb from groin to tip of the fourth toe
15	Tibia length	TL	aTL	The length between the knee and tibiotarsal

16	Tarsus length	TTL	aTTL	articulation The length between tibiotarsal articulation and the fourth toe
17	Inner metatarsal tubercle length	iMTL	aiMTL	The maximal diameter of inner metatarsal tubercle
18	Toe 1st length, 1TL	1TL	a1TL	The length from the end to first toe tip to the base of the toe
19	Toe 4th length	4TL	a4TL	The length from the end to fourth toe tip to the base of the toe

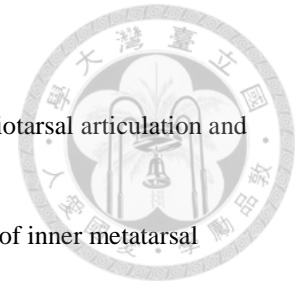


Table 3. The results of generalized linear mixed model (GLMM) on acoustic traits of short calls (n= 251). Ecological factors were treated as covariates in GLMM.

Effect	Num. DF	Den. DF	F	<i>p</i> value
Call duration				
Region	5	9	5.25	0.02
Eco	1	235	0.03	0.87
Call rise time				
Region	5	9	5.25	0.02
Eco	1	235	0.03	0.87
Call fall time				
Region	5	9	0.29	0.91
Eco	1	235	0.06	0.80
Dominant frequency				
Region	5	9	4.92	0.02
Eco	1	235	0.03	0.87
IQR f.				
Region	5	9	0.11	0.99
Eco	1	235	0.00	0.99

Num.: numerator; Den.: denominator; Eco: the principal component of temperature and humidity.

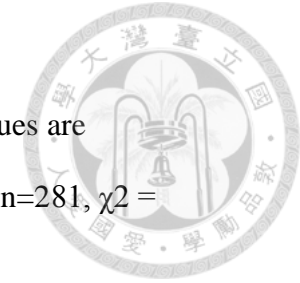


Table 4. Contingency table for testing the association between long call types and regions. Observed and expected values are significantly different when the absolute values of adjusted standized residuals test (adjusted standized r.) exceed 2.0 (n=281, $\chi^2 = 185, p < 0.001$).

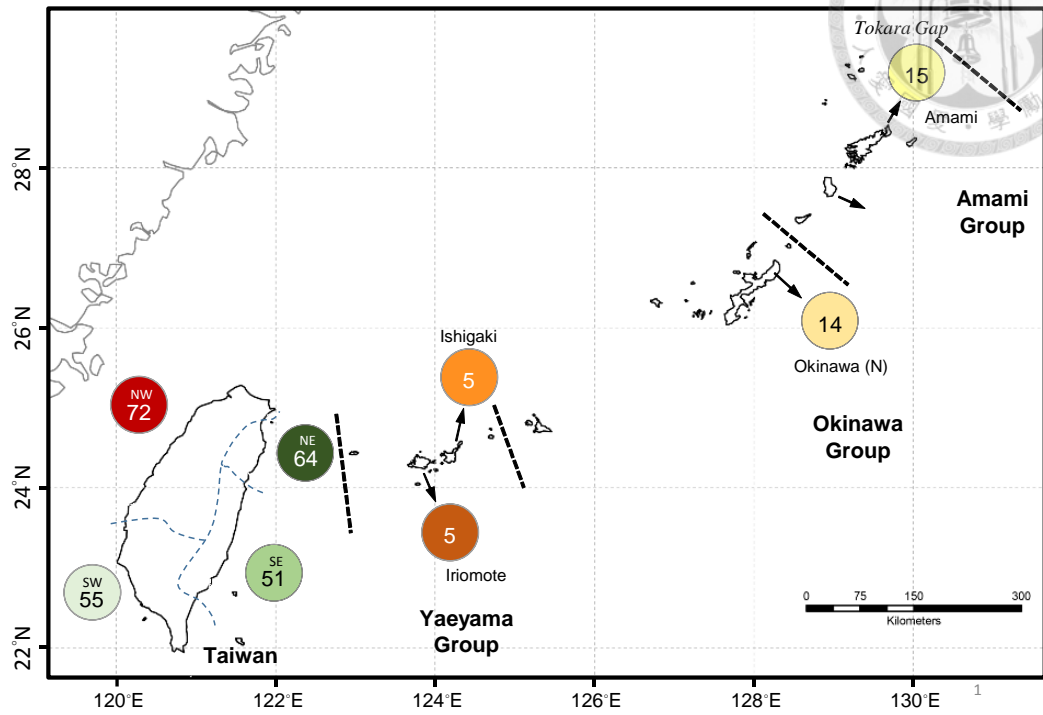
Call Type	Group							
	AM	OKI	ISG	NW	NE	SW	SE	total
no Type 2 count	15.0	14.0	10.0	72.0	14.0	13.0	24.0	162
expected count	8.6	8.1	5.8	41.5	36.9	31.7	29.4	
total%	5.3	5.0	3.6	25.6	5.0	4.6	8.5	
adjusted standized r.	3.4	3.3	2.8	8.4	-6.6	-5.7	-1.7	
Type 2 count	0.0	0.0	0.0	0.0	50.0	42.0	27.0	119
expected count	6.4	5.9	4.2	30.5	27.1	23.3	21.6	
total%	0.0	0.0	0.0	0.0	17.8	15.0	9.6	
adjusted standized r.	-3.4	-3.3	-2.8	-8.4	6.6	5.7	1.7	
Total	15	14	10	72	64	55	51	281



Table 5. Generalized linear mixed model on morphometric traits.

Effect	Num. DF	Den. DF	F value	P value
PC1				
Clade	1	14	0.98	0.34
PC2				
Clade	1	14	15.85	0.0014
PC3				
Clade	1	14	1.39	0.26
PC4				
Clade	1	14	2.72	0.12
PC5				
Clade	1	14	1.73	0.21
PC6				
Clade	1	14	1.34	0.27

(1a)



(1b)

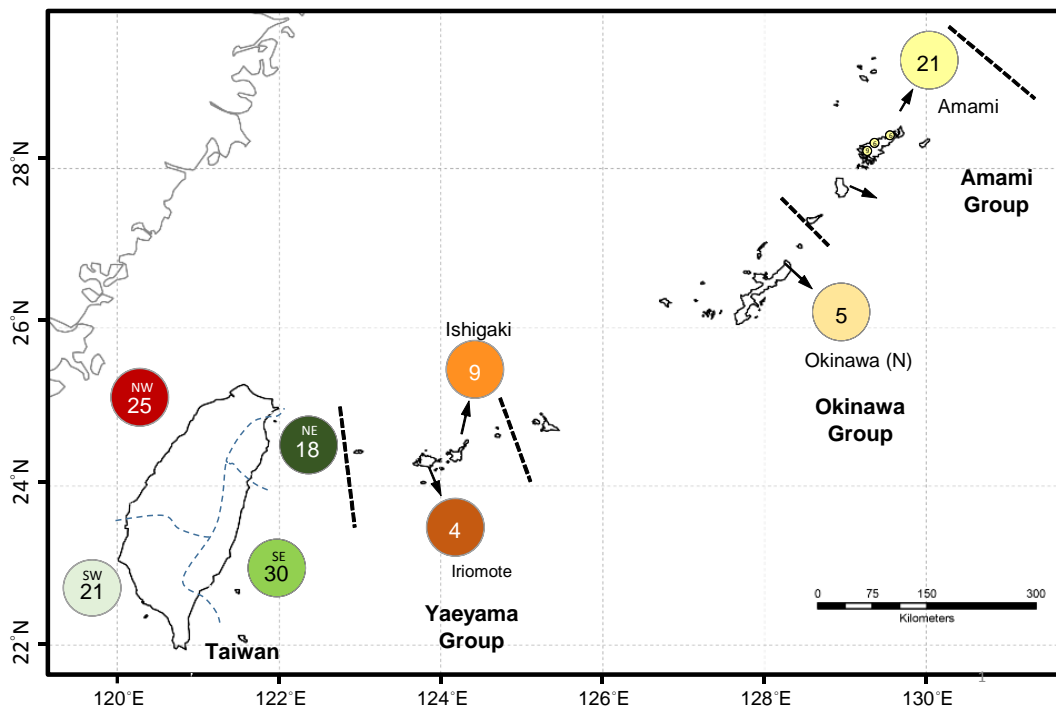


Figure 1. Large-scaled sample regions for (a) acoustic and (b) morphological characters of *Buergeria japonica* from Ryukyu and Taiwan. See Table 1 for the details of each region.

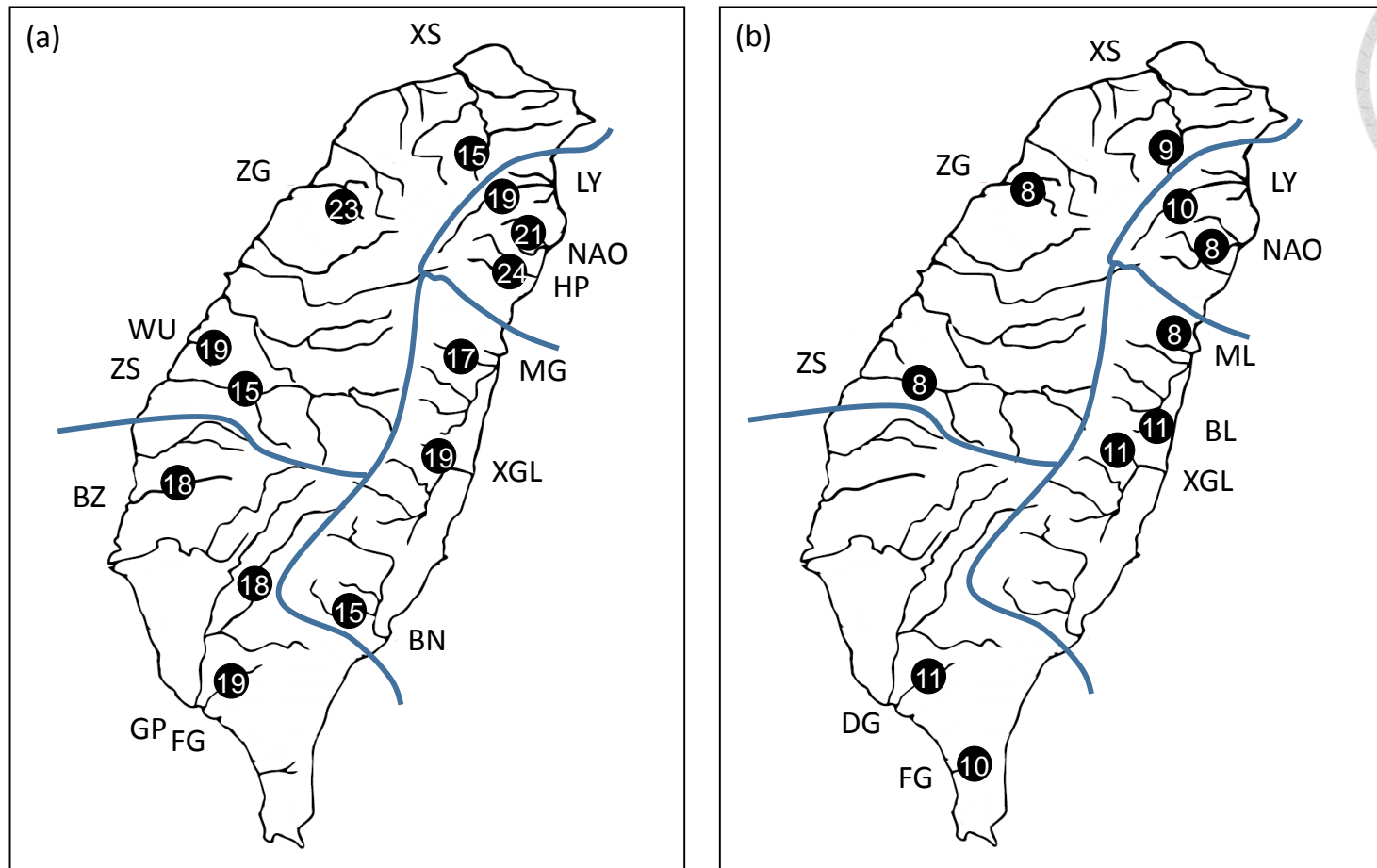


Figure 2. Fine-scaled sample localities for (a) acoustic and (b) morphological characters of *Buergeria japonica* in Taiwan. Number in the circles denote sample size in each locality. Also see Table 1 for abbreviations of each locality.

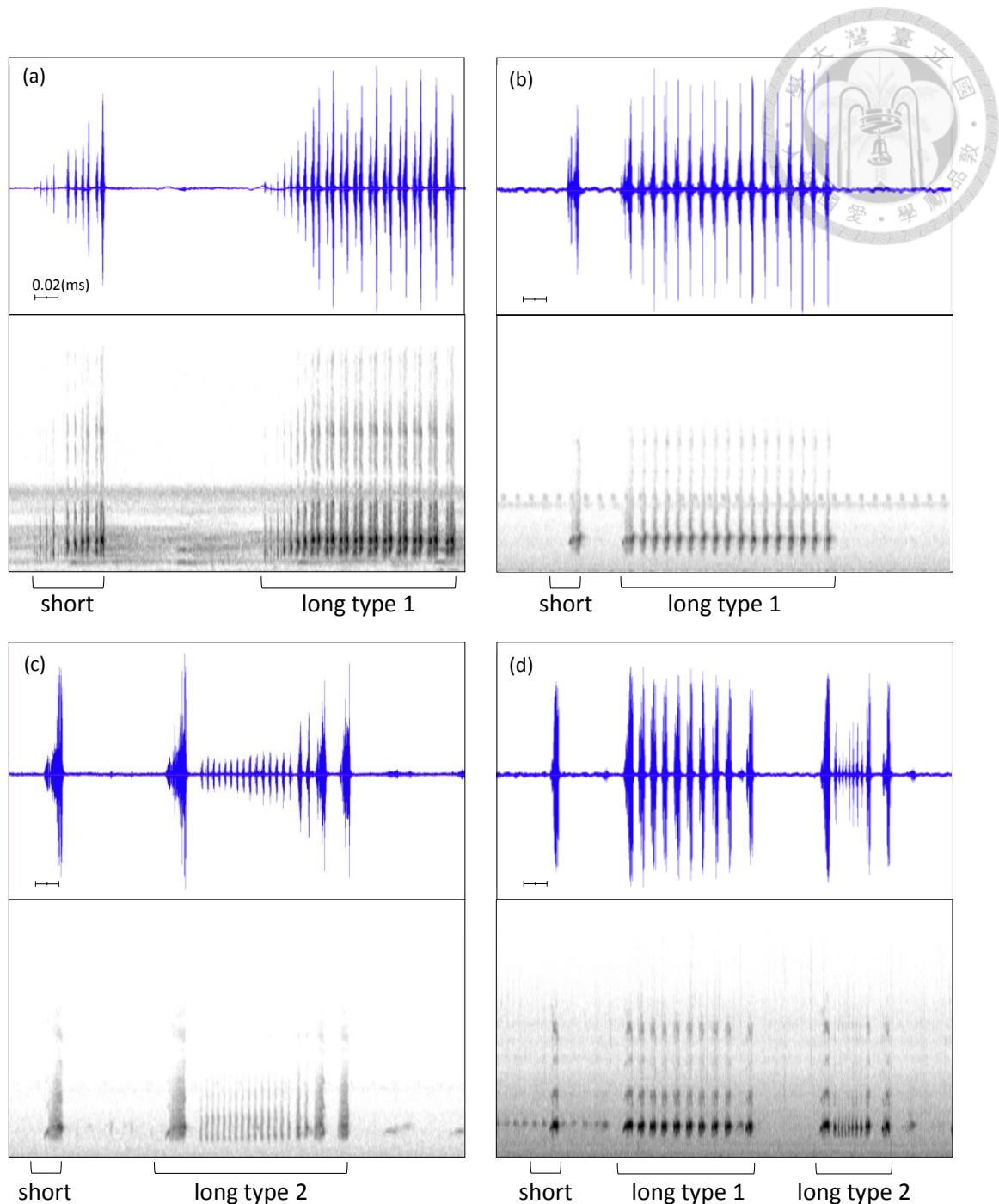


Figure 3. Entire advertisement call sections of *Buergeria japonica*. (a) A typical waveform and spectrum of short and long calls (type 1) produced by populations from Ryukyu and northwestern Taiwan; (b) a waveform and a spectrum from eastern and southwestern Taiwan, with a short call followed by a type 1 long call; (c) eastern and southwestern Taiwan, a short call followed by a type 2 long call; (d) eastern Taiwan, a combination of short, type 1, and type 2 long calls.

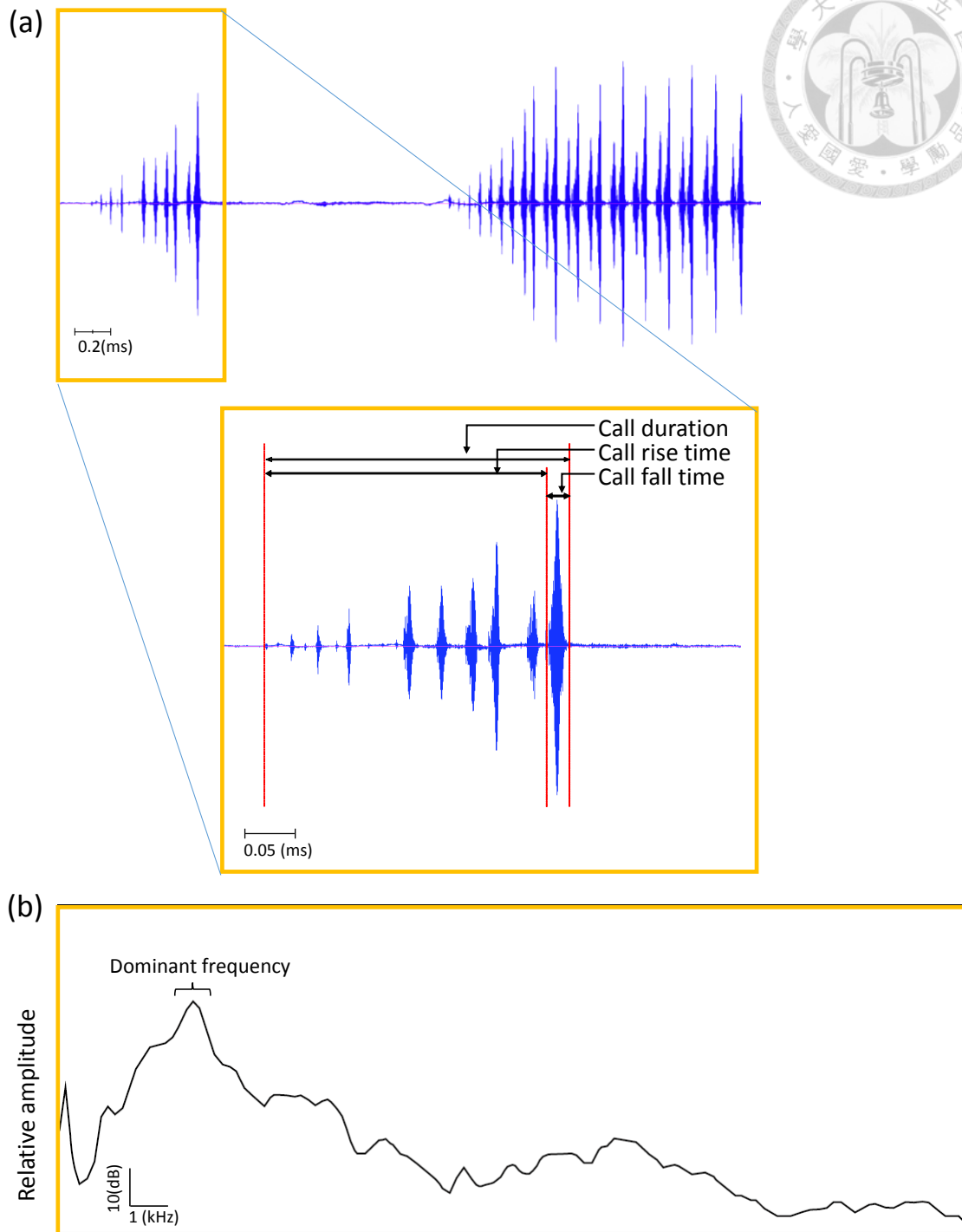


Figure 4. Definition of temporal and spectral call properties from a short call of *Buergeria japonica*. (a) A typical waveform of a short call with three temporal traits (DT, RT, and FT); and (b) power spectrum of this short call. See context for the abbreviation of these traits.

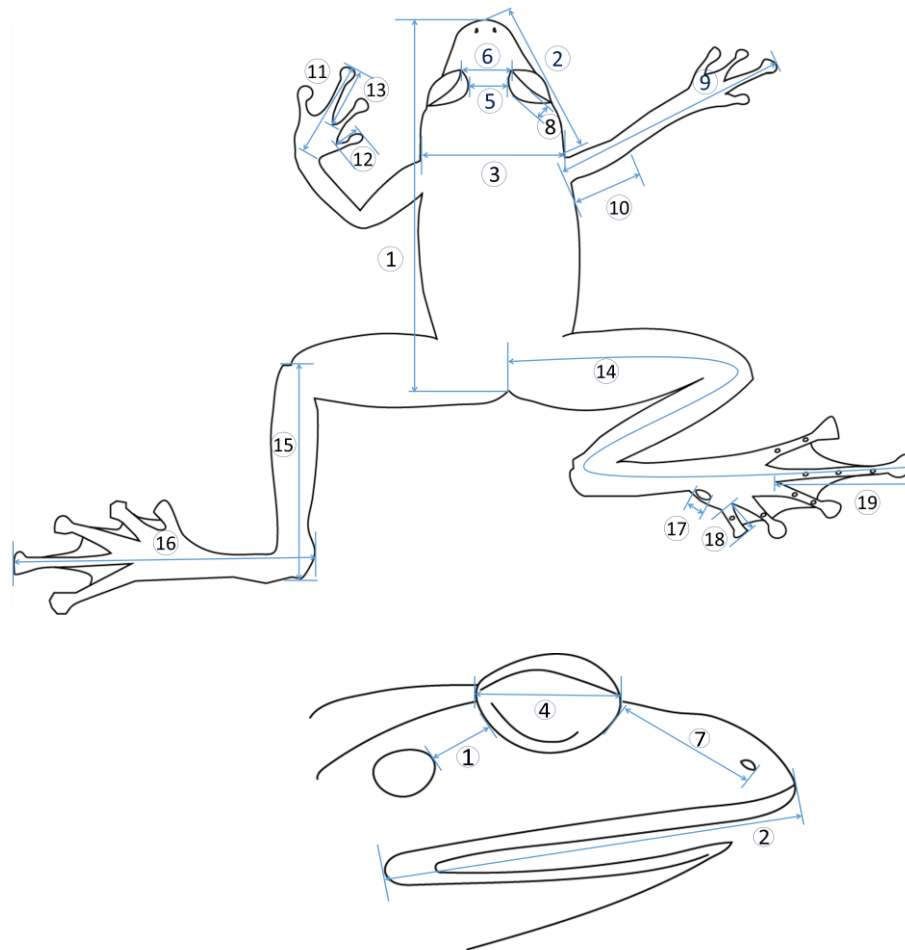


Figure 5. The 19 morphometric traits of *Buergeria japonica* used in this study.

Measurements:

1	Snout-vent length	SVL
2	Head length	HL
3	Head width	HW
4	Eye length	EL
5	Interorbital distance	IO
6	Intercanthal distance	IC
7	Eye-nose length	END
8	Width of upper eyelid	UEW
9	Forelimb length	FLL
10	Forelimb arm length	FAL
11	Length of hand	HAL
12	Finger 1 st length	1FL
13	Finger 3 rd length	3FL
14	Hindlimb length	HLL
15	Tibia length	TL
16	Tarsus length	TTL
17	Inner metatarsal tubercle length	iMTL
18	Toe 1 st length	1TL
19	Toe 4 th length	4TL



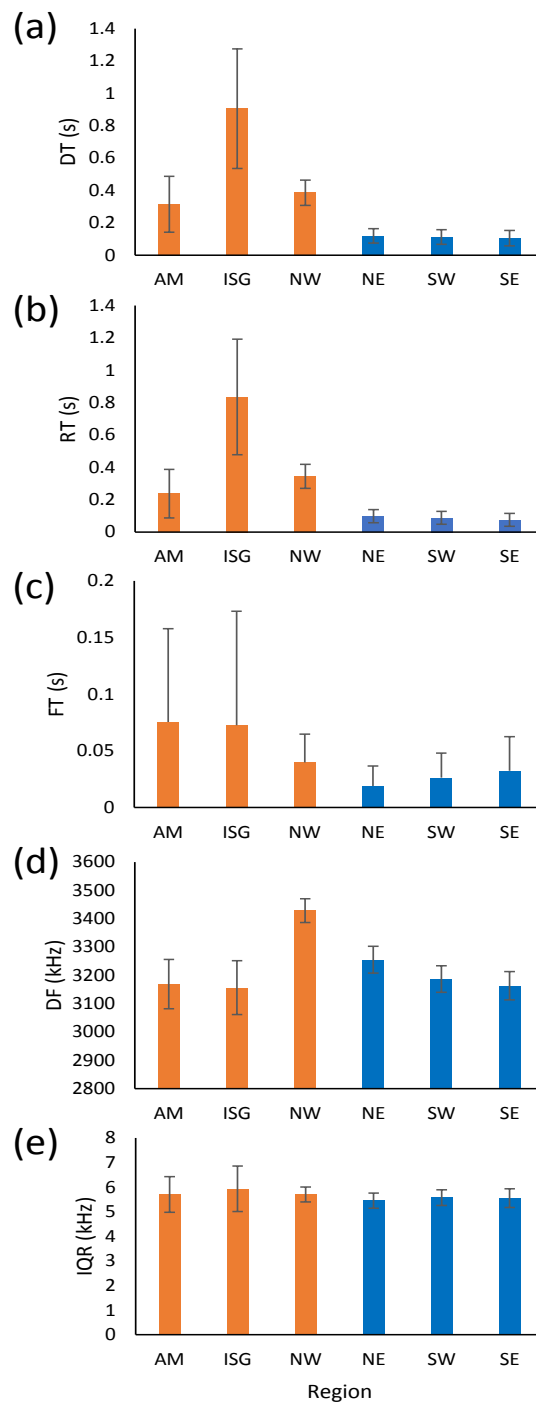


Figure 6. Statistic comparison of short call temporal traits among six regions of *Buergeria japonica*, with ecological parameters as cofactors. The bar which presented with orange is from the Japanese clade and with blue is from the Taiwanese clade. (a) Call duration ($p=0.02$); (b) call rise time ($p=0.02$); (c) call fall time ($p=0.9$); (d) dominant frequency ($p=0.02$); and (e) IQR bandwidth of frequencies ($p=0.99$).

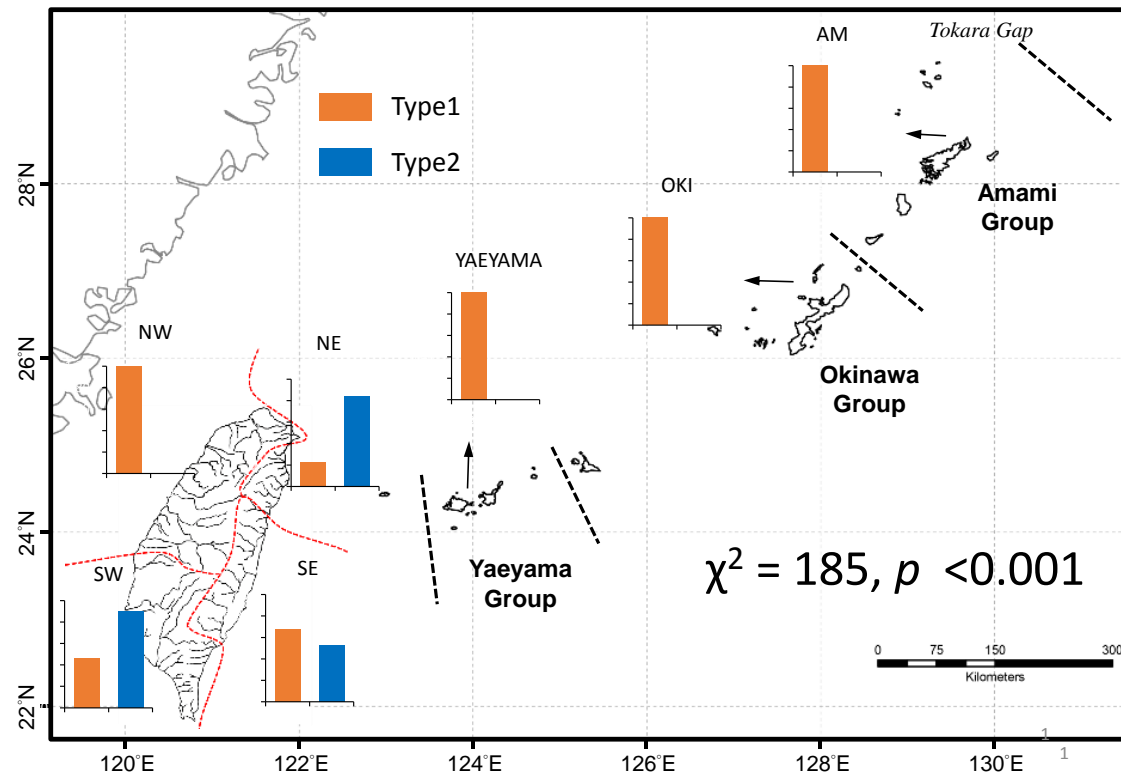


Figure 7. The percentage of the two types of long calls occurring from the seven geographic regions across Ryukyu and Taiwan. Orange bars: the proportion of type 1 long calls; blue bars: the proportion of type 2 long calls (proportion = the number of individuals producing this call type/all individuals in this area). Some individuals in eastern and southeastern Taiwan are able to produce both types of long calls, yielding to exceeding of 100% in these regions.

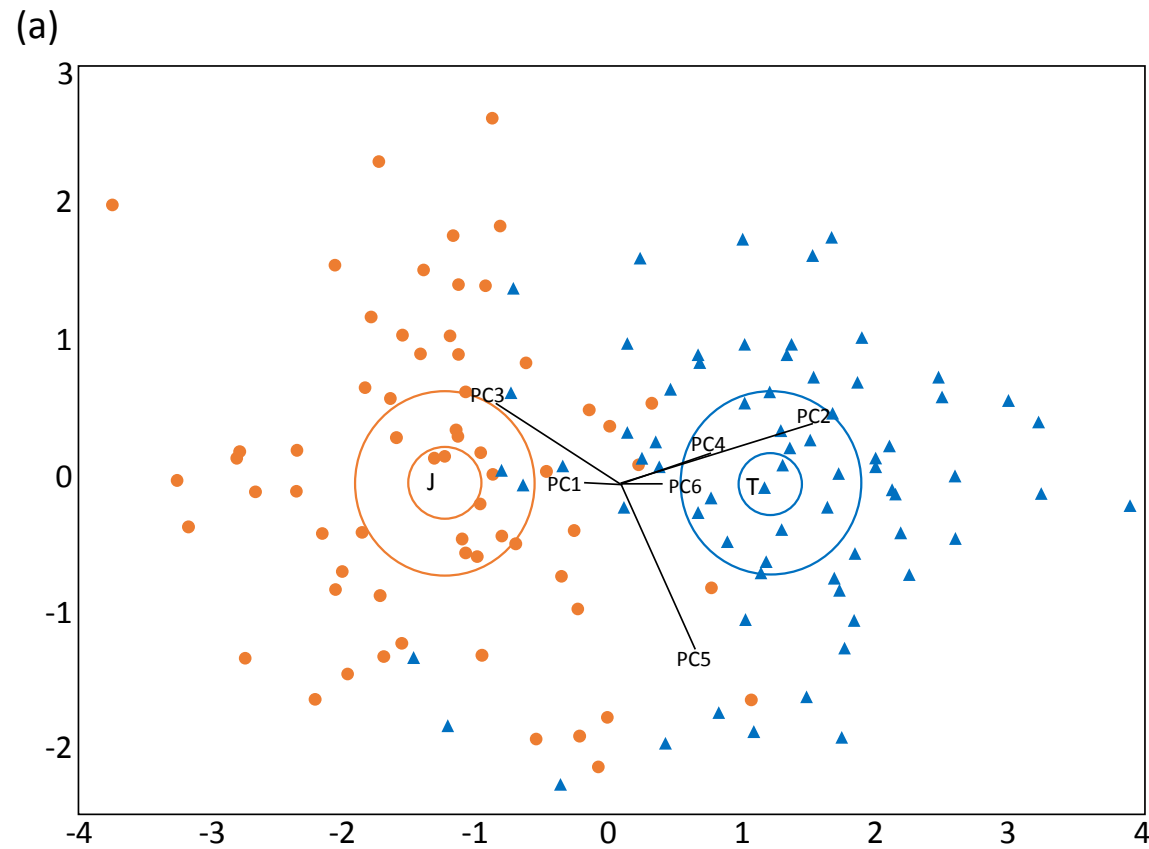


Figure 8. (a) Discriminant analysis of principal component on morphometric traits from *Buergeria japonica* in Ryukyu and Taiwan when defined as two groups. This is also the grouping design which obtained the highest statistic score.

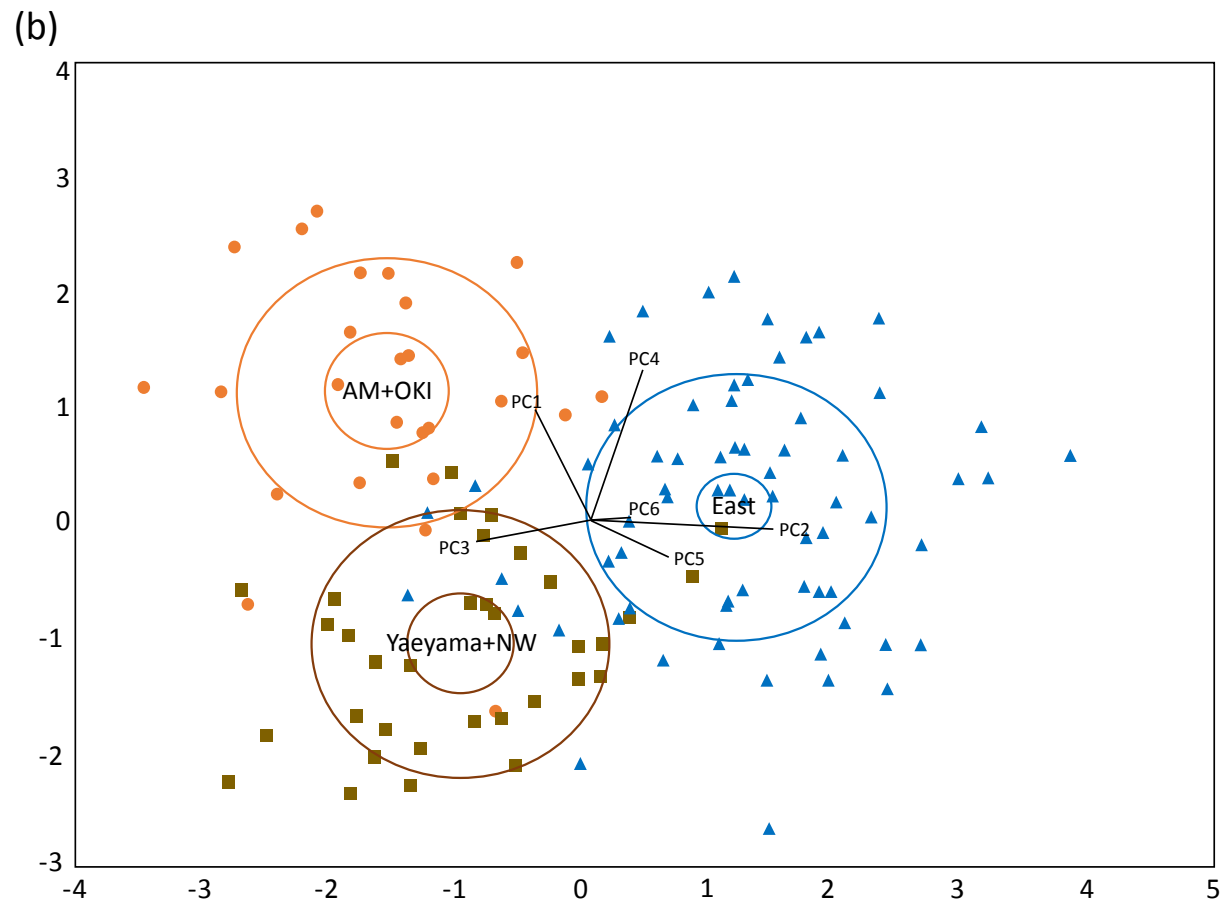


Figure 8 (continued). (b) Discriminant analysis of principal component on morphometric traits from *Buergeria japonica* in Ryukyu and Taiwan when defined as three groups.

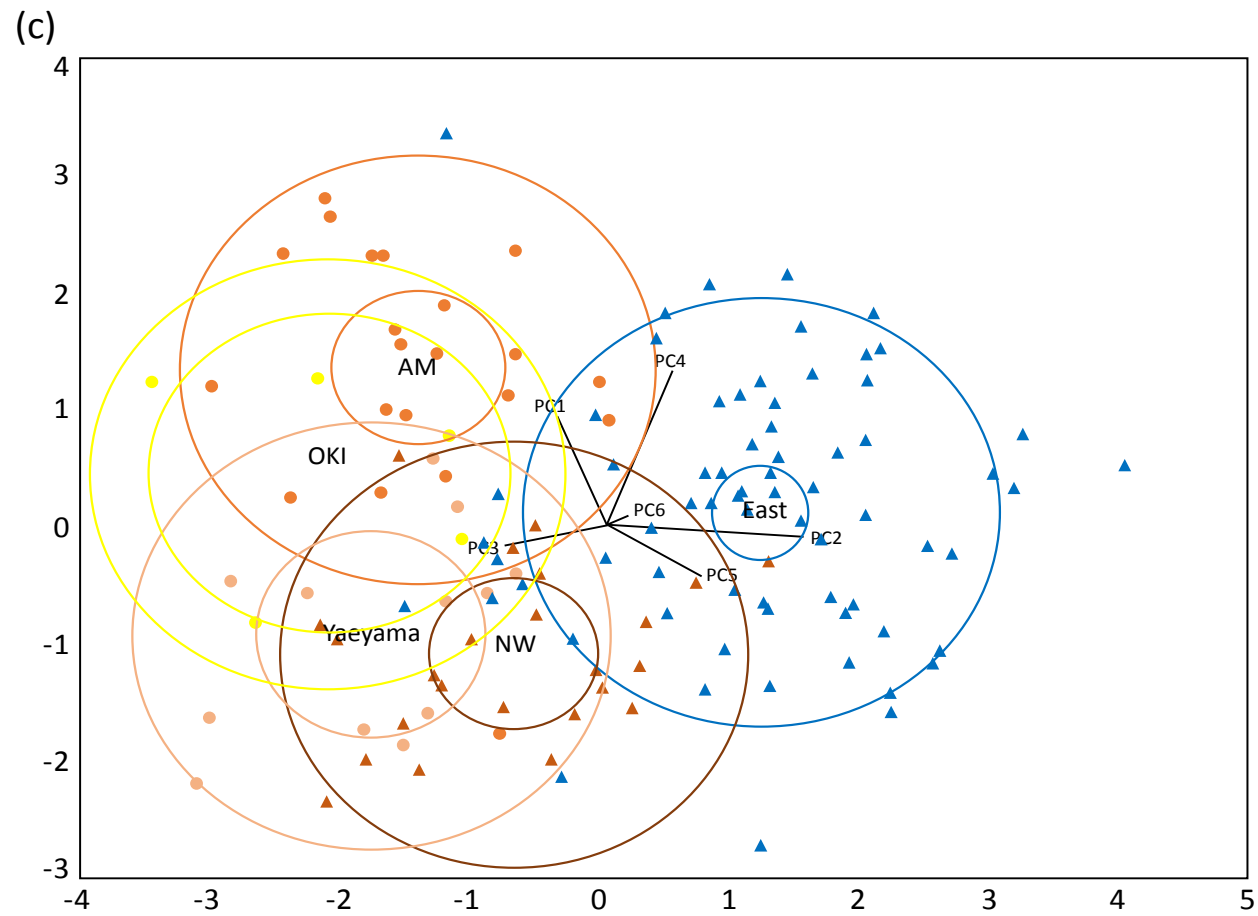


Fig. 8 (continued). (c) Discriminant analysis of principal component on morphometric traits from *Buergeria japonica* in Ryukyu and Taiwan when defined as five groups.

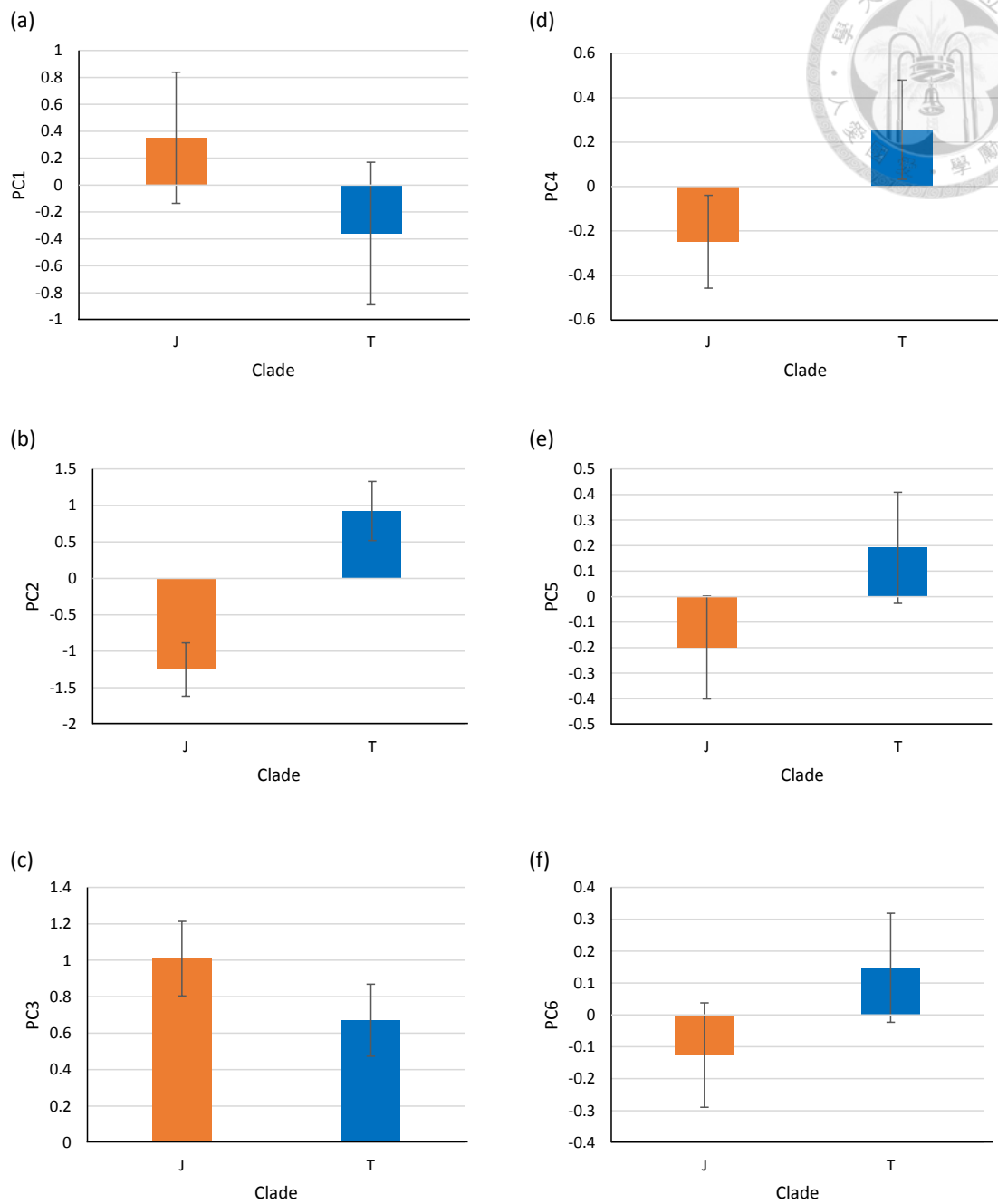


Figure 9. The association between principal component of morphometric traits and genetic/acoustic clades. (a) – (f) denote PC1 – PC6.



Appendix

Appendix 1.

The principle components of ecological factor for generalized linear mixed model of acoustic characteristics of short call. PC1 was chose as ambient factor.

		Temperature	Humidity	Variance explained (%)
ambient	PC1	-0.70711	0.70711	72.70
	PC2	0.70711	0.70711	27.30

Appendix 2.

The list and information of the excluded individuals from discriminant analysis.

ID	Location	aHL	aHW	aEL	aIOD	aIC	aEND	aUEW	aFLL	aFAL	aHAL	a1FL	a3FL	aHLL	aTL	aTTL	aiMTL	a1TL	a3TL
150705_02#0	WL	0.40	0.37	0.18	0.09	0.12	0.09	0.10	0.69	0.24	0.30	0.09	0.18	2.02	0.64	0.82	0.06	0.14	0.43
150705_06#0	WL	0.38	0.37	0.17	0.09	0.15	0.11	0.10	0.70	0.24	0.30	0.10	0.18	1.89	0.61	0.81	0.06	0.15	0.41
150601_01	ML	0.35	0.30	0.12	0.08	0.12	0.09	0.10	0.80	0.29	0.28	0.05	0.17	1.88	0.59	0.77	0.02	0.12	0.46
150911_05	ISG	0.36	0.32	0.13	0.09	0.16	0.11	0.09	0.58	0.09	0.31	0.10	0.19	2.01	0.61	0.85	0.04	0.14	0.41
151027_2-9	XGL	0.38	0.36	0.13	0.10	0.17	0.09	0.09	0.83	0.30	0.31	0.08	0.19	2.05	0.63	0.88	0.03	0.09	0.39

Appendix 3

The principal components of morphological traits included SVL and 18 standardized measurements for discriminant analysis and generalized linear mixed model.

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	5.55	3.21	1.52	1.27	1.06	1.01
% variance	29.23	16.91	7.99	6.68	5.55	5.31
SVL	-0.169	-0.143	0.158	0.419	0.133	-0.001
aHL	0.179	0.406	0.180	-0.045	-0.058	0.015
aHW	0.210	0.377	0.083	0.111	0.001	-0.134
aEL	0.120	0.252	0.182	-0.368	-0.263	-0.097
aIOD	0.065	0.342	-0.101	0.219	0.377	0.136
aIC	0.087	0.376	-0.032	0.371	0.113	0.084
aEND	0.167	0.271	0.216	0.291	-0.144	0.152
aUEW	0.136	0.162	0.006	-0.405	-0.088	0.498
aFLL	0.327	-0.014	-0.400	-0.085	0.195	-0.040
aFAL	0.200	0.070	-0.500	-0.226	0.373	-0.103
aHAL	0.325	-0.084	-0.129	0.187	-0.112	0.009
a1FL	0.134	-0.221	0.208	0.007	0.455	0.038
a3FL	0.271	-0.188	-0.116	0.294	-0.099	0.085
aHLL	0.360	-0.124	0.088	-0.017	-0.082	-0.118
aTL	0.357	-0.103	0.120	-0.056	-0.077	-0.154
aTTL	0.361	-0.187	0.090	0.058	-0.106	-0.018
aiMTL	0.079	0.048	0.455	-0.171	0.394	-0.511
a1TL	0.087	-0.148	0.332	-0.137	0.351	0.590
a3TL	0.277	-0.262	0.115	0.078	-0.132	0.084