

# Same-sex sexual behavior in *Xenogryllus marmoratus* (Haan, 1844) (Grylloidea: Gryllidae: Eneopterinae): Observation in the wild from YouTube

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## Abstract

Searching on the internet for supplementary information about species of interest can be helpful when trying to document a quite common but under studied species. While revising the genus *Xenogryllus* Bolívar, 1890, we used this method to find additional references about the species *Xenogryllus marmoratus* (Haan, 1844). We were extremely interested in one video showing an interaction between two males of this species. The video shows a small male of *X. marmoratus* mounting a larger male of the same species, and eating the product of the metanotal glands. Meanwhile, the larger male tries to transfer his spermatophore to the small one. Same-sex matings have been described in other insect species and are frequently observed in many animal groups. Most of the reported cases of same-sex sexual behavior documented in other species of insects are consistent with two causes: mistakes in sexual recognition and lack of experience. However, there are several hypotheses to explain same-sex sexual behavior that vary according to the context in which the behavior occurs. Here, we describe and discuss this video content in terms of reproductive strategies, in a context of evolution and sexual selection.

## Key words

male-male sexual interactions, reproductive strategies, sexual selection

## Introduction

While searching on the internet for additional references about species of interest, one can sometimes find little natural history treasures. Images from websites such as Flickr (<https://www.flickr.com/>) or Projectnoah ([www.projectnoah.org](http://www.projectnoah.org)) can provide rich information about the animal's distribution, in addition to high-quality pictures of our favorite animals. Similarly, sound recordings can be found in online sound libraries, even if orthopterans are usually far less covered by these databases than are birds or frogs and are difficult to identify based on sound only. A good compromise is to look for video files on popular websites such as YouTube (<http://www.youtube.com>). With video

media, it is common to get information about the location where the video was made, along with sounds and images that facilitate identification. Online videos can be particularly helpful when trying to document a species that is relatively common, but under studied. Recently, the study of such under studied species of eneopterine crickets has revealed the existence of a system of communication never reported before in crickets. This new system of communication involves high-frequency calling songs emitted by the male (Robillard and Desutter-Grandcolas 2004), absence of phonotaxis and vibrational response by the female (ter Hofede et al. 2015). Such findings challenge the stereotyped model of communication of crickets and their mating strategies. It suggests that detailed investigations should focus on biological traits of as many species as possible in a phylogenetic context (Desutter-Grandcolas and Robillard 2004).

While revising the tribe Xenogryllini Robillard, 2004 (Jaiswara et al. in prep), we used online videos to find additional information about the species *Xenogryllus marmoratus* (Haan, 1844). This species is very common in Japan and China, where it is named Matsumushi (Japanese = pine insect) or bao ta ling (Chinese = pagoda bell), respectively. Because *X. marmoratus* is commonly observed in the field by local naturalists, it was likely to occur in the list of species that can benefit from online resources (Robillard and Tan 2013).

We found several videos where the species can be heard and/or seen, mostly from Japan, and sometimes recorded with high-quality equipment allowing us to document the details of wing movements during sound production (for example see the videos of “naturalistempus”: <https://www.youtube.com/watch?v=TxlWDRbWh2Y>, or “kiokuima”: <https://www.youtube.com/watch?v=kGIj7m9L7ys>). Among these interesting data, we were intrigued by one particular video showing a very uncommon interaction in the wild between two males of this species.

The mating behavior of *X. marmoratus* has not been studied in detail yet, but basic observations made in the laboratory (T. Robillard pers. obs.) suggest that its basic structure is similar to the mating behavior documented in other species of crickets (Alexander

and Otte 1967), including the eneopterine *Nisitrus* sp. (Preston-Mafham 2000): when the first spermatophore is ready to transfer, the male turns his back while raising his forewings perpendicularly to the body, which exposes the glandular structures located on the metanotum below the base of the forewings. Dorsal glands are common in males of ensiferan insects, in which the female mounts the male's back during mating (Alexander and Brown 1963, Gwynne 1984, Vahed 1998). In many species of crickets, the female feeds on the secretions from the male's metanotal glands before, during and after copulation (Boldyrev 1915, Hohorst 1937, Alexander and Otte 1967, Walker 1978, Funk 1989, Brown 1997). These kinds of nuptial feeding include the transfer of any form of nutrient from the male to the female during, or directly after, mating. The nutritional gifts are attractive for females and could represent a paternal investment: nutrients from the nuptial gift are used by the female to increase the fitness of the male's offspring (Trivers 1972, Thornhill 1976, Gwynne 1984, Vahed 1998). Also, it is known that the nuptial feeding can increase the duration of copulation to maximize ejaculate transfer (Hohorst 1937, Bidochka and Snedden 1985).

As mentioned above, the video content studied here shows an interaction between two males of the same species, and includes glandular feeding by one of the males. Same-sex sexual behavior is widespread among animals and it has been reported in most insect orders (Bagemihl 1999). In many cases, this sexual behavior is a by-product of other reproductive strategies, which are positively correlated to fitness (Bailey and Zuk 2009, Han and Brooks 2015). However, the causes, consequences and evolution of this behavior are still unknown in most groups. In this short communication, we describe the video content and discuss the implications of the observed behavioral interactions in terms of reproductive strategies.

## Materials and methods

The video content analyzed here was recorded in Japan, in the prefecture of Yamaguchi in Hofu, by the YouTube user "kiokuima", and added on the public website YouTube on 20 October 2014, under the address <https://www.youtube.com/watch?v=C9hF0Fu5A64> (Kiokuima 2014). It was retrieved by typing the key word *Xenogryllus* in the search window. The author of the video is an experienced naturalist who has been posting videos of Japanese fauna since 2011. The video content is cited here as a reference and remains the property of its author.

The video was extracted for study using the online application Fluvore (<http://www.fluvore.com/>). Screenshots of the behavioral interactions were extracted from the video using Windows Movie Maker Version 2012 (Microsoft) (Fig. 1).

## Results

The video lasts 6:08 min and consists of several takes fused together. It was recorded at night in the field. The songs of several species of orthopterans can be heard in the background noise, including calls of *X. marmoratus* (at least two distinct males).

*Behavioral description.*— 0:00–0:54 min. – A male of *X. marmoratus* is sitting on dry herbaceous vegetation (Fig. 1A). It turns around, stops, then walks left out of the frame, while moving the antennae.

0:54–1:12 min. – Same male, seen from above, motionless on plant stems.

1:12 min. – Male raises its wings perpendicularly to the body, adopting the usual mating position and exhibiting the metanotal glands (Fig. 1B).

1:20 min. – Another cricket's antennae clearly appear at the male's back (Fig. 1B, red circle). Both individuals' antennae briefly touch each other, causing a subtle startle of the first male's body (1:26), but it keeps its forewings raised. The second cricket slowly approaches from behind (Fig. 1C).

1:37 min. – The second individual puts one leg on the back of the first one (red circle on Fig. 1D). At this point, the viewer is prepared to observe a mating sequence, but when the second individual moves forward on the first individual's back, it becomes clear that this is not a female, but a second male of *X. marmoratus* (Fig. 1E) (hereafter male 2).

1:37–1:48 min. – Male 2 mounts the back of male 1 up to the exposed metanotal glands (Fig. 1F).

1:48–3:07 min. – Male 2 feeds on the glands of male 1 (Fig. 1F–G), even pushing forward male 1's forewings with its head.

3:08 min. – Male 1 reacts by moving the whole body, then becomes still again while male 2 continues feeding on its glands.

4:10 min. – Slightly different angle, probably shortly after. Male 2 is still eating from male 1's glands but the latter moves, as if trying to remove male 2 from its back (Fig. 1H).

4:24 min. – The intentions of male 1 become clearer as the apex of its abdomen enters the frame of the video: its genitalia are raised and a spermatophore is ready to be transferred (Fig. 1I). Male 1 attempts mating with male 2.

4:45 min. – Slightly different angle, probably shortly after. This angle shows that male 2 is clearly shorter than male 1, as it is entirely sitting on the abdomen of male 1 (Fig. 1J). Both males are motionless, but male 2 continues feeding on male 1's glands.

5:20–6:08 min. – Multiple new attempts at mating. Male 1 repeatedly bends its abdomen apex dorsally, but fails to reach male 2, which calmly continues feeding on the other's glands (Fig. 1J).

