Ultrasonic songs and stridulum anatomy of *Asiophlugis* crystal predatory katydids (Tettigonioidea: Meconematinae: Phlugidini)

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Abstract

The behavioural ecology of ultrasonic-singing katydids is not well understood, and the general bioacoustics, barely known for a few Neotropical Meconematinae, tends to be overlooked for species from Southeast Asia. These include Asiatic species of Phlugidini, commonly known as crystal predatory katydids. One of its genera, *Asiophlugis* consists of 16 species for which acoustic signals and stridulum anatomy are broadly unknown. These characters can be used to understand species boundaries. Here, we sampled *Asiophlugis* from five sites in Malay Peninsula and Borneo Island, recorded the acoustic signals of five species plus one subspecies using ultrasound sensitive equipment, and examined their stridulum anatomy. The calling songs of the taxa involved were documented for the first time. We found that the stridulum anatomy (e.g., tooth distributions, tooth length and tooth density) is distinct between species, but less so between subspecies. In contrary, songs of different taxa are different based on acoustic parameters (e.g., pulse duration, peak frequency) and descriptive patterns, even between the subspecies. We also did not observe that song signals are more different in sympatry than in allopatry. Whether this can be generalised requires further sampling, highlighting the need for more research on the ultrasonic acoustic communication in Asiatic katydids.

Key words: bioacoustics, carrier frequency, Southeast Asia, stridulatory file, taxonomy, predator.

Introduction

In katydids (Insecta: Orthoptera: Tettigonioidea), ~70% of the species investigated so far communicate using calls with ultrasonic components, and these extend to the extreme ultrasonic frequency of 150 kHz (Montealegre-Z et al. 2006, 2017). Sound production in katydids is achieved by tegmino-tegminal stridulation, where a scraper in the right wing or tegmina is swept over a serrated vein (the stridulatory file) on the left forewing (Montealegre-Z 2009). Since males produce sound to attract conspecific females, calling song parameters can be used to understand species boundaries. The use of acoustics for examining species boundaries is more prominent for species from the Neotropics than that of relatives from the similarly diverse Southeast Asia. This is probably an artefact of the limited sampling and a lack of acoustic experts working in this region, leading to many species which are known only from their original description (Tan et al. 2017). Considering that many katydid clades are more congruent with biogeography (Mugleston et al. 2018), lacking knowledge on the acoustics of Southeast Asian species impedes our understanding on the evolution (and biomechanics) of acoustic communication.

One group of poorly known katydids from Southeast Asia belongs to the Phlugidini (Meconematinae). These crystal predatory katydids, which comprise of about 100 species globally (Cigliano et al. 2019), are characterised by large protruding eyes and movable spines on anterior legs. They have diversified more in South America, with as many as 59 species known to science (Cigliano et al. 2019). Except for the acoustic behaviour of two species of *Phlugis* (see Chamorro-Rengifo et al. 2014; Chamorro-Rengifo and Braun 2016; Sarria-S et al. 2017), very little is known about the acoustics and biomechanics of sound production in this group.

This is also the case for their relatives from Southeast Asia, which include species from the genus *Asiophlugis* Gorochov, 1998 (Fig. 1). *Asiophlugis* currently consists of 16 described species (Cigliano et al. 2019) but calling song was known for only one species (Helfert and Sänger 1998). Species are delimited using morphological characters on the abdominal apex, pronotum and tegmina length (Kevan and Jin 1993; Gorochov 1998, 2012, 2013, 2019; Gorochov and Tan 2011; Tan 2011). Unlike relatives from the Neotropics (see Nickle 2003, 2005; Chamorro-Rengifo and Olivier 2017), their stridulatory apparatus are rarely examined and used in taxonomy (but see Helfert and Sänger 1998; Gorochov and Tan 2011).

To address the lack of information on the bioacoustics of *Asiophlugis*, we collected specimens from the Malay Peninsula (which includes Peninsular Malaysia and Singapore) and Borneo (i.e., Brunei Darussalam and Sabah), recorded the songs, analysed and described the acoustic signatures, and examined and compared their stridulum anatomy. Then, we attempt to answer the following research question: Can the call structure and stridulum anatomy assist with inferring species identity? We predict that the acoustic parameters in the domains of frequency (e.g., peak frequency) and time (e.g., pulse repetition rates) and/or organisation of stridulatory file (i.e., inter-tooth distance, tooth length and tooth density) can reflect the species and subspecies delimited by morphology since males should produce species-specific acoustic signals and that stridulum anatomy is responsible for sound production. Alternatively, songs are also sexual signals, which tend to be more different in sympatry than in allopatry (Fitzpatrick and Gray 2001; Izzo and Gray 2004). If this is true, we can expect syntopic species from Singapore to have very different songs than allopatric species from Peninsular Malaysia, Brunei Darussalam and Sabah.

Materials and Methods

Collection and Husbandry of Katydids

Surveys were conducted by MKT in the lowland forests of (1) Singapore; (2) Pulau Tioman, Pahang, Peninsular Malaysia; (3) Ulu Gombak Field Studies Centre, Selangor, Peninsular Malaysia; (4) Andulau Forest Reserve, Belait, Brunei Darussalam; and (5) Rainforest Discovery Centre, Sandakan, Sabah, East Malaysia (Fig. 2) between July 2018 and March 2019. Opportunistic collection was conducted at night time (1930 to 2300 hours). The katydids were then housed in standardised insect cages (25 cm in diameter and 33 cm tall) with nylon cover. They were fed with living fruit flies and regularly sprayed to avoid dehydration. Fresh plants, typically *Dillenia suffruticosa*, were also provided. The katydids typically survive in captivity between two to four weeks.

Acoustic Recordings and Analysis

All recordings were obtained in laboratory conditions or in biological stations. Calling songs of males in the cages were recorded using a portable ultrasound recorder Echo Meter Touch (Wildlife Acoustics, Inc. USA, Massachusetts), attached to an iPhone, and using a sampling frequency of 256 kHz-samples/s. The Echo Meter Touch 1 (based on Knowles FG sensor) was placed at about 1 m horizontally away from the cage. We observed that the katydid usually (but not always) sing while clinging onto the side of the cage (thus positioning with dorsal surface facing the recording device). Triggered recording was used with the Trigger Minimum Frequency set at 20 kHz. However, this is only a trigger and will not affect lower frequencies to be recorded. As temperature can influence frequency of calls, a temperature-humidity meter (Smartsensor AR867) was used to record the ambient temperature (°C) and relative humidity (%) at the time of recording. All recordings were done at an ambient temperature between 27.0 °C to 29.8 °C.

The recorded signals were saved in 12-bit WAV format. All acoustic analyses were done using the open source R package WarbleR version 1.1.14 (Araya-Salas and Wright 2017) in the R software version 3.5.1 (R Development Core Team 2018). To filter out low or high background noise before performing measurements, the lower and upper limits of a frequency bandpass filter (in kHz) were set at 20 kHz and 120 kHz respectively so measurements of the peak frequency and mean frequency were obtained only between the frequency range. The limits are beyond the frequency range of the *Asiophlugis* songs and will not affect the measurements of the frequencies. The peak frequency (frequency with highest energy from the mean spectrum, in kHz) and the mean frequency (weighted average of frequency by amplitude) were quantified using the function ‘specan’. Power spectra were generated using custom-designed Matlab code in Matlab (Version 9.5.0.1049112, R2018b; The MathWorks, Inc., Natick, MA, USA). The following parameters associated with the time domain of the songs were also measured: (1) pulse duration, which refers to duration of the rapid-decay pulse, (2) pulse repetition rate (number of discrete pulses per second), and (3) down time, which refers to the silent interval between calls and/or pulses. All sound files were uploaded to the Orthoptera Species File Online Version 5.0/5.0 (Cigliano et al. 2019).

Specimen Identification and Curation

Identification was done using descriptions and keys: Hebard (1922); Gorochov (1998, 2013, 2019); Gorochov and Tan (2011); Tan (2011). The specimens were first preserved in absolute analytic-grade ethanol and later pinned and dry-preserved (after dissection of the tegmen). A single hind leg was preserved in absolute analytic-grade ethanol for future molecular work.

Material Examined

Asiophlugis longiuncus *Gorochov, 2013*

Material with acoustic data (n = 3). 1 male (BRU.19.17), Brunei Darussalam, Belait, Andulau Forest Reserve, mixed dipterocarp forest, N4.62488, E114.51228, 76.0±6.6 m, 25 February 2019, coll. M.K. Tan and H. Yeo; 2 males (BRU.19.29, 30), same locality, N4.62502, E114.51218, 90.1±8.0 m, 26 February 2019, coll. M.K. Tan and H. Yeo.

Additional material examined (n = 3). 1 male (BRU.19.36), same locality, N4.63355, E114.51096, 75.6±6.6 m, 27 February 2019, coll. M.K. Tan and H. Yeo; 2 males (BRU.19.49, 50), same locality, N4.28437, E114.41873, 39.1±6.5 m, 1 March 2019, coll. M.K. Tan and H. Yeo.

Asiophlugis rete *Gorochov, 1998*

Material with acoustic data (n = 1). 1 male, Singapore, Dairy Farm Nature Park, along Wallace Trail, abandoned plantation, 17 August 2018, coll. M.K. Tan and H. Yeo.

Additional material examined (n = 1). 1 male (UG.17.14), Peninsular Malaysia, Selangor, Ulu Gombak Field Studies Centre, secondary lowland forest, N3.32344, E101.74861, 267.3±6.5m, 24 February 2017, M.K. Tan, D.M Belabut, Sofwan Badr.

Asiophlugis temasek temasek *Gorochov and Tan, 2011*

Material with acoustic data (n = 3). 1 male, Singapore, Rifle Range Link, secondary lowland forest, 13 July 2018, coll. M.K. Tan; 1 male, Singapore, Dairy Farm Nature Park, along Wallace Trail, 16 November 2018, coll. M.K. Tan and H. Yeo; 1 male, Singapore, Upper Seletar Trail, secondary lowland forest, 16 January 2019, coll. M.K. Tan, H. Yeo and X.B. Jin.

Additional material examined (n = 4). 1 male, Singapore, Rifle Range Link, secondary lowland forest, 13 July 2018, coll. M.K. Tan; 1 male, Singapore, Dairy Farm Nature Park, near Dairy Farm Quarry, 16 September 2018, coll. M.K. Tan; 1 male, Singapore, Dairy Farm Nature Park, near Dairy Farm Quarry, 6 November 2018, coll. M.K. Tan and H. Yeo; 1 male, Singapore, Dairy Farm Nature Park, near Dairy Farm Quarry, 16 September 2018, coll. M.K. Tan

Asiophlugis temasek tioman *Gorochov, 2011*

Material with acoustic data (n = 1). 1 male, Peninsular Malaysia, Pahang, Pulau Tioman, Paya Village, N2.785, E104.1236, coastal secondary forest, 8 August 2018, coll. M.K. Tan and H. Yeo.

Additional material examined (n = 3). 3 males (PT.17.01, 06, 14), same locality, coastal secondary forest, N2.78524, E104.12331, 30.3±6.2 m, N2.78481, E104.12357, 26.4±6.0 m and N2.78463, E104.12400, 28.2±6.1 m, 5 April 2017, coll. M.K. Tan and S.T. Toh

Asiophlugis thaumasia *(Hebard, 1922)*

Material with acoustic data (n = 2). 2 males, Singapore, Dairy Farm Nature Park, near Car Park A, abandoned plantation, 9 December 2018 and 31 January 2019, coll. M.K. Tan.

Additional material examined (n = 2). 2 males, Singapore, Dairy Farm Nature Park, near Car Park A, abandoned plantation, 21 August 2018, coll. M.K. Tan.

Asiophlugis trusmadi *Gorochov, 2011*

Material with acoustic data (n = 2). 2 males (SDK.19.64, 65), East Malaysia, Sabah, Sandakan, Rainforest Discovery Centre, secondary/ primary lowland forest, N5.87469, E117.94057, 53.9±6.9 m and N5.87529, E117.94171, 50.7±5.7 m, 12 January 2019, coll. M.K. Tan and S.T. Toh.

Measurement of Morphological Traits

The left tegmen was removed using micro-scissors for imaging and measurements of the tooth distribution. Photomicrograph of the stridulatory apparatus was done using a Canon EOS 50D digital SLR camera with an Olympus OM system extension tube and an Olympus NFK 6.7 × LD (125) lens attached to Olympus BH2 research compound light microscope. Canon Macro Ring Lite MR-14EX were used for lighting and flash. Image-editing and measurements were accomplished using Adobe Photoshop CC 2014 (Adobe Systems Incorporated, San Jose, CA, USA) and/or ImageJ 1.51j8 (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) respectively:

- The **number of teeth** on the file was counted.
- The **inter-tooth distance** (mm) was measured from the edge of the cusp of one tooth to the cusp of the next one (Montealegre-Z and Mason, 2005), from the anal end to the last tooth at the basal end of the left stridulatory file.
- The **tooth length** (mm) as an indication of tooth size was also measured from the anterior to the posterior ends of the cusp of each tooth (Fig. 8).
- The **tooth density** was calculated by dividing the summation of inter-tooth distance by the number of teeth.

Data Analysis

To describe the songs for each *Asiophlugis*, we obtained the arithmetic means and standard deviations of pulse duration, pulse repetition rate, down time, mean peak frequency and mean frequency. To compare these acoustics parameters, we fitted the linear mixed effects models

(LMMs) for pulse duration, pulse repetition rate, down time and mean peak and peak frequencies using the ‘lmer’ function from the R package ‘lme4’ (Bates et al. 2014). The frequency and time parameters were natural log-transformed. In each model, we used species as a fixed effect and individual katydid as a random intercept since more than one recording were obtained for each individual. Temperature was also used as a random slope since it is known to affect the acoustic parameters. Estimated marginal means (or least-squares means) were computed as the sampling was imbalanced. These were done using the ‘emmeans’ function from the R package ‘emmeans’ (Lenth 2018). To compare the stridulum anatomy of *Asiophlugis*, we compared the inter-tooth distance and tooth length between the different *Asiophlugis* by fitting trend lines using generalised additive models to visualise the tooth distributions on the stridulatory file based on inter-tooth distances and tooth length variation for each species. We also compared the tooth density between taxa using Kruskal-Wallis rank sum test.

Results

Part I: Calling Songs

We analysed the songs of *Asiophlugis* from a total of 163 sound files: *A. longiuncus* (n = 42), *A. rete* (n = 2), *A. temasek temasek* (n = 24), *A. temasek tioman* (n = 24), *A. thaumasia* (n = 42) and *A. trusmadi* (n = 29). The songs for the six *Asiophlugis* are similar in that they consist of rapid-decay pulses with broad-based spectrum (Fig. 3). Such rapid-decay pulse consists of a train of complex waves showing rapid decay after only one or a few cycles at maximum amplitude. The songs are also completely inaudible to the human ear (Fig. 5). We excluded

A. rete for the statistical modelling of the calling songs since there were only two recordings, much fewer than other species.

A. longiuncus song is made up of a rapid-decay pulse, within which impulses typically paired in time (Fig. 3a). Each pulse consisting of 2 rapid-decay impulses is about 16.7±2.8 ms in duration (Fig. 4a). The second impulse is of much lower amplitude than the first one (Figs. 3a, 4a). The average pulse repetition rate is 5.1±1.5 s^{-1}. The average down time between consecutive pulses is 175.5±84.9 ms. The mean peak frequency is 55.5±1.4 kHz and mean frequency is 58.1±1.3 kHz (Fig. 5a).

A. rete song consists of a rapid-decay pulse which repeats over time (Fig. 3b). Each pulse is 2.7–4.4 ms in duration (Fig. 4b). The average pulse repetition rate is 4–9 s^{-1}. The average down time between consecutive pulses is 22.8 ms. The mean peak frequency is 33.3–35.3 kHz and mean frequency is 47.9–52.0 kHz. (Fig 5b)

A. temasek temasek song similar to *A. rete* song. It is made up of a rapid-decay pulse and repeats in a series (Fig. 3c). Each pulse is about 7.7±3.5 ms in duration (Fig. 4c). The song has two modes, even within the same individual (Figs. 6a, b). The first mode has a high average pulse repetition rate of 52.2±28.1 s^{-1}, and low average down time between pulses of 13.5±8.0 ms. The second mode has a low average pulse repetition rate of 12.3±4.0 s^{-1}, and high average down time between pulses of 67.6±22.7 ms. The mean peak frequency is 57.8±2.9 kHz and mean frequency is 57.2±1.2 kHz (Fig. 5c).

A. temasek tioman song is also made up of a rapid-decay pulse and repeats in a series (Fig. 3d). We only observed one mode for this subspecies (Figs. 6c, d), unlike subspecies *temasek*.

Each pulse is about 5.0±0.9 ms in duration (Fig. 4d), shorter than that of subspecies *temasek*. The average pulse repetition rate is 43.1±21.6 s^{-1}. The average down time between consecutive pulses is 9.3±5.1 ms. The mean peak frequency is 46.2±6.2 kHz and mean frequency is 50.9±2.1 kHz, lower than that of subspecies *temasek* (Fig 5d).

A. thaumasia song is quite distinct from that of the above taxa in that it consists of a series of 4 rapid-decay pulses of very similar amplitude (Fig. 3e). The duration of each series is 166.9±49.5 ms and consecutive series are separated by a longer and highly variable down time of average 744.8±378.4 ms. Each pulse is about 30.0±4.3 ms in duration (Fig. 4e), distinctively higher than the other taxa. The average pulse repetition rate is 4.3±1.1 s^{-1}. The average down time between consecutives rapid-delay pulses within a series is about 10.9±3.2 ms. The mean peak frequency is 44.8±2.1 kHz and mean frequency is 48.3±0.9 kHz (Fig. 5e).

A. trusmadi song, similar to *A. longiuncus*, is made up of a rapid-decay pulse, within which impulses typically occurs in triplet in time (Fig. 3f). Each pulse consisting of 3 rapid-decay impulses is about 18.5±3.0 ms in duration (Fig. 4f). The average pulse repetition rate is 1.5±0.5 s^{-1}. The average down time between consecutive pulses is 687.5±303.2 ms. The mean peak frequency is 59.5±5.2 kHz and mean frequency is 61.7±0.7 kHz (Fig. 5f).

The time domain of the songs was not different between the two subspecies of *A. temasek* and between *A. longiuncus*, *A. thaumasia* and *A. trusmadi*. The pulse duration of *A. longiuncus, A. thaumasia* and *A. trusmadi* were longer than that of *A. temasek* (Fig. 7a, Table 1). Pulse repetition rate was highly variable for *A. temasek*, which is drastically different from the other species, and greater than the other species (Fig. 7b, Table 1). This can be attributed to the presence of two song modes for *A. temasek temasek*. Pulse repetition rate also did not appear to be different between *A. longiuncus*, *A. thaumasia* and *A. trusmadi* (Fig. 7b, Table 1). We observed similar patterns for down time since longer down time is associated with higher pulse repetition rate (Fig. 7c, Table 1). Down time for *A. longiuncus* and *A. thaumasia* was also highly variable compared to the other taxa (Fig. 7c, Table 1), which can be explained by the irregular calling behaviour in these two species.

The frequency domain showed different patterns with the time domain. The frequency domain between the two subspecies of *A. temasek* were drastically different (Fig. 7d, Table 1). Yet, there was no evidence of such difference between *A. temasek temasek* and *A. longiuncus* and *A. trusmadi*; and there was no evidence of such difference observed between *A. temasek tioman* and *A. thaumasia* (Fig. 7d, Table 1). Both peak and mean frequencies exhibited similar patterns between the *Asiophlugis* (Fig. 7e, Table 1).

Syntopic species from Singapore had different song signatures but there was weak evidence that song signatures of sympatric species are more different than allopatric species. The time and frequency domains of the Bornean species (i.e., *A. logniuncus* and *A. trusmadi*) were very similar (Fig. 7) although they should exhibit greater differences than with allopatric species from the Malay Peninsula. Moreover, frequency domain of *A. temasek temasek* was just as different as that of sympatric and allopatric species from Malay Peninsula (Figs. 7d, 7e) and the time domain of *A. temasek temasek* was more different in sympatric species than allopatric *A. temasek tioman* (Figs. 7a–c).

Part II: Stridulum Anatomy

Stridulatory files on the left tegmen are visibly different between the six *Asiophlugis* species (Fig. 8). The stridulatory files are generally straight and slender, although that of *A. longiuncus* (Fig. 8a) and *A. trusmadi* (Fig. 8f) are slightly sigmoidal.

Tooth distributions based on inter-tooth distances (Fig. 9) and tooth lengths (Fig. 10) between subspecies of *A. temasek* are similar. The inter-tooth distances of *A. temasek temasek* and *A. temasek tioman* are fairly even throughout the file, in contrast to other species in which the inter-tooth distance tends to increase to a maximum before decreasing drastically (Fig. 9). Likewise, *A. temasek temasek* and *A. temasek tioman* have evenly-sized teeth, except at both ends of the files (Fig. 10). This was also observed in *A. rete*, whereas *A. longiuncus*, *A. thaumasia* and *A. trusmadi* have a few very large teeth in the middle and much smaller teeth at both ends of the files (Fig. 10). Tooth density was significantly different between all species (χ = 13.3, p-value = 0.021), but the differences are slightly smaller between the two subspecies of *A. temasek* (Fig. 11).

Discussion

Songs of Asiophlugis

We recorded and analysed the calling songs of six taxa of *Asiophlugis* (among 16 known species), where previously the song of only one species from the Asiatic genera of Phlugidini was known (see Helfert and Sänger 1998). The calling frequencies used by all species recorded here is ultrasonic (>40 kHz) and have broad distribution of carrier frequencies (as shown by the spectrograms). We also examined whether ultrasonic songs and stridulum anatomy of *Asiophlugis* and Phlugidini are useful evidence for species delimitation, which has been challenging so far.

The songs of Phlugidini are similar in that they are entirely ultrasonic, supporting the fact that Meconematinae species usually employ ultrasonic calls (Helfert and Sanger 1998; Montealegre-Z et al. 2006, 2017; Chamorro-Rengifo et al. 2014; Chamorro-Rengifo and Braun 2016; Sarria-S et al. 2017). *A. rete* (*A. thai* junior synonym) sings at frequencies between 30 and 50 kHz (Helfert and Sanger 1998) and neotropical species such as *Phlugis ocraceovittata* Piza, 1960 also sings at frequencies between 40 and 60 kHz (Chamorro-Rengifo and Braun 2016). The call of species of the tribes Phlugidini and Phisidini (e.g., *Phugis* spp, *Arachnoscelis*, *Arachnoides*, *Supersonus* spp.) incorporates a sequence of short impulses or very discrete pulses. These impulses likely result from scraper pauses along the file teeth, its deformation, storage of elastic energy, and slipping free and forward drive over a few file teeth at higher rate that likely exceeds the normal scraper velocity being driven by muscles in a sustained closing wing phase. The elastic energy that was stored then released as kinetic energy leads to increase in scraper velocity, thus enhancing extremely fast tooth strikes, which end in ultrasonic frequency generated beyond what the wing muscle alone can generate (Montealegre-Z et al. 2006). Different from other singing Ensifera species, like crickets (Koch et al 1988) or other katydids (Montealegre-Z and Mason 2005; Montealegre-Z and Postles 2010) where a systematic distribution of teeth is necessary for maintaining a constant tooth strike rate and a coherent tonal pulse, such systematic organisation is not required in the extreme ultrasonic Phlugidini. Instead, a few large and separated teeth seem to be appropriate for scraper distortion and recoiling at high speeds.

Can stridulum anatomy assist with inferring species identity?

Since the tooth distributions are similar between the two subspecies of *A. temasek*, but different between species of *Asiophlugis* (especially for inter-tooth distance), the morphology of stridulatory files seems to be useful characters in differentiating species and subspecies. Prior to this study, the stridulatory files of only *A. rete* (as *A. thai* junior synonym) and *A. temasek temasek* were described and illustrated using scanning electron microscopy and hand-drawing, respectively (Helfert and Sanger, 1998; Gorochov and Tan 2011). However, they were never used for species identification. Here, we support previous work on other katydids (e.g., Naskrecki and Rentz 2010; Sarria-S et al. 2014; Heller and Hemp 2018) that propose that the morphology of the stridulatory file is congruent with general morphology in delimiting species in Phlugidini.

Can the call structure assist with inferring species identity?

Overall, both the descriptive comparison and statistical analysis of the song parameters indicate that song signatures of the six *Asiophlugis* are all different, even though the time and frequency domains do not necessarily differ between the taxa in a similar manner. Furthermore, songs of *A. longiunus* and *A. trusmadi* differ by the paired and tripled rapid-decay impulses respectively, although they are not different in the other acoustic parameters, including the frequency domain and pulse duration and repetition rate. The songs between the two subspecies of *A. temasek* can also be considered as different as congeneric differences, as corroborated by the differences in the frequency domain and the presence of two modes in only the subspecies *temasek.* These underscore that one acoustic parameter cannot give an accurate and reliable indication for species identity and it is crucial to examine as many parameters as possible to avoid biases when interpreting the songs.

The difference in the frequency domain of the *A. temasek* subspecies songs signifies that song signatures do not entirely reflect the greater similarities between subspecies and greater differences between species we initially predicted and have observed for the morphology and stridulum anatomy. The incongruency between song signatures and stridulum anatomy is not unexpected since the differences in stridulum anatomy do not necessarily produce songs of different frequencies. This has been observed in the *Aerotegmina* katydids (e.g., Heller and Hemp 2018). Carrier frequency is mainly dictated by the resonator anatomy (mirror and harp but see; Montealegre-Z and Postles 2010; Montealegre-Z 2012; Montealegre-Z et al. 2017).

Subspecies status of* A. temasek *based on songs and stridulum anatomy

This led us to question whether the two subspecies of *A. temasek* should more rightly be considered as two different species. Although differences in their morphology (see Gorochov and Tan 2011) and stridulum anatomy are probably not as clear-cut as species differences in *Asiophlugis*, one can also argue that the differences in the calling songs may be considered species specific. Our hypothesis is that *A. temasek temasek* and *A. temasek tioman* probably represent two recently diverged sister species. But there is currently a lack of phylogenetic analysis that may inform about their relationships. We also only know that the subspecies *temasek* occurs in Singapore and subspecies *tioman* occurs in Pulau Tioman. The differences in their songs may be an artefact of sympatry but there is currently no way to confirm this and infer species boundaries until sampling in Malay Peninsula between these two islands and congeneric data from other parts of the genus' distribution (e.g., east of Wallace Line) have been done. Thus, to avoid confusion, we tentatively keep the status of the two taxa as status quo until we have more evidence to suggest otherwise.

The song signatures of syntopic species from Singapore (they can be found in the same forest patch of Dairy Farm Nature Park) are also drastically different, even though there was no clear evidence that songs are more different in sympatry than allopatry. Such difference in the songs of *A. temasek temasek*, *A. thaumasia* and *A. rete* can be a crucial pre-mating isolating mechanism to avoid hybridisation between these syntopic species. We have also observed the occurrence of syntopic species within Brunei Darussalam, Pulau Tioman and Sandakan but could not find the males and/or record their songs. This implies that further sampling is needed to validate whether the song differences between syntopic species observed in Singapore can be generalised.

Conclusions

The present study provides the most in-depth investigation into the bioacoustics of Asiatic Phlugidini to date. This is despite our study spanning only across *Asiophlugis* species from Malay Peninsula and Borneo. There are other representatives of the Asiatic Phlugidini, including *Papuaphlugis* Gorochov, 2012 and *Stenophlugis* Gorochov, 2012, but of which we do not know anything about their acoustics. These signify that the study of acoustic communication in Asiatic Phlugidini is still in its early stage. We demonstrate that the morphology and acoustics can provide additional meaningful evidence in species delimitation. As such, we recommend that species description and species delimitation exercises in the future should include acoustic information, since it is expected that more species (see Gorochov 2019) and perhaps genera of Phlugidini to be discovered in the understudied regions of Southeast Asia (Tan et al. 2017). Our study aims to provide a baseline for more research on ultrasound communication in *Asiophlugis*, Phlugidini and other understudied ultrasonic-singing katydids.

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Author Contributions

MKT and FMZ contribute equally to this study.

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Table

Table 1. Pairwise comparison of the song signatures between the five taxa of *Asiophlugis*. The values indicate the ratio of the parameter between two taxa. *A. rete* was excluded from the LMMs since there were only two recordings, much fewer than other species Significance is denoted as follows: * P < 0.05; ** P < 0.01; *** P < 0.001; ^{ns} not significant.

	Borneo	Malay Peninsula			Borneo
	A. longiuncus	*A. t. temasek*	*A. t. tioman*	*A. thaumasia*	*A. trusmadi*
Pulse duration					
A. longiuncus		3.1 ***	4.0 ***	0.6 ***	0.9 ^{ns}
A. t. temasek			1.3 ^{ns}	0.2 ***	0.3 ***
A. t. tioman				0.1 ***	0.2 ***
A. thaumasia					1.6 ^{ns}
Pulse repetition rate					
A. longiuncus		0.1 **	0.1 ***	0.9 ^{ns}	2.7 ^{ns}
A. t. temasek			0.4 ^{ns}	6.9 *	19.4 ***
A. t. tioman				16.2 **	45.4 ***
A. thaumasia					2.8 ^{ns}
Down time					
A. longiuncus		8.32 ***	17.93 ***	0.40 ^{ns}	0.27 ^{ns}
A. t. temasek			2.16 ^{ns}	0.05 ***	0.03 ***
A. t. tioman				0.02 ***	0.01 ***
A. thaumasia					0.66 ^{ns}
Mean peak frequency					
A. longiuncus		1.0 ^{ns}	1.2 ***	1.3 ***	1.0 ^{ns}
A. t. temasek			1.2 ***	1.3 ***	1.0 ^{ns}
A. t. tioman				1.1 ^{ns}	0.8 ***
A. thaumasia					0.8 ***
Mean frequency					
A. longiuncus		1.0 ^{ns}	1.2 ***	1.2 ***	0.9 ^{ns}
A. t. temasek			1.1 ***	1.2 ***	0.9 ^{ns}
A. t. tioman				1.1 ^{ns}	0.8 ***
A. thaumasia					0.8 ***

Figure captions

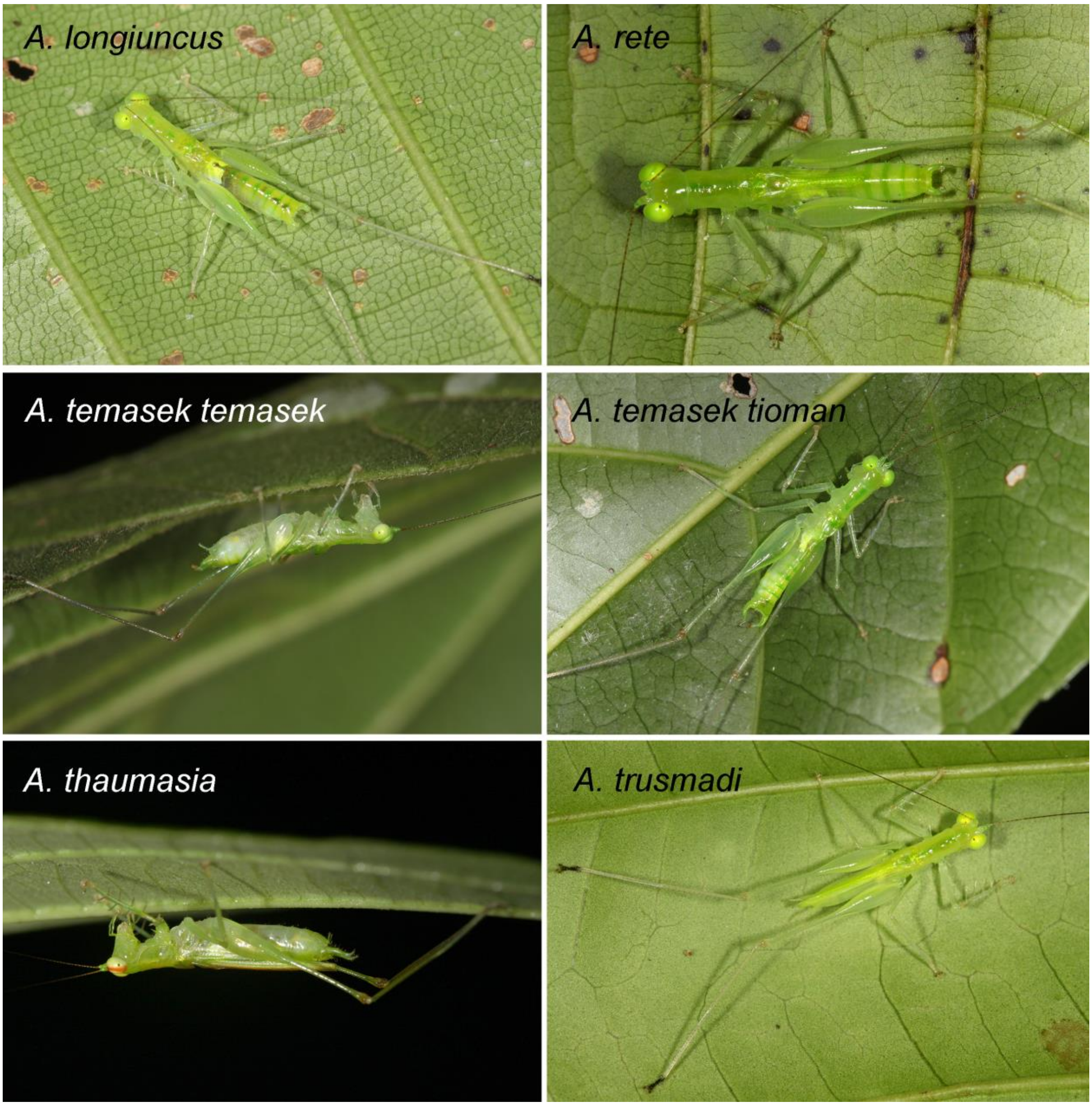

Figure 1. Habitus of the *Asiophlugis* taxa studied.

Figure 2. A map of Malay Peninsula and Borneo indicating the sampling sites for *Asiophlugis*.

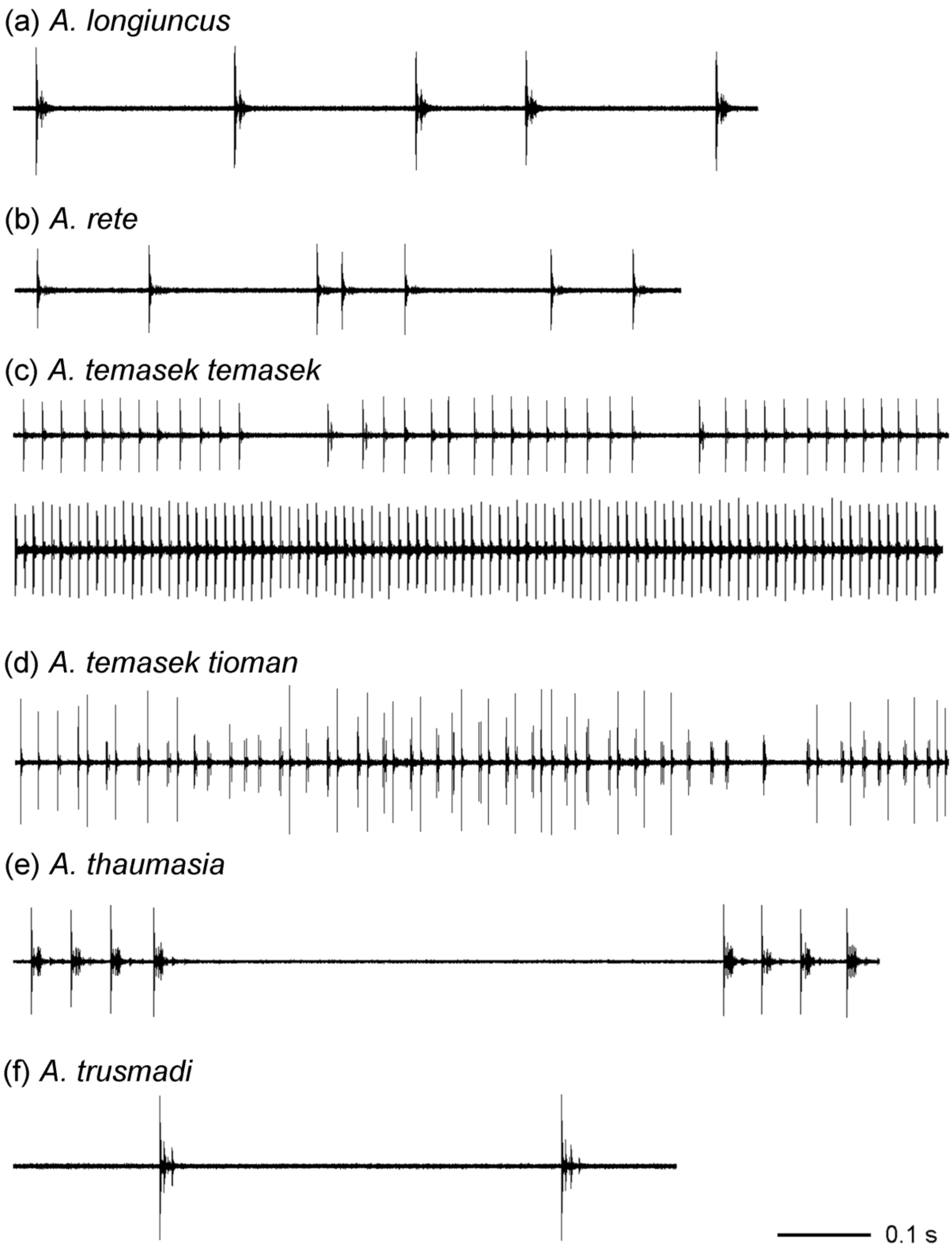

Figure 3. Oscillograms of the *Asiophlugis* songs: *A. longiuncus* (a), *A. rete* (b), *A. temasek temasek* (c), *A. temasek tioman* (d), *A. thaumasia* (e), and *A. trusmadi* (f). Scale bar: 0.1 s.

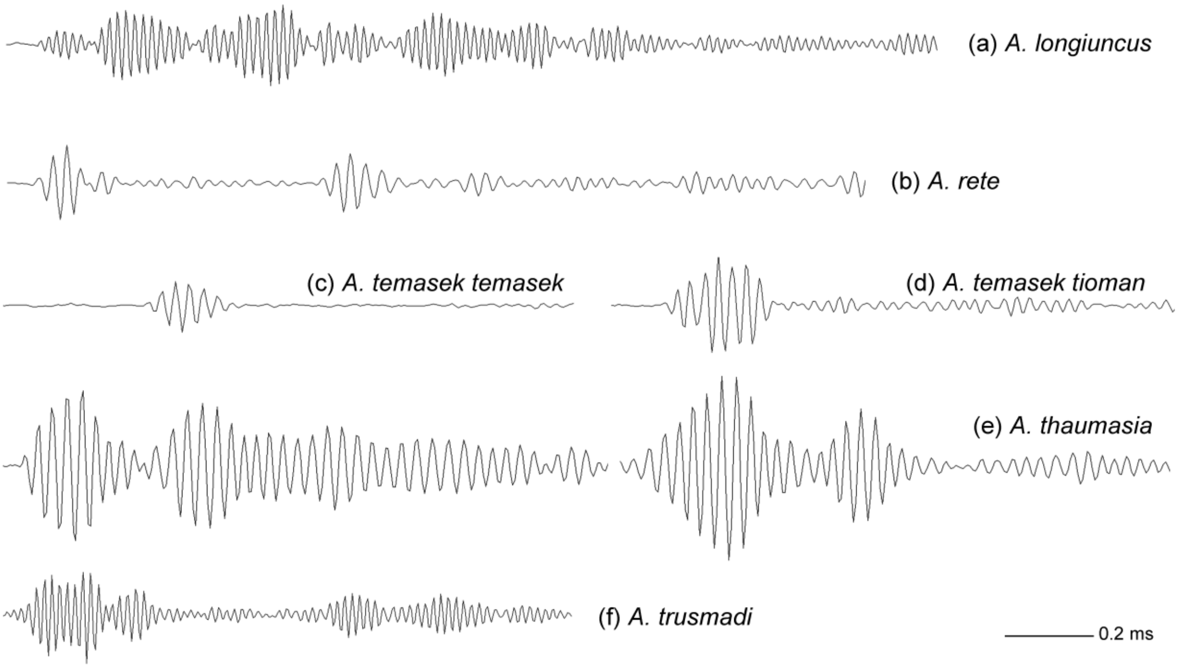

Figure 4. Pulse structure of the *Asiophlugis*: *A. longiuncus* (a), *A. rete* (b), *A. temasek temasek* (c), *A. temasek tioman* (d), *A. thaumasia* (e), and *A. trusmadi* (f). Scale bar: 0.2 ms.

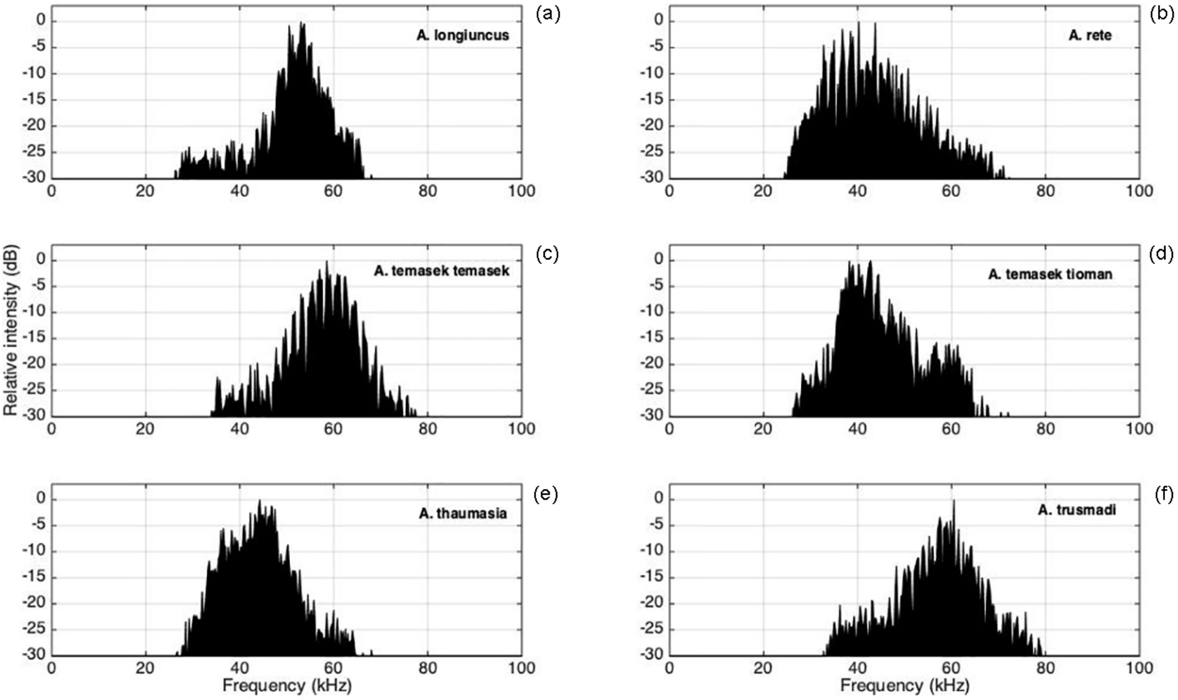

Figure 5. Power spectra of the *Asiophlugis* songs: *A. longiuncus* (a), *A. rete* (b), *A. temasek temasek* (c), *A. temasek tioman* (d), *A. thaumasia* (e), and *A. trusmadi* (f).

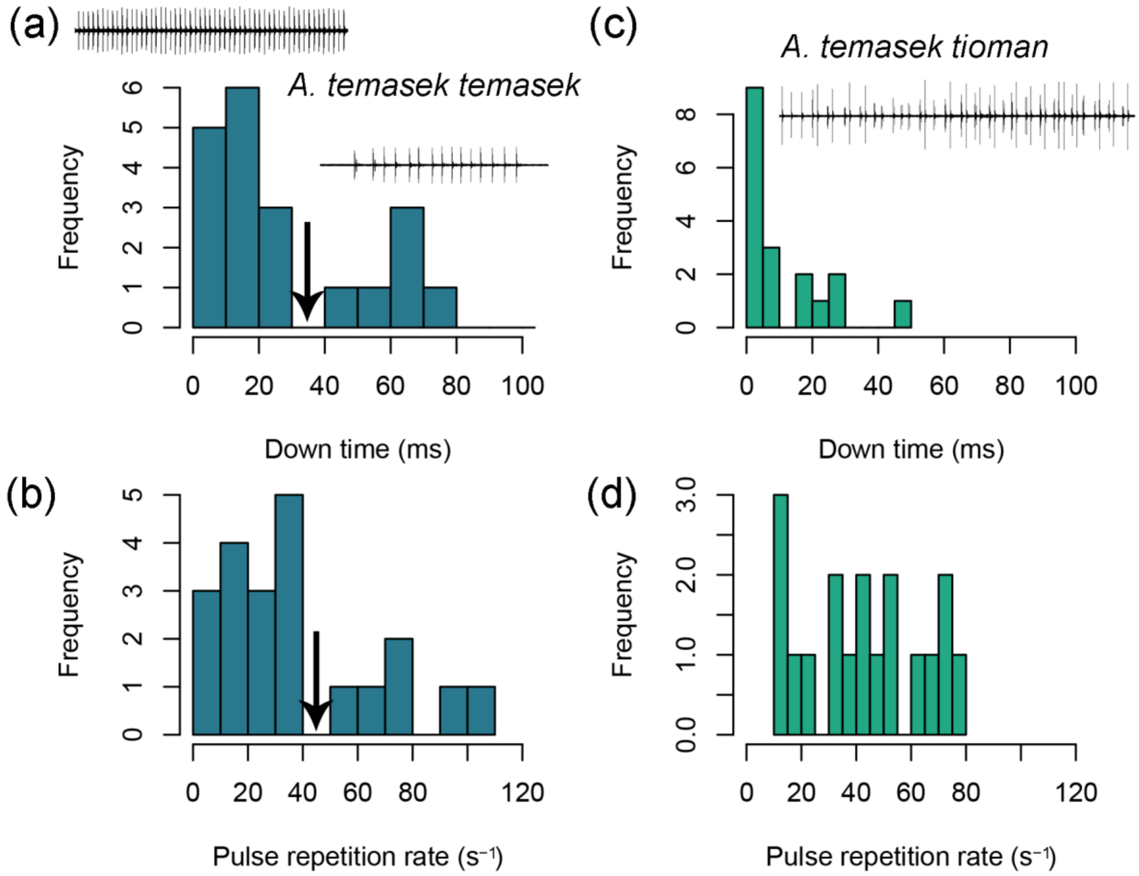

Figure 6. Histograms of down time and pulse repetition rate of *A. temasek temasek* (a, b) and *A. temasek tioman* (c, d), Arrow indicates a gap between the two modes of song.

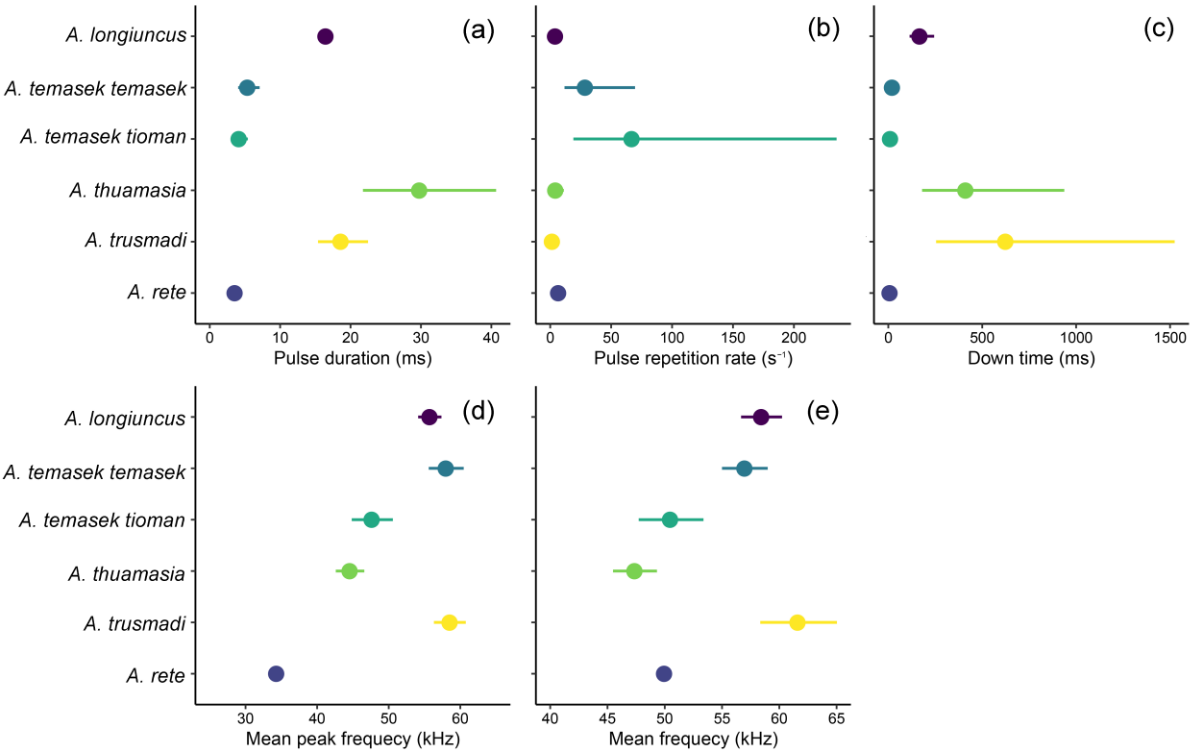

Figure 7. Mean plots on the comparison of the time and frequency domains of the *Asiophlugis* song. The dot represents the least-square means and the horizontal line represents the 95% confidence interval (CI). The mean of each parameter was included for *A. rete* but the 95% CIs were not included since it was excluded from the statistical analysis.

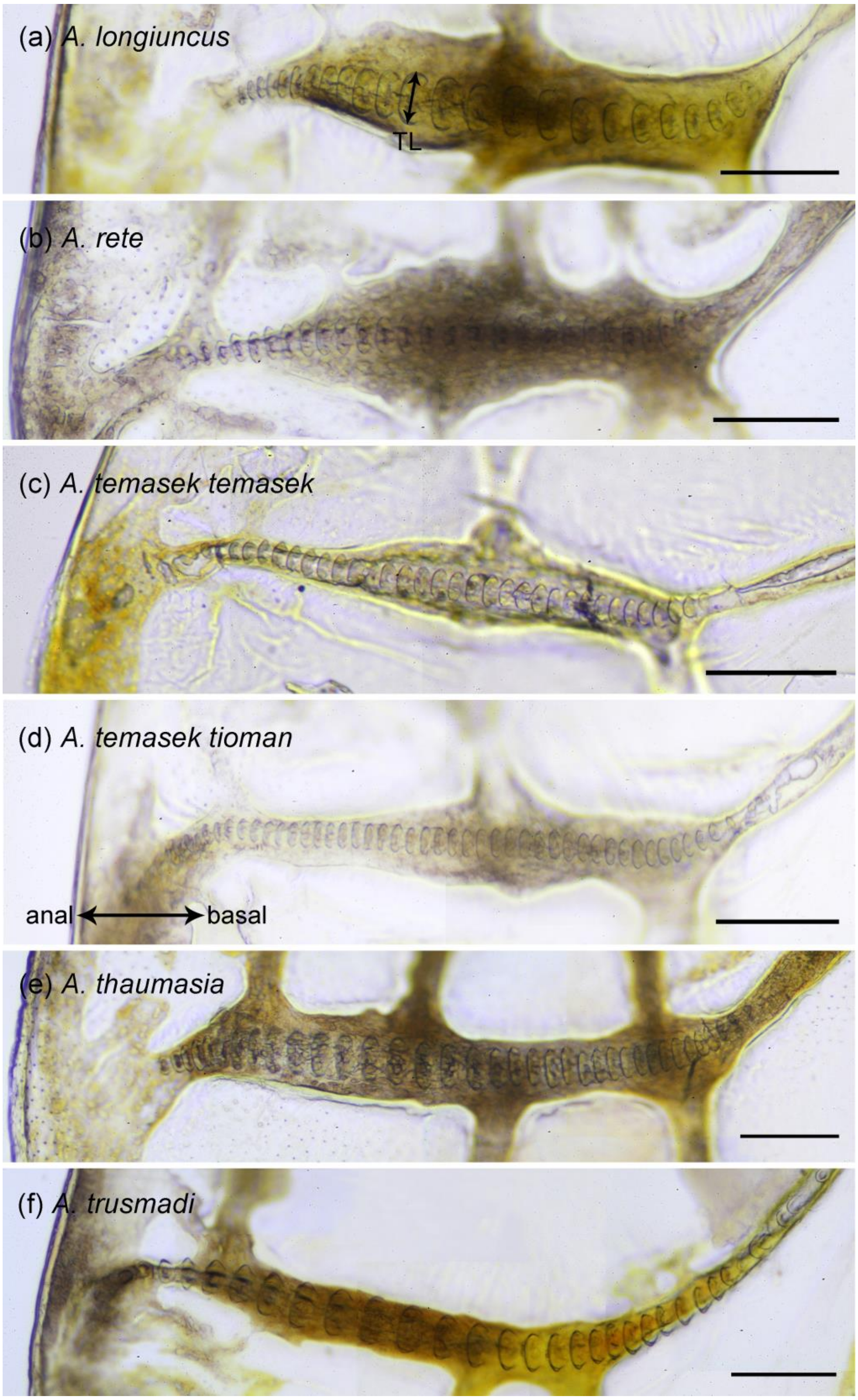

Figure 8. Stridulatory file on the left tegmen of *Asiophlugis*: *A. longiuncus* (a), *A. rete* (b), *A. temasek temasek* (c), *A. temasek tioman* (d), *A. thaumasia* (e), and *A. trusmadi* (f). TL refers to tooth length. Scale bars: 0.1 mm.

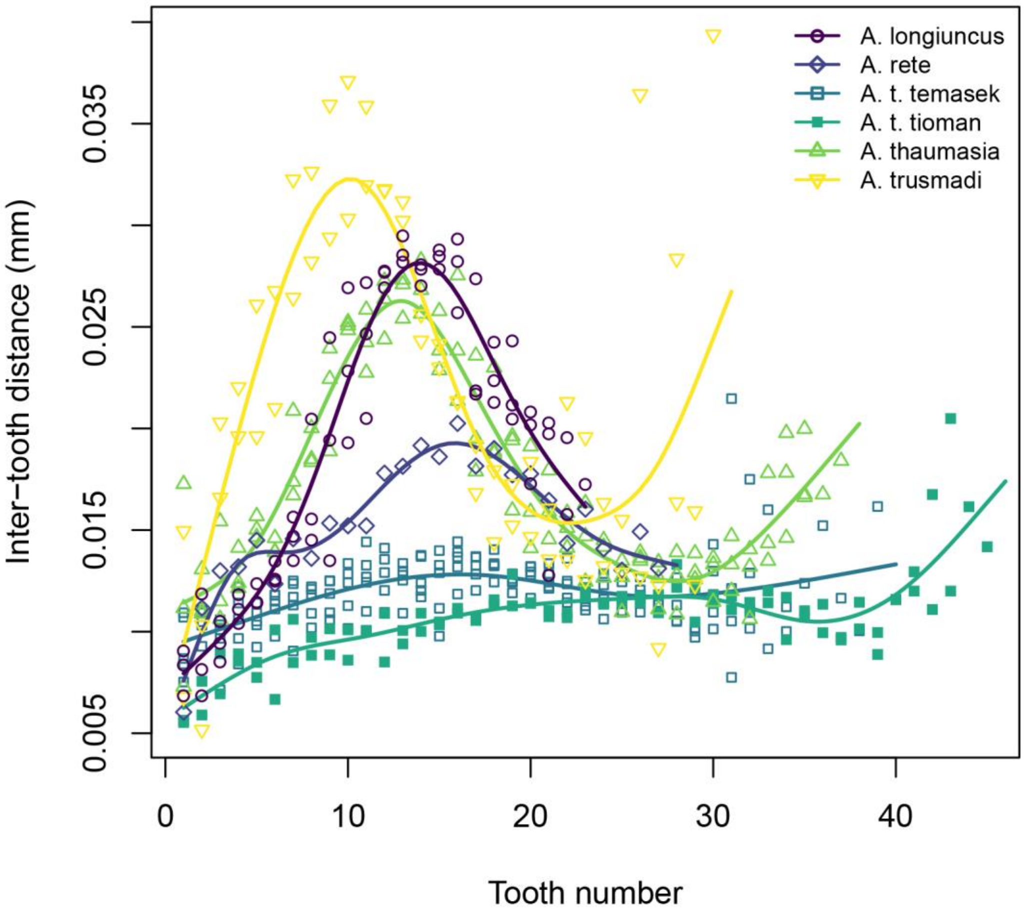

Figure 9. Tooth distribution on the stridulatory file of *Asiophlugis* based on inter-tooth distances. Trend lines are generalised additive model fits. *A. longiuncus*, $r^2 = 0.91$; *A. rete*, $r^2 = 0.89$; *A. temasek temasek*, $r^2 = 0.28$; *A. temasek tioman*, $r^2 = 0.69$; *A. thaumasia*, $r^2 = 0.87$; and *A. trusmadi*, $r^2 = 0.63$.

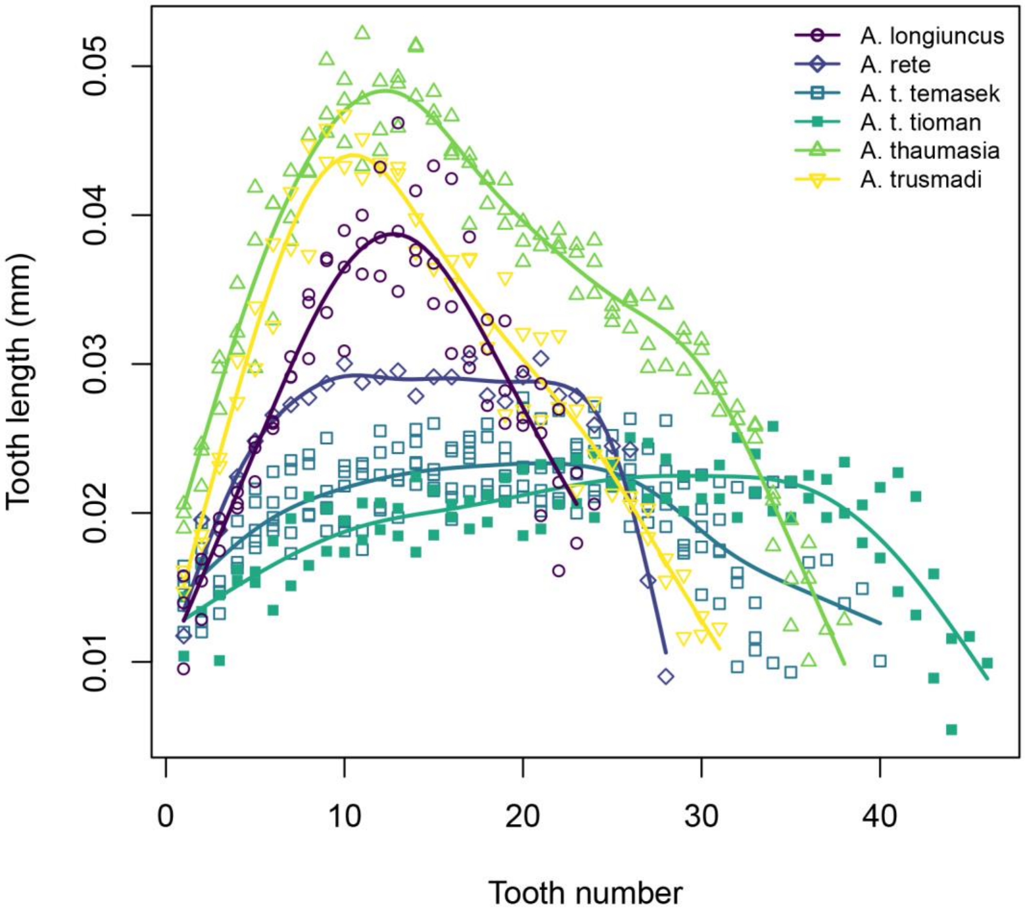

Figure 10. Tooth distribution on the stridulatory file of *Asiophlugis* based on tooth length variation. Trend lines are generalised additive model fits. *A. longiuncus*, $r^2 = 0.88$; *A. rete*, $r^2 = 0.94$; *A. temasek temasek*, $r^2 = 0.57$; *A. temasek tioman*, $r^2 = 0.70$; *A. thaumasia*, $r^2 = 0.95$; and *A. trusmadi*, $r^2 = 0.95$.

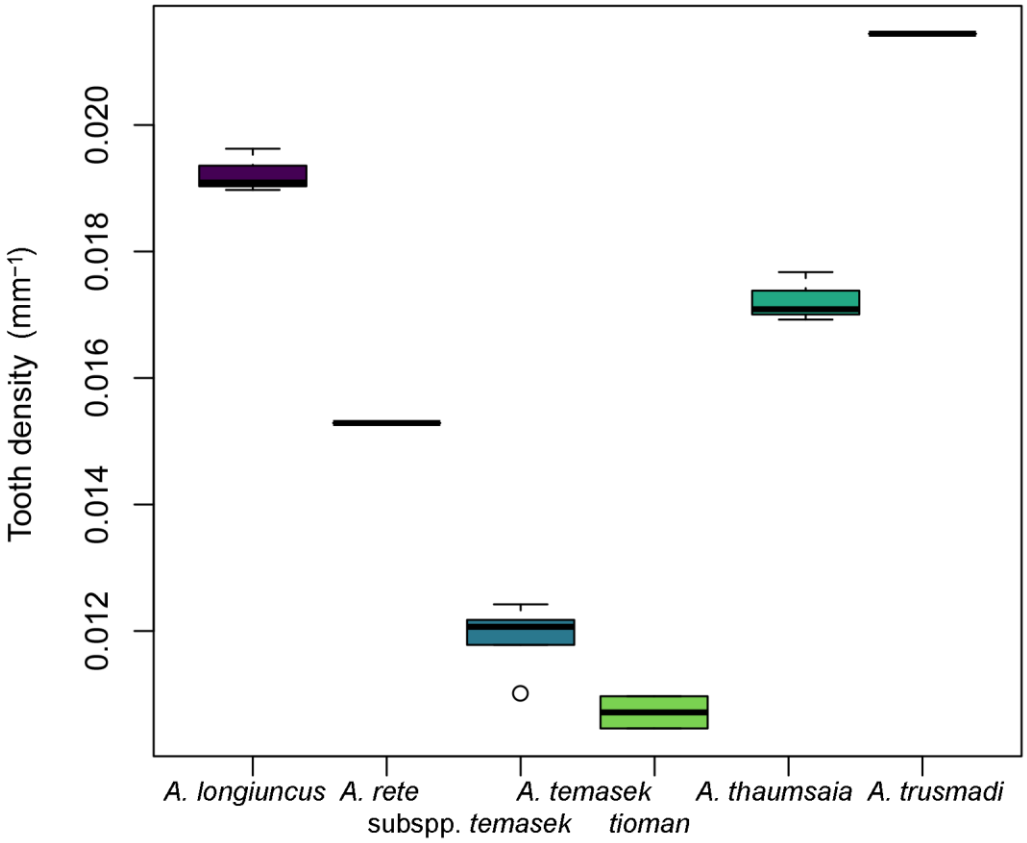

Figure 11. Tooth density on the stridulatory file of *Asiophlugis*. The whiskers refer to the minimum and maximum; lower margin, thick bar and upper margin of the box refer to the 25th, 50th and 75th quartile, respectively.