## Discussion

The video clearly shows a small male of *X. marmoratus* mounting a larger male of the same species, and eating the product of the metanotal glands. Meanwhile, the larger male tries to transfer his spermatophore to the small one. The hypothesis that males of two sympatric species, a large one and a smaller, may be interacting in the video can be discarded for several reasons: first, the acoustic background of the video lets us hear only the song of *X. marmoratus* and no other song that could be due to another species of this genus. Second, we believe that, if there was a new species of large cricket in Japan, it would have been described long ago by local taxonomists. Third, the ongoing revision of *Xenogryllus* led us to examine many *Xenogryllus* specimens from Japan from several collections of natural history museums; this study suggests that *X. marmoratus* is the only species of the genus distributed in Japan (T. Robillard pers. obs.; Jaiswara et al. in prep.).

The fact that the video content was obtained from YouTube might call into question its authenticity. It is obviously the result of video editing from several video takes, which means that the overall timing could not be completely trusted. However, it is clear that the same insects have been filmed in the wild more or less continuously, according to the acoustic background and the similarity of the vegetation visible during the whole sequence. The longevity and high-quality of activity of the author on his YouTube channel (ca. 650 followers) dedicated to the fauna of Japan attests to the reliability of his videos and precludes the possibility that this video could be faked. Consequently, even if this video should be treated with caution, the rarity of observations of same-sex mating in the wild deems this video worthy of discussion here.



Figure 1. Screenshots of the video showing same-sex sexual behavior between males of *Xenogryllus marmoratus* (Haan, 1844). For details, see the results section.

Same-sex matings are frequently observed in many animal groups (Thornhill and Alcock 1983, Maklakov and Bonduriansky 2009) and have been described in other insect species, for example in *Drosophila melanogaster* (McRobert and Tompkins 1988), the weevil *Diaprepes abbreviatus* (Harari et al. 2000), the damselfly *Ischnura elegans* (van Gossium et al. 2005), the seed beetle *Acanthoscelides obtectus*, the flour beetle *Tribolium castaneum* (Castro et al. 1994), and also in field crickets (Bailey and French 2012). However, in most cases, same-sex mating function is difficult to define (Vasey and Sommer 2006).

Same-sex sexual behavior is treated differently according to the context and the social environment where it occurs, such as sex-ratio, competition between males for females, mating experience of the males, etc. (Burgevin et al. 2013, Han and Brooks 2015). Therefore, there are a variety of hypotheses to explain its evolution in male insects. Genetic studies in model organisms have demonstrated that there are common patterns in the evolution of these behaviors (Bailey and Zuk 2009) and most of the reported cases of same-sex sexual behavior documented in other species of insects are consistent with two causes: 1) mistakes in sexual recognition and 2) lack of experience (Scharf and Martin 2013). The first hypothesis posits that mistakes in sexual recognition could come from errors in processing the sensorial information commonly involved in courtship (e.g. visual, acoustic, chemical signals) or because individuals resemble the opposite sex in some way (Bailey and French 2012). In some cases, males use body size to distinguish between the sexes. For example, in species of butterflies or grasshoppers in which females are larger than males, larger individuals are mounted more often, independent of their sex (Bland 1991, Solensky 2004). On the other hand, immature males of some species show absence of sex-specific characteristics (McRobert and Tompkins 1988) or phenotypes more typical of females (Ruther and Steiner 2008), which may cause interacting partners to confuse their identity (McRobert and Tompkins 1988, Bailey and French 2012). In crickets, it is known that during mating, males deposit sexual pheromones on the body of the females. These pheromones can be detected by other individuals in subsequent sexual interactions (Thomas and Simmons 2009). In the same way, in other groups of insects, female pheromones are attached to the male's cuticle during mating and those males consequently become attractive for other males (Barrows and Gordh 1978, Wendelken and Barth 1985). This could result in cases of same-sex sexual behavior by the receptive males (Wendelken and Barth 1985). On the other hand, the second hypothesis posits that the need to recognize mates successfully could cause immature individuals to attempt to mate with same-sex individuals. In this way, they would learn from unsuccessful matings to identify the opposite sex correctly (Dukas 2006).

Mistaken identification was suggested in 80% of the cases as the mechanism responsible for same-sex sexual behavior in insects (Scharf and Martin 2013). In the video, we can observe how the larger male lifts the forewings to expose its metanotal gland to the smaller male and then tries to transfer the spermatophore. From the point of view of the larger male, there is a possibility that it confuses the smaller one with a female, making plausible the hypothesis of a recognition mistake. This explanation, however, is not possible if males distinguish females based on body size, since the dimorphism is usually the contrary in crickets (females are commonly larger than males), including in *Xenogryllus* species (TR, pers. obs.).

Nevertheless, we have only one observation of the behavior in the species and we do not know the conditions or the environment in which the individuals were found. If the males here are

experiencing a male-biased sex ratio and strong scramble competition, it would be important for males to find females rapidly before another male does (Han and Brooks 2013a, b). Consequently, males could benefit from increasing the rate of mating attempts, even if they sometimes choose the wrong partners. This strategy would be less costly than missing a chance to mate with an available female (Han and Brooks 2015).

Finally, another possible explanation for this observation could be that the males are isolated and have no possibility of interacting with females. Long-term exposure to individuals of the same sex or absence of mating opportunities can be the cause of another mechanism leading to these sexual behaviors. The lack of potential mates could increase the occurrence of same-sex sexual behaviors due to learned changes in preferences. In some species, the preferences for same-sex partners after isolation from opposite sex partners can even persist after opposite sex partners are available (Field and Waite 2004, Bailey and French 2012).

From the point of view of the smaller male, which deliberately feeds on the larger male's metanotal glands, the hypothesis of recognition mistake is not permitted. This behavior could however be compared to satellite male strategy documented in field crickets (e.g. Cade 1979, Bailey 1991). Here, the smaller male would not only act as a satellite male waiting to silently intercept potential females, but could also use nutritive resources produced by the larger male and destined for females, to benefit its own reproduction.

In conclusion, we document a case of same-sex sexual behavior in the cricket species *Xenogryllus marmoratus* based on one wild observation, including courtship behaviors and even the production of the spermatophore. More data and experiments are obviously necessary to test the hypotheses raised by this observation and to understand the conditions in which these sexual behaviors occur. However, this video content offers valuable information about a sexual behavior that had not been reported before for this species, and rarely for crickets in general. This can demonstrate that casual observational data could offer important information for the analysis of unaddressed aspects of the biology. The information obtained in this work will help provide a more complete knowledge of this behavior in crickets, in the context of evolution and sexual selection.

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# Grasshopper species composition shifts following a severe rangeland grasshopper outbreak

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## Abstract

Little is known about how grasshopper species abundances shift during and following severe outbreaks, as sampling efforts usually end when outbreaks subside. Grasshopper densities, species composition and vegetation have infrequently been sampled during and after a severe outbreak in the western U.S., which is needed to better understand the cause of outbreaks and population declines. In this study, grasshopper densities, species composition and vegetation were monitored at a northern mixed rangeland site from 1999 to 2003 where densities reached 130 per m<sup>2</sup> during a severe outbreak. *Phoetaliotes nebrascensis* (Acrididae: Melanoplinae) comprised 79% of the outbreak in 2000, but declined to 3% by 2003. The dramatic shifts in proportional and actual abundance of *P. nebrascensis* over a 5 year period illustrate that species dominance can change rapidly, even for a highly dominant outbreak species. The difficulty of fully understanding factors causing shifts in grasshopper populations is illustrated by population declines in all species observed in 2002 and 2003. The data can help predict the intensity and decline of outbreaks and points to the critical importance of long term simultaneous monitoring of grasshopper densities, species composition and vegetation for outbreak prediction.

## Key words

*Phoetaliotes*, Melanoplinae, rangeland, Acrididae, prairie

## Introduction

Grasshoppers are often the dominant herbivore in western U.S. grasslands (Belovsky and Slade 2000, Branson et al. 2006), with both cyclical regional grasshopper outbreaks and localized outbreaks of 30 to 90 per m<sup>2</sup> in western North America (Nerney and Hamilton 1969, Hewitt and Onsager 1983, Belovsky 2000, Onsager 2000). Despite this, the mechanisms underlying grasshopper outbreak dynamics remain poorly understood (Joern 2000, Onsager 2000, Branson et al. 2006, Jonas and Joern 2007, Powell et al. 2007, Jonas et al. 2015). Even less is known about shifts in the relative abundance of species during and following outbreak periods, as sampling efforts either fail to monitor species composition or end when outbreaks or chemical control efforts subside (Onsager 2000). Little, if any, data exist where grasshopper densities, species composition and vegetation were sampled during and after a severe outbreak, which is needed to better understand the cause of

outbreaks and population declines in the western U.S. (Branson et al. 2006, Branson and Haferkamp 2014). Given species differences in food preference and phenology, grasshopper species should differentially respond to abiotic and biotic conditions (Joern 2000, Onsager 2000, Branson et al. 2006). In this study, grasshopper densities and species composition were sampled at a northern mixed prairie site during and after a severe outbreak.

## Materials and methods

Grasshopper sampling occurred in a large livestock enclosure at the USDA, Agricultural Research Service, Fort Keogh Livestock and Range Research Lab located near Miles City, Montana, U.S., from 1999 through 2003. Cattle were the only mammal excluded from the site and insects were not controlled. The site consisted of mixed grass prairie, with Western Wheatgrass (*Pascopyrum smithii*) initially comprising over 90% of vegetation (Branson 2008, Branson and Haferkamp 2014). In the area of the study site, greater than 90% of plant production typically occurs by July 1 (Heitschmidt and Vermeire 2005), with annual precipitation highly variable but averaging ~34 cm (Heitschmidt and Vermeire 2006).

Grasshopper density was estimated by counting grasshoppers flushing from within eight, 0.1 m<sup>2</sup> wire rings by tapping in the ring (Onsager and Henry 1977). To allow more accurate density estimates with the high grasshopper densities in 2000, ten 0.05 m<sup>2</sup> rings were used in each plot. Densities and species composition were assessed multiple times each year. A matched random catch sweep sample, consisting of an equal number of faster sweeps in the plant canopy and slower sweeps near ground level, was taken to assess grasshopper species composition (Larson et al. 1999, Berry et al. 2000). Samples were frozen for later identification to species. Species composition was combined with densities to provide species specific densities (Joern 2004). Peak density refers to the highest yearly density measured and typically occurs shortly after the majority of numerically dominant species hatch, given high rates of early mortality.

Vegetation was sampled in mid and late summer from 1999 to 2001, but only during mid-summer in 2002 and 2003. Plots were clipped after randomly tossing five to ten 0.1 m<sup>2</sup> rings on rangeland. Green vegetation was separated by grasses and forbs, dried,

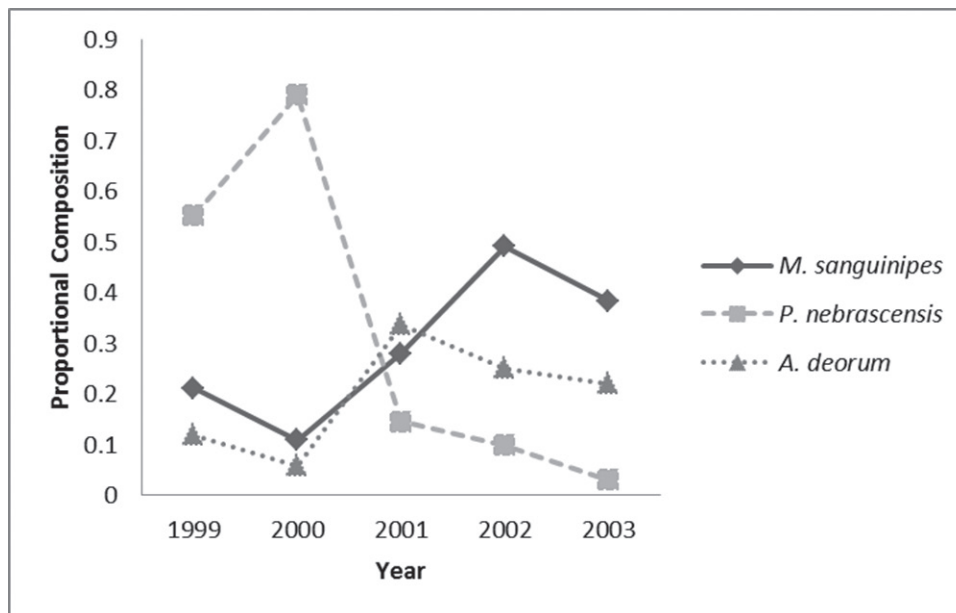
weighed, and ground. Percentage total nitrogen content of grass was assessed using a dry combustion C/N analyzer and used as an index of plant quality.

## Results and discussion

Grasshopper densities at sites >2 km from the study site ranged from 8 to 17 per m<sup>2</sup> in 1997 and 13 to 31 per m<sup>2</sup> in 1998 (Branson unpublished data). At the study site, peak grasshopper densities in 1999 were 30.9 per m<sup>2</sup> (Table 1). A severe grasshopper outbreak occurred in 2000, with peak densities increasing 320% to ~130 per m<sup>2</sup> (Table 1). Grasshopper densities declined 84% in 2001 and continued to decline in 2002 and 2003 (Table 1). During high density years, species composition at the site was dominated by *Phoetaliotes nebrascensis* (Thomas), *Melanoplus sanguinipes* (Fabricius), and *Ageneotettix deorum* (Scudder). All common species of grasshoppers at the site were egg overwintering species that hatched in early summer, although phenology differed between species (Pfadt 2002). *Phoetaliotes nebrascensis* typically hatches ap-

proximately two to four weeks later than *A. deorum* (Pfadt 2002, Branson unpublished data). Although *M. sanguinipes* usually begins to hatch slightly after *A. deorum*, it often has a prolonged hatch period (Pfadt 2002). All common species feed on grasses, but *M. sanguinipes* is a polyphagous grass and forb feeder (Pfadt 2002). Although the density of other uncommon species at the site increased by 1.45 per m<sup>2</sup> from 2002 to 2003, in general sub-dominant species were not consistently abundant enough to examine density shifts over time.

Proportional species composition shifted during and following the grasshopper outbreak, with species composition initially dominated by *P. nebrascensis* (Fig. 1). Community composition of *P. nebrascensis* increased from 55% in 1999 to 79% in 2000, when densities increased 488% to 100 per m<sup>2</sup> (Fig. 1, Table 1). *P. nebrascensis* declined from 79% to 15% of the grasshopper community in 2001, when densities declined by 97% (Fig. 1, Table 1). By 2003, *P. nebrascensis* densities had declined from 100 per m<sup>2</sup> to 0.15 per m<sup>2</sup> and comprised less than 3% of the grasshopper community (Fig. 1, Table 1).



**Figure 1.** Proportional composition for each of the three major grasshopper species averaged across random catches in a given year from 1999 to 2003.

**Table 1.** A. Peak total and common species grasshopper densities from 1999 to 2003. B. Green grass biomass (g/m<sup>2</sup>) in late June or early July (Mid-summer) in all years and late August or early September (Late-summer) from 1999 to 2001. C. Grass percent nitrogen content in mid-summer and late summer. \* Indicates missing data.

A. Grasshopper density (#/m <sup>2</sup> )	1999	2000	2001	2002	2003
<i>M. sanguinipes</i>	6.52	13.67	5.86	2.33	1.97
<i>P. nebrascensis</i>	17.03	100.31	3.05	0.48	0.15
<i>A. deorum</i>	3.68	7.36	7.08	1.19	1.13
Total grasshopper density	30.90	130.00	21.00	4.75	5.15
B. Grass biomass (g/m <sup>2</sup> )					
Mid-summer	148.4	53.0	123.4	66.8	98.4
Late-summer	52.3	4.2	104.0	*	*
C. % Nitrogen content					
Mid-summer	0.94	1.48	1.55	1.25	1.07
Late summer	1.84	0.82	0.71	*	*



Densities of both *A. deorum* and *M. sanguinipes* doubled from 1999 to 2000 (Table 1), even though their proportional composition declined (Fig. 1). However, community composition of the two species that were subdominant during the outbreak increased between 2000 and 2001 (Fig. 1). *M. sanguinipes* increased from 11 to 28% of the grasshopper community, despite a 57% density decline (Fig. 1). By contrast, *A. deorum* maintained a relatively stable density and increased from 6% to 35% of the community (Table 1, Fig. 1). When peak densities declined to 4.75 and 5.15 per m<sup>2</sup> in 2002 and 2003, proportional composition of *M. sanguinipes* increased to ~43%, while proportional composition of *A. deorum* declined to ~23%. Species specific responses to food limitation during the outbreak likely affected the proportional abundance of these two species. *A. deorum* accepts more plant litter in its diet than most grasshoppers (Pfadt 2002), which together with its earlier hatching phenology likely contributed to a stable population density from 2000 to 2001 despite a lack of late summer grass biomass in 2000 (Table 1). As the slightly later hatching *M. sanguinipes* declined 57% in 2001, late summer conditions and food availability in 2000 appeared to more strongly affect it.

The large positive and negative shifts in the dominant species *P. nebrascensis* from 1999 to 2001 matched patterns of late season food availability. Above average August precipitation in 1998 and 1999 led to elevated grass nitrogen content (Heitschmidt et al. 2005, Table 1) and *P. nebrascensis* reproduction (Branson 2008). The 97% decline in *P. nebrascensis* densities in 2001 were associated with low late season food availability and nitrogen content in 2000 (Table 1) that reduced survival and reproduction (Branson and Haferkamp 2014). The larger proportional reduction in *P. nebrascensis* in 2001 compared to *M. sanguinipes* and *A. deorum* (Table 1) at least partially resulted from its later phenology. Mid-season green grass biomass and nitrogen content were variable and not obviously related to shifts in grasshoppers (Fig. 1, Table 1), likely because vegetation was collected when grasshoppers were still nymphs. The reason for the continued population decline from 2001 to 2003, when food availability was presumably not as limiting, is not clear and illustrates the complexity of understanding the many interacting biotic and abiotic factors causing shifts in grasshopper populations (Joern 2000, Branson et al. 2006, Jonas et al. 2015).

There is a paucity of data where concurrent grasshopper density and species composition sampling combined with vegetation sampling occurred during and after a grasshopper outbreak. The dramatic shifts in proportional and actual abundance of *P. nebrascensis* over a 5 year period illustrate that species dominance can change rapidly, even for a species that was highly dominant in a severe outbreak. Precipitation timing is important, as peak plant biomass production in this system is driven by spring and early summer moisture (Heitschmidt and Vermeire 2005, Haferkamp et al. 2005), while late summer rains are required to sustain quality late summer vegetation quality that led to the community dominance by *P. nebrascensis* (Heitschmidt and Vermeire 2006, Branson 2008, 2016). Therefore, late-season food availability and thermal conditions seem the most useful correlates to predict both outbreaks and population crashes of later hatching species such as *P. nebrascensis*, as outbreaks are likely associated with availability of high quality food during the late summer reproductive period (Branson 2008, 2016). Although statistical inference is precluded by the inability to replicate grasshopper outbreaks, the data can help predict the intensity and decline of outbreaks and point to the critical importance of long term simultaneous monitoring of grasshopper densities, species composition and vegetation to facilitate outbreak prediction.

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# Phylogenetic relationship of Japanese Podismini species (Orthoptera: Acrididae: Melanoplinae) inferred from a partial sequence of cytochrome c oxidase subunit I gene

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## Abstract

Members of the tribe Podismini (Orthoptera: Acrididae: Melanoplinae) are distributed mainly in Eurasia and the western and eastern regions of North America. The primary aim of this study is to explore the phylogenetic relationship of Japanese Podismini grasshoppers by comparing partial sequences of cytochrome c oxidase subunit I (COI) mitochondrial gene. Forty podismini species (including nineteen Japanese species) and thirty-seven species from other tribes of the Melanoplinae (Dactyloini, Dichroplini, Melanoplinae, and Jivarini) were used in the analyses. All the Japanese Podismini, except *Anapodisma*, were placed in a well-supported subclade. However, our results did not correspond with the classification on the basis of morphological similarity for the status of Tonkinacridina. This group of Japanese species constituted a single clade with other species of Miramellina and Podismina, while Eurasian continental species of Tonkinacridina were placed in other separate clades. This incongruence might have resulted from historical migratory events between continent and ancient islands and subsequent convergent/parallel evolution in morphology. Some remarks on phylogenetic positions in Podismini and other tribes were also made in terms of reconstructed phylogeny.

## Key words

grasshoppers, polymorphism, mitochondrial DNA

## Introduction

The tribe Podismini Jacobson, 1905 is one of the five tribes belonging to the acridid subfamily Melanoplinae Scudder, 1897 (Cigliano et al. 2017). Podismini genera are distributed in the Palearctic and Nearctic region (Vickery 1987). They usually occur in grassland and scrub formations. Although morphology is rather variable between species, most species are clearly definable (Ito 2015). According to morphological features, Podismini is currently divided into three subtribes: Miramellina (Rehn & Randell, 1963), Podismina (Jacobson, 1905), and Tonkinacridina (Ito, 2015). The genus group Bradynotae (Rehn & Randell, 1963) and another 21 genera have also been considered as members of this tribe, but they have not yet been included in the subtribes (Cigliano et al. 2017).

Because of the substantial variability in morphology and even in karyotype, the taxonomy of Podismini has been excessively confused. Based on the reexamination of characters, Japanese Podismini consists of 22 species in nine genera (Ito 2015), while the phylogenetic relationship between species in the tribe is still ambiguous. The first molecular study of Podismini examined one mitochondrial gene (COII) and three ribosomal nuclear and mitochondrial genes (ITS1, 12S, and 16S) for 25 species of Podismini (Chintauan-Marquier et al. 2014). In this study, nine Japanese species of seven genera [*Parapodisma dairisama* (Scudder, 1897), *P. mikado* (Bolivar, 1890), *P. subastris* Huang, 1983; *Sinopodisma punctata* Mistshenko, 1954; *Ognevia longipennis* (Shiraki, 1910); *Podisma kanoi* (Storozhenko, 1994); *Zubovskya koepfeni parvula* (Ikonnikov, 1911); *Fruhstorferiola okinawaensis* (Shiraki, 1930); *Tonkinacris* sp. (Carl, 1916)] were also examined. Four of seven genera (*Parapodisma* Mistshenko, 1947, *Sinopodisma* Chang, 1940, *Tonkinacris* Carl, 1916, *Fruhstorferiola* Willemsse, 1921) constituted a clade with moderate statistical support, two of seven (*Podisma* Berthold, 1827, *Ognevia* Ikonnikov, 1911) composed another clade, and *Zubovskya* Dovnar-Zapolsky, 1932 did not comprise a clade with any other genera.

The Japanese archipelago is composed of a multitude of smaller islands in addition to the four main islands (Hokkaido, Honshu, Kyushu, and Shikoku). The isolation of the Japanese archipelago from the Eurasian continent presumably began in Miocene (ca. 23 Myr ago), and the present form of the archipelago was reached in the approximate end of Pleistocene (Iijima and Tada 1990, Tada 1994, Yonekura et al. 2001). Interestingly, land bridges between the continent and some of the islands were formed at least three times during geochronologic periods between the Pliocene and Pleistocene as a result of changes in sea level during ice ages (Dobson and Kawamura 1998), which may have permitted back-and-forth movement of animals via the bridges. These complex geological events have probably shaped the present fauna and flora in Japan. The present Japanese Podismini had also presumably been derived in part from continental species group which evolved uniquely at a new place.



Figure 1. A map of Japan with the distribution of nine genera of Japanese Podismini.

The Japanese archipelago in broad sense consists of Hokkaido, Honshu, Shikoku, Kyushu, south Kuril Islands, and chain of islands extending from southwestern Kyushu to northern Taiwan (i.e. "Nansei Islands"). The brief distribution of nine genera in Podismini is shown in Fig. 1. Three of nine genera, *Fruhstorferiola*, *Sinopodisma*, and *Tonkinacris* are distributed only in Nansei Islands, presumably derived directly from continental species of the same genera. The genus *Anapodisma* Dognar-Zapolskii, 1933 is found only in Tsushima Island, the southern vicinities of Korean Peninsula. The distribution of *Prumna* Motschulsky, 1859, *Zubovskya*, and *Podisma* is localized in a northern part of Japan (central - northern Honshu, Hokkaido and Kunashir Island) and the habitat tends to be highly fragmented especially in mountain districts. *Ognevia* shows the broadest distribution range among Japanese Podismini and is distributed in high altitude areas. Although other genera are apterous or have reduced forewings, flight organs are fully developed in this genus. The genus *Parapodisma* comprises 11 species (50% of the Japanese podismine species) including two subspecies in Japan, and shows a variety of morphology such as body colors, genitalic characters and forewings, which has sometimes confounded their taxonomic status (Ito 2015). Although Vickery (1977) suggested that *Sinopodisma*, *Fruhstorferiola* and *Parapodisma* comprise Miramellina together with *Zubovskya* and *Miramella* Dognar-Zapolskii, 1933,

Ito (2015) proposed that the first three genera with *Tonkinacris* should be settled in a new subtribe, Tonkinacridina based on the cladistic assessment of 23 morphological traits.

The principal aim of the present study is to examine whether Ito's (2015) hypothesis still holds if the relationship is assessed using mitochondrial DNA sequences. We utilized a partial sequence of the cytochrome c oxidase subunit I (COI) mitochondrial gene for this purpose because the sequence is used as standard in DNA barcoding and thus is feasible for comparing species other than Japanese Podismini. In order to test the hypothesis of a close affinity between all Japanese taxa, other Melanoiplinae species from Eurasia and America were also drawn from GenBank and included in the analysis.

#### Materials and methods

*Taxa studied.*— A total of 82 species and subspecies were included in the analysis. All genetic sequences were acquired from GenBank except Podismini species in Japan (Table 1). The in-group consisted of 20 Podismini species and subspecies from Japan (new data) and 21 species from Eurasia and America. We included members of four other tribes of Melanoiplinae: Melanoiplini (19 species), Dactylotini (3 species), Dichroplini (13 species), and Jivarini (2 species). As an outgroup, we included four species of subfam-

Table 1. Taxonomic information and GenBank accession numbers for taxa included in this study.

Taxa	Sampling locality and year	Accession No.	Reference
<b>outgroup</b>			
<b>Subfamily: Catantopinae</b>			
<i>Xenocatantops humilis</i> (Serville, 1838)	China	EU366111	Wang and Jiang (unpublished)
<i>Catantops erubescens</i> (Walker, 1870)	Pakistan	KJ672128	Nazir et al. (unpublished)
<i>Diabolocatantops innotabilis</i> (Walker, 1870)	Pakistan	KJ672135	Nazir et al. (unpublished)
<i>Goniaea vocans</i> (Fabricius, 1775)	Australia	JX033911	Chapco 2013
<b>Subfamily: Melanoplinae</b>			
<b>Tribe: Dactylotini</b>			
<i>Dactylotum bicolor bicolor</i> Charpentier, 1845	North America	KJ531421	Woller et al. 2014
<i>Liladownsia fragile</i> Fontana, Mariño-Pérez, Woller & Song, 2014	North America	KJ531423	Woller et al. 2014
<i>Perixerus squamipennis</i> Gerstaecker, 1873	North America	KJ531427	Woller et al. 2014
<b>Tribe: Dichroplini</b>			
<i>Atrachelacris unicolor</i> Giglio-Tos, 1894	South America	FJ829334	Dinghi et al. 2009
<i>Atrachelacris gramineus</i> Bruner, 1911	South America	AY014360	Amédégnato et al. 2003
<i>Baeacris pseudopunctulata</i> (Ronderos, 1964)	South America, Argentina	DQ083452	Colombo et al. 2005
<i>Chlorus bolivianus</i> Brunner, 1913	South America	FJ829333	Dinghi et al. 2009
<i>Dichromatos lilloanus</i> (Liebermann, 1948)	South America	FJ829336	Dinghi et al. 2009
<i>Dichroplus obscurus</i> Bruner, 1900	South America	DQ084357	Dinghi et al. 2009
<i>Dichroplus pratensis</i> Brunner, 1900	South America, Argentina	DQ083459	Colombo et al. 2005
<i>Leiotettix pulcher</i> Rehn, 1913	South America, Argentina	DQ083464	Colombo et al. 2005
<i>Neopedies noroestensis</i> Ronderos, 1991	South America	AF539852	Amédégnato et al. 2003
<i>Pseudoscopas nigrigena</i> (Rehn, 1913)	South America	FJ829342	Dinghi et al. 2009
<i>Ronderosia bergii</i> (Stål, 1878)	South America, Argentina	DQ083467	Colombo et al. 2005
<i>Ronderosia forcipata</i> (Rehn, 1918)	South America, Argentina	DQ083468	Colombo et al. 2005
<i>Scotussa daguerrei</i> Liebermann, 1947	South America, Argentina	DQ083469	Colombo et al. 2005
<b>Tribe: Jivarini</b>			
<i>Jivarus americanus</i> Giglio-Tos, 1898	South America	DQ389233	Chapco 2006
<i>Jivarus gurneyi</i> Ronderos, 1979	South America	DQ389231	Chapco 2006
<b>Tribe: Melanoplini</b>			
<i>Hypochlora alba</i> (Dodge, 1876)	North America, USA	AF260548	Chapco et al. 2001
<i>Melanoplus bivittatus</i> (Say, 1825)	North America, Canada	KR141481	Hebert et al. 2016
<i>Melanoplus borealis</i> (Fieber, 1853)	North America, Canada	KR142429	Hebert et al. 2016
<i>Melanoplus bowditchi</i> Scudder, 1878	North America, Canada	KM535226	Dewaard et al. (unpublished)
<i>Melanoplus bruneri</i> Scudder, 1897	North America, Canada	KM535553	Dewaard et al. (unpublished)
<i>Melanoplus cinereus</i> Scudder, 1878	North America, Canada	KR141925	Hebert et al. 2016
<i>Melanoplus dawsoni</i> (Scudder, 1875)	North America, Canada	KM537453	Dewaard et al. (unpublished)
<i>Melanoplus deceptus</i> Morse, 1904	North America, Canada	KR140464	Hebert et al. 2016
<i>Melanoplus differentialis</i> (Thomas, 1865)	North America	KJ531425	Woller et al. 2014
<i>Melanoplus femurrubrum</i> (De Geer, 1773)	North America, Canada	KM536630	Dewaard et al. (unpublished)
<i>Melanoplus gladstoni</i> Scudder, 1897	North America, Canada	KR140625	Hebert et al. 2016
<i>Melanoplus infantilis</i> Scudder, 1878	North America, Canada	KM537809	Dewaard et al. (unpublished)
<i>Melanoplus mexicanus</i> (Saussure, 1861)	North America	KJ531426	Woller et al. 2014
<i>Melanoplus montanus</i> (Thomas, 1873)	North America, Canada	KM536558	Dewaard et al. (unpublished)
<i>Melanoplus oregonensis</i> (Thomas, 1875)	North America, Canada	KR140837	Hebert et al. 2016
<i>Melanoplus packardii</i> Scudder, 1878	North America, Canada	KM537414	Dewaard et al. (unpublished)
<i>Melanoplus punctulatus</i> (Uhler, 1862)	North America, Canada	KR140511	Hebert et al. 2016
<i>Melanoplus sanguinipes</i> (Fabricius, 1798)	North America, Canada	KR143225	Hebert et al. 2016
<i>Phoetaliotes nebrascensis</i> (Thomas, 1872)	North America, Canada	KM535800	Dewaard et al. (unpublished)

Taxa	Sampling locality and year	Accession No.	Reference
<b>Tribe: Podismini</b>			
<b>Subtribe: Miramellina</b>			
<i>Anapodisma beybienkoi</i> Rentz & Miller, 1971	Tsushima, Nagasaki, Japan, 2016	KY558890	This study
<i>Anapodisma miramae</i> Dovnar-Zapolskij, 1932	China	KM362650	Kang et al. 2016
<i>Zubovskya koeppeni parvula</i> (Ikonnikov, 1911)	Mt. Daisetsu, Hokkaido, Japan, 2015	KX440513	This study
<i>Zubovskya koeppeni parvula</i> (Ikonnikov, 1911)	Mt. Daisetsu, Hokkaido, Japan, 2015	KX440514	This study
<i>Zubovskya koeppeni parvula</i> (Ikonnikov, 1911)	Mt. Daisetsu, Hokkaido, Japan, 2015	KX440515	This study
<i>Zubovskya koeppeni parvula</i> (Ikonnikov, 1911)	Mt. Daisetsu, Hokkaido, Japan, 2015	KX440516	This study
<b>Subtribe: Podismina</b>			
<i>Ognevia longipennis</i> (Shiraki, 1910)	China	JQ301452	Lü and Huang 2012
<i>Ognevia sergii</i> Ikonnikov, 1911	Russia	KC261364	Bugrov et al. (unpublished)
<i>Podisma kanoi</i> Storozhenko, 1994	Mt. Yokote, Nagano, Japan, 2014	KX440484	This study
<i>Podisma kanoi</i> Storozhenko, 1994	Mt. Yokote, Nagano, Japan, 2014	KX440485	This study
<i>Podisma sapporensis</i> Shiraki, 1910	Kamishihoro, Hokkaido, Japan, 2015	KY558881	This study
<i>Podisma sapporensis</i> Shiraki, 1910	Nukabira, Hokkaido, Japan, 2015	KY558882	This study
<i>Podisma tyatiensis</i> Bugrov & Sergeev, 1997	Russia	KC261368	Bugrov et al. (unpublished)
<i>Yunnanacris yunnaneus</i> (Ramme, 1939)	China	KX223964	Guan and Xu (unpublished)
<b>Subtribe: Tonkinacridina</b>			
<i>Fruhstorferiola huayinensis</i> Bi & Xia, 1980	China	KC139873	Huang et al. 2013
<i>Fruhstorferiola kulinga</i> (Chang, 1940)	China	KC139885	Huang et al. 2013
<i>Fruhstorferiola okinawaensis</i> (Shiraki, 1930)	Kunigami, Okinawa, Japan, 1998	KX440482	This study
<i>Fruhstorferiola okinawaensis</i> (Shiraki, 1930)	Kunigami, Okinawa, Japan, 1998	KY558871	This study
<i>Fruhstorferiola tonkinensis</i> (Willemse, 1921)	China	KC139890	Huang et al. 2013
<i>Parapodisma awagatakenis</i> Ishikawa, 1998	Kanaya, Shizuoka, Japan, 2015	KY558873	This study
<i>Parapodisma awagatakenis</i> Ishikawa, 1998	Kanaya, Shizuoka, Japan, 2015	KY558874	This study
<i>Parapodisma caelestis</i> Tominaga & Ishikawa, 2001	Mt. Kamikouchi, Nagano, Japan, 2016	KY558875	This study
<i>Parapodisma caelestis</i> Tominaga & Ishikawa, 2001	Mt. Kamikouchi, Nagano, Japan, 2016	KY558876	This study
<i>Parapodisma caelestis</i> Tominaga & Ishikawa, 2001	Mt. Kamikouchi, Nagano, Japan, 2016	KY558877	This study
<i>Parapodisma dairisama</i> (Scudder, 1897)	Kofu, Tottori, Japan, 2005	KX440478	This study
<i>Parapodisma dairisama</i> (Scudder, 1897)	Kofu, Tottori, Japan, 2005	KX440479	This study
<i>Parapodisma dairisama</i> (Scudder, 1897)	Kofu, Tottori, Japan, 2005	KX440480	This study
<i>Parapodisma dairisama</i> (Scudder, 1897)	Kofu, Tottori, Japan, 2005	KX440481	This study
<i>Parapodisma mikado</i> (Bolívar, 1890)	Kami-sugo, Furukawa, Japan	KY558878	This study
<i>Parapodisma niihamensis hiurai</i> Tominaga & Kano, 1987	Kawachi-nagano, Osaka, Japan, 2015	KX440483	This study
<i>Parapodisma niihamensis niihamensis</i> Inoue, 1979	Yoshinogawa, Tokushima, Japan, 2015	KX440486	This study
<i>Parapodisma niihamensis niihamensis</i> Inoue, 1979	Yoshinogawa, Tokushima, Japan, 2015	KX440487	This study
<i>Parapodisma niihamensis niihamensis</i> Inoue, 1979	Yoshinogawa, Tokushima, Japan, 2015	KX440488	This study
<i>Parapodisma setouchiensis</i> 1 Inoue, 1979	Mima, Tokushima, Japan, 2015	KX440498	This study
<i>Parapodisma setouchiensis</i> 1 Inoue, 1979	Mima, Tokushima, Japan, 2015	KX440499	This study
<i>Parapodisma setouchiensis</i> 2 Inoue, 1979	Minamiasakawa, Hachioji, Japan, 2015	KX440489	This study
<i>Parapodisma setouchiensis</i> 2 Inoue, 1979	Sefuriyama, Fukuoka, Japan, 2015	KX440490	This study
<i>Parapodisma setouchiensis</i> 2 Inoue, 1979	Sefuriyama, Fukuoka, Japan, 2015	KX440491	This study
<i>Parapodisma setouchiensis</i> 3 Inoue, 1979	Toyooka, Hyogo, Japan, 2014	KY558872	This study
<i>Parapodisma subastris</i> 1 Huang, 1983	Oe, Kyoto, Japan, 2014	KX440494	This study
<i>Parapodisma subastris</i> 1 Huang, 1983	Oe, Kyoto, Japan, 2014	KX440495	This study

Taxa	Sampling locality and year	Accession No.	Reference
<i>Parapodisma subastris</i> 2 Huang, 1983	Oe, Kyoto, Japan, 2014	KX440496	This study
<i>Parapodisma subastris</i> 2 Huang, 1983	Oe, Kyoto, Japan, 2014	KX440497	This study
<i>Parapodisma subastris</i> 2 Huang, 1983	Oe, Kyoto, Japan, 2014	KX440492	This study
<i>Parapodisma subastris</i> 2 Huang, 1983	Oe, Kyoto, Japan, 2014	KX440493	This study
<i>Parapodisma tenryuensis</i> 1 Kobayashi, 1983	Oyama, Shizuoka, Japan, 2015	KY558883	This study
<i>Parapodisma tenryuensis</i> 1 Kobayashi, 1983	Oyama, Shizuoka, Japan, 2015	KY558884	This study
<i>Parapodisma tenryuensis</i> 2 Kobayashi, 1983	Mt. Chausu, Shizuoka, Japan, 2016	KY558885	This study
<i>Parapodisma tenryuensis</i> 2 Kobayashi, 1983	Mt. Chausu, Shizuoka, Japan, 2016	KY558886	This study
<i>Parapodisma tenryuensis</i> 2 Kobayashi, 1983	Mt. Chausu, Shizuoka, Japan, 2016	KY558887	This study
<i>Parapodisma yasumatsui</i> Yamasaki, 1980	Sefuriyama, Fukuoka, Japan, 2015	KX440500	This study
<i>Parapodisma yasumatsui</i> Yamasaki, 1980	Mitsuse, Saga, Japan, 2015	KX440501	This study
<i>Sinopodisma aurata</i> Ito, 1999	Kohama Island, Okinawa, Japan, 2016	KY558888	This study
<i>Sinopodisma aurata</i> Ito, 1999	Kohama Island, Okinawa, Japan, 2016	KY558889	This study
<i>Sinopodisma houshana</i> Huang, 1982	China	KC139919	Huang et al. 2013
<i>Sinopodisma kodamae</i> (Shiraki, 1910)	Kukuan, Taiwan, 1998	KX440502	This study
<i>Sinopodisma kodamae</i> (Shiraki, 1910)	Kukuan, Taiwan, 1998	KX440503	This study
<i>Sinopodisma lofaoshana</i> (Tinkham, 1936)	China	KC139936	Huang et al. 2013
<i>Sinopodisma lushiensis</i> Zhang, 1994	China	KC139925	Huang et al. 2013
<i>Sinopodisma punctata</i> Mistshenko, 1954	Tatsugo, Kagoshima, Japan, 1997	KX440504	This study
<i>Sinopodisma punctata</i> Mistshenko, 1954	Tatsugo, Kagoshima, Japan, 1997	KX440505	This study
<i>Sinopodisma punctata</i> Mistshenko, 1954	Tatsugo, Kagoshima, Japan, 1997	KX440506	This study
<i>Sinopodisma punctata</i> Mistshenko, 1954	Tatsugo, Kagoshima, Japan, 1997	KX440507	This study
<i>Sinopodisma punctata</i> Mistshenko, 1954	Tatsugo, Kagoshima, Japan, 1997	KX440508	This study
<i>Sinopodisma punctata</i> Mistshenko, 1954	Tatsugo, Kagoshima, Japan, 1997	KX440509	This study
<i>Sinopodisma rostellocerna</i> You, 1980	China	KC139947	Huang et al. 2013
<i>Sinopodisma tsinlingensis</i> Zheng, 1974	China	KC139903	Huang et al. 2013
<i>Sinopodisma wulingshanensis</i> Bi, Huang & Liu, 1992	China	KC139909	Huang et al. 2013
<i>Tonkinacris ruficerus</i> Ito, 1999	Kunigami, Okinawa, Japan, 1998	KX440510	This study
<i>Tonkinacris ruficerus</i> Ito, 1999	Kunigami, Okinawa, Japan, 1998	KX440511	This study
<i>Tonkinacris yaeyamaensis</i> Ito, 1999	Mt. Omoto, Okinawa, Japan, 1998	KX440512	This study
<b>genus group Bradynotae</b>			
<i>Asemoplus montanus</i> (Bruner, 1885)	North America, Canada	KM535587	Dewaard et al. (unpublished)
<i>Bradynotes obesa</i> (Thomas, 1872)	North America	KJ531419	Woller et al. 2014
<b>Other members of Podismini – do not assign into any subtribe</b>			
<i>Prumna arctica</i> (Zhang & Jin, 1985)	China	KC139971	Huang et al. 2013
<i>Prumna fauriei</i> (Bolívar, 1890)	Mt. Gassan, Yamagata, Japan, 2014	KY558879	This study
<i>Prumna fauriei</i> (Bolívar, 1890)	Mt. Gassan, Yamagata, Japan, 2014	KY558880	This study
<i>Prumna mandshurica</i> Ramme, 1939	China	FJ531676	Zhao et al. (unpublished)
<i>Prumna primnoa</i> (Motschulsky, 1846)	Russia	KX272717	Sukhikh et al. (unpublished)
<i>Qinlingacris choui</i> Li, Wu & Feng, 1991	China	FJ531684	Zhao et al. (unpublished)

ily Catantopinae [*Xenocatantops humilis* (Serville, 1838), *Catantops erubescens* (Walker, 1870), *Diabolocatantops innotabilis* (Walker, 1870), and *Goniaea vocans* (Fabricius, 1775)]. We did not include Japanese species of the genus *Ognevia*; instead, an existing sequence for *O. longipennis* from China was examined in this paper (Lü and Huang 2012).

**DNA extraction, amplification, and sequencing.**— Total genomic DNA was extracted with the DNeasy Tissue Kit (QIAGEN, Hilden, Germany).

Partial gene sequences were amplified by PCR using the following primers: forward UEA7 (TACAGTTGGAATAGACGTTGATAC) and reverse UEA10 (TCCAATGCACATAATCTGCCATAITA) (Lunt et al. 1996). PCR was conducted in a 20 µl volume containing 1 µl of DNA, 2 µl 10 × Ex Taq Buffer (Mg<sup>2+</sup> free; Takara Bio Inc., Shiga, Japan) with 10 µM each primer, 10 mM dNTPs, 25 mM MgCl<sub>2</sub>, and 5 U/µl of Ex Taq polymerase (Takara Bio Inc., Shiga, Japan). The mitochondrial COI fragment was amplified under the following temperature profile: initial activation at 94 °C for 3 min, 30 cycles of denaturation at

94 °C for 1 min, annealing at 45 °C for 1 min, and elongation at 72 °C for 2 min, and a final elongation step at 72 °C for 7 min. PCR products were purified by using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Samples were sequenced in both directions by using the same primers as those used for PCR and the chain termination reaction method (Sanger et al. 1977). The sequencing was carried out in a total volume of 10 µl by using the Genome Lab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Brea, California, USA), with a cycle-sequencing profile of 40 cycles of 96 °C for 20 s, 50 °C for 20 s, and 60 °C for 3 min. Sequencing was performed using GenomeLab GeXP™ (Beckman Coulter, Brea, California, USA) at the Laboratory of Entomology in the Faculty of Agriculture, University of the Ryukyus, Japan. Sequences were deposited in GenBank under the accession numbers provided in Table 1.

*Sequence alignment and phylogenetic analyses.*— DNA sequences were aligned by using MUSCLE (Edgar 2004) with default parameters. In order to identify numts (Bensasson et al. 2001, Song et al. 2008), mitochondrial COI sequences were translated into amino acid sequences with MEGA 6 (Tamura et al. 2013) using the standard invertebrate mitochondrial genetic code. The substitution model of evolution was estimated by using the program jModelTest (Guindon and Gascuel 2003, Darriba et al. 2013). The Akaike information criterion was preferred over the hierarchical likelihood ratio test to compare the various models as recommended by Posada and Buckley (2004). The data matrices were subjected to Bayesian analysis (BI) with MrBayes v3.1. (Huelsenbeck and Ronquist 2001, Huelsenbeck et al. 2001). Bayesian analyses were performed with 10 000 000 generations, with a sampling of trees every 100 generations. Likelihood values were observed with Tracer v.1.4 (Rambaut and Drummond 2007); all the trees created before stability in likelihood values were discarded as a 'burn-in' (first 1200 trees). Maximum likelihood (ML) analysis was implemented in Phyml (Guindon and Gascuel 2003). For the bootstrapping analyses 1000 pseudoreplicates were generated. FigTree 1.4.0 (Rambaut and Drummond 2012) was used to visualize the trees.

## Results

The total alignment of the COI gene consisted of 646 bp including 53% variable sites and 48% parsimony-informative sites. The analysis of the partial mitochondrial COI gene amplified from 59 individuals revealed 20 different haplotypes. Among them individuals were identical for 14 species except *Parapodisma subastris*, *P. setouchiensis*, and *P. tenryuensis*. The model F81 + G (gamma distribution shape parameter  $G = 0.6220$ ) was determined to be the most justified.

The Bayesian inference and maximum likelihood analyses resulted in similar trees, the only differences between them being the degree of statistical support for the recovered nodes (Fig. 2). Nodal supports were generally poor across all backbone nodes. ML bootstrap percentages were lower than BI posterior probabilities. The relationship between Podismini and the related tribes were not fully resolved and varied depending on the nodes.

Melanoplinae were divided into six distinctive lineages and appeared as a polytomy of four clades (II – VI). Dactylotini (I) was placed as sister to the other five lineages. The second and third lineages (II and III) consisted of two genera (seven species) and one genus (three species) of Podismini, respectively. The fourth clade (IV) clustered the genera of Dichroplini. Within clade five (V),

Melanoplinae formed a monophyletic group with strong support [posterior probability (PP) = 1.00, bootstrap value (BV) = 77]. The sixth clade (VI) was constituted of the rest of the members of Podismini and Jivarini.

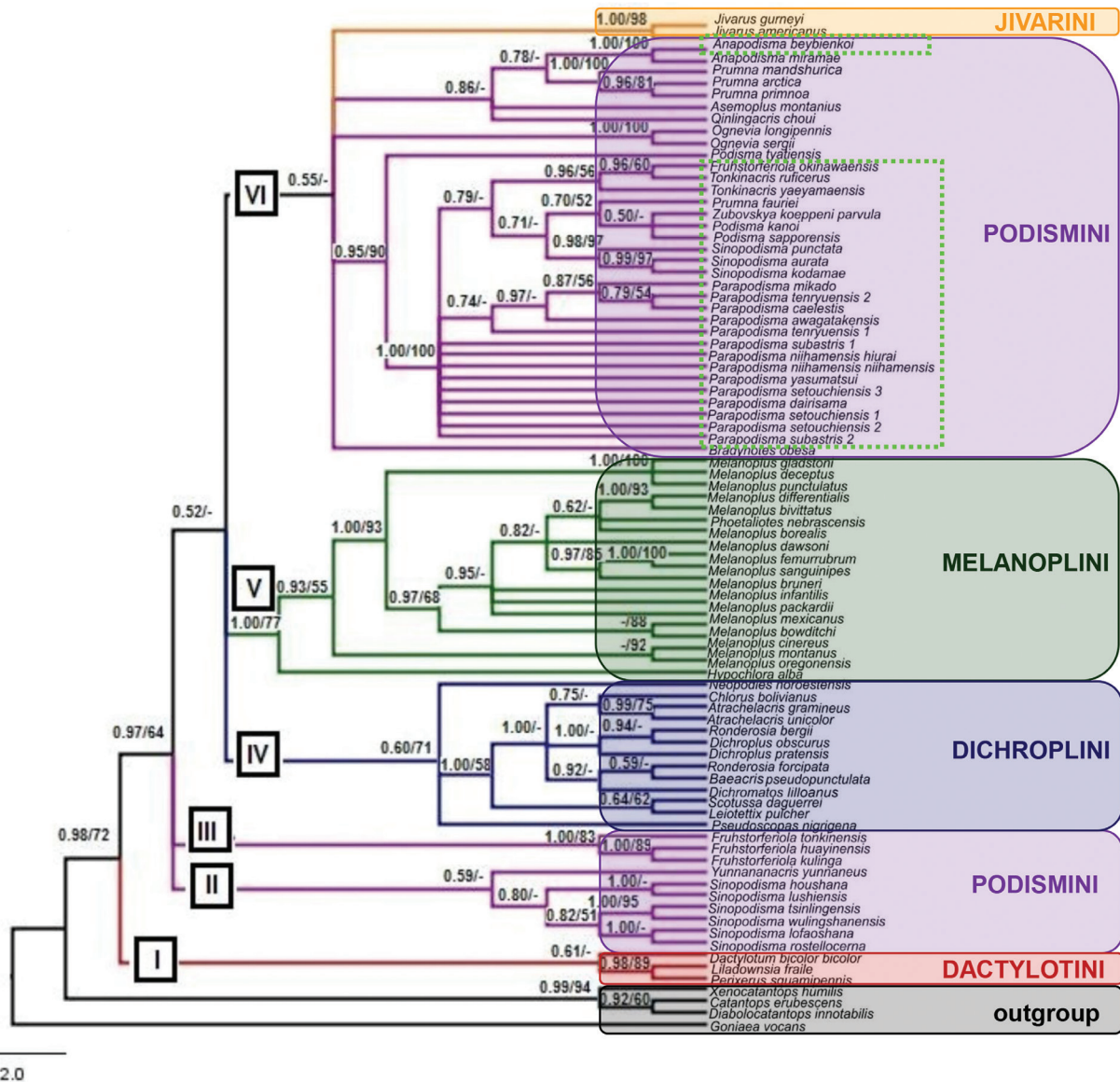
Thirteen genera of Podismini included in this study formed three separate clades. The Japanese Podismini, except *Anapodisma beybienkoi* Rentz & Miller, 1971, were placed in a well supported subclade with high nodal support (PP = 1.00, BV = 100) within clade VI. Nine species of *Sinopodisma* and four species of *Fruhstorferiola* included in the analysis nested in different clades. The majority of Podismini species formed clade VI together with Jivarini species. The basal relationships within clade VI were not resolved. Clade VI consisted of 13 branches with a single terminal taxon: nine species of *Parapodisma*, *Bradynotes obesa* (Thomas, 1827), *Podisma tyatiensis* Bugrov & Sergeev, 1997, *Qinlingacris choui* Li & Feng, 1991 and *Asemoplus montanus* (Bruner, 1885), and five subclades including members of three podismine subtribes. Tonkinacridina comprising *Parapodisma*, *Tonkinacris*, *Fruhstorferiola* and *Sinopodisma* did not constitute a single clade. Among 11 species of *Parapodisma*, *P. tenryuensis* Kobayashi, 1983 (two haplotypes), *P. caelestis* Tominaga & Ishikawa, 2001, *P. mikado*, and *P. awagatakenensis* Ishikawa, 1998 were clustered together with moderate statistical support (Fig. 2).

## Discussion

The present study obtained some interesting results with respect to the relationships within Japanese Podismini. The subclade of Japanese Podismini within clade VI (indicated with light green frames in Fig. 2) included genera which have been attributed to three podismine subtribes, but Tonkinacridina did not form a single clade. Two different methodological inferences on phylogeny (BI, ML) yielded mostly congruent nodes, but the trees were poorly resolved (Fig. 2). Most taxa were determined within a large polytomy of Podismini, in which only a few clades have been recovered. Support remained generally low for the deeper nodes, as was expected for a phylogeny constructed using COI only, but some more derived nodes had higher values (Fig. 2).

Our results are compared with tree inferred by Chintauan-Marquier et al. (2014) who were the first to show molecular phylogeny of Eurasian Podismini including nine Japanese species. The most important finding is that Podismini did not constitute monophyly as previously suggested in Chintauan-Marquier et al. (2014), but there are some incongruent patterns between the two. In the present results, most of the species of Japanese Podismini, except *Anapodisma*, constituted a single clade (Fig. 2), whereas species belonging to Podismina and Miramellina constituted separate clades from Tonkinacridina in the previous molecular study (Chintauan-Marquier et al. 2014). Although the statistical support was not very strong, a monophyly of Tonkinacridina was supported in the previous study, a view concordant with morphological inspection (Ito 2015). On the contrary, our data placed the continental species of Tonkinacridina in different clades (clade II and III in Fig. 2) from Japanese Tonkinacridina. Of course, strict comparisons between these studies are impossible at this stage since continental Tonkinacridina was not included in the previous dataset (Chintauan-Marquier et al. 2014). The view of monophyly in Tonkinacridina is quite doubtful. We can postulate that the observed continental and Japanese species of Tonkinacridina assigned in different clusters reflect somewhat historical migration events coupled with geological processes described above and subsequent convergent/parallel evolution has eventually accumulated





**Figure 2.** Phylogenetic tree of Podismini based on the Bayesian analysis (BI) of concatenated COI sequences. BI posterior probability (PP) and maximum likelihood bootstrap values (BV) are shown near resolved branches (only support values above 50% are shown) as PP/BV. The respective clades are marked with a square and Roman numeral. We examined *Ognevia longipennis* from China because of the availability and thus did not treat this specimen as Japanese Podismini (see also text). Light green frames denote the Japanese Podismini analyzed in the present study.

in morphology. This conjecture could be evaluated by estimating coalescent time of clades using a mitochondrial clock.

In the genera compared, *Parapodisma* is particularly interesting because this includes a vast variety of morphological variation in genital and external characters (Kawakami 1999, Kawakami and Tatsuta 2010), while almost no variation in karyotype exists in contrast to morphology (Inoue 1985). Even in the same species, various forms in forewings and body colors are often found and thus have caused synonymous species/subspecies (Kawakami 1999). This taxonomic disorder still continues in this group partly because there is no robust phylogenetic tree that enables to disentangle “genuine” relationships from homoplasy in morphology. Unfortunately, most species constituted polytomy because of a lack of statistical power, a subclade comprised of closely related species, *Parapodisma mikado*, *P. tenryuensis*,

*P. caelestis*, and *P. awagatakensis* was detected (Fig. 2). While *P. mikado* shows an extended distribution from vicinities of northern Japan and Russia such as Sakhalin, Kunashir, and Hokkaido to the middle of Honshu, the other three species are distributed in narrower regions in Honshu. In particular, populations of *P. caelestis* are limited to narrow habitats such as flower fields with a variety of wild grass and alpine flora on the top of mountains and *P. awagatakensis* inhabits patchy forest edges with very low population density; thus are considered to be vulnerable to unexpected environmental degradation. According to the cladistic assessment in morphology, *P. awagatakensis* was clustered together with *P. mikado* and *P. dairisama*, whereas *P. tenryuensis* constituted holophyly with *P. caelestis* and *P. takeii* (Takei, 1914) (this species is not included in our study) (Ito 2015), a result dissimilar to the present molecular relationship. Rigorous character sampling

with additional molecular data is definitely required for resolving the complex relationship between morphological and genetic similarity. We also have to pay attention to possible hybridization between partly sympatric species, while no clear evidence for this has been obtained even in closely related species (Kawakami and Tatsuta 2010).

The genus *Sinopodisma* emerged as a highly paraphyletic group in which species did not appear closely related and nested in different clades. Likewise, although *Sinopodisma punctata* resembles *S. kodamae* (Shiraki, 1910) in several morphological features such as body color and genital appendages in comparison with *S. aurata* Ito, 1999, the inferred tree supports the closer relationship between *S. aurata* and *S. kodamae*. Furthermore, most of the continental species of *Sinopodisma* are distinguished from *S. punctata* and *S. aurata* in respect of the features in pronotum and cerci (Ito 2015). We postulate that the morphological similarities within *Sinopodisma* are the result of convergent evolution; further intensive studies based on molecular data are definitely necessary for the reliable underpinning of phylogenetic relationships.

The present investigation generated additional evidence for the relationships within Melanoplineae. In present trees, Dichroplini species were recovered as a monophyletic group, in agreement with the analysis of Chapco (2006) and Woller et al. (2014). On the other hand, Chintauan-Marquier et al. (2011) found the paraphyly of Dichroplini. In our analysis, Dactyloptini and Melanopline species each formed a monophyletic clade. Previous studies of Dactyloptini including *Hesperotettix viridis* Thomas, 1872 discovered that this tribe is paraphyletic (Chapco 2006, Chintauan-Marquier et al. 2011, Woller et al. 2014). The prior analysis of the melanopline tribes placed Jivarini in a basal position in the subfamily (Amédégno et al. 2003, Woller et al. 2014). In our results, Jivarini species were clustered together with Podismini representatives. Different studies (Litzenberger and Chapco 2001, Chintauan-Marquier et al. 2014, Woller et al. 2014) recovered Podismini as a monophyletic group, while Litzenberger and Chapco (2003) hypothesized a paraphyly of Podismini.

Although a single mitochondrial gene may lead to a half answer for the whole picture of relationships of higher taxa, the present study provides some significant implications of phylogenetic position. One of the great merits of this study is that the gene has extensively been used for DNA barcoding studies in insects, including grasshoppers, which enables us to examine a store of sequences in a global database (Cameron 2014). The selected mitochondrial COI gene allowed us to estimate intra- and interspecies relationships because of the presence of both variable and conserved regions as well as a heterogeneous evolutionary rate across the gene (Lunt et al. 1996). Simultaneously, we also should keep in mind that the shorter COI gene sequences may include paralogous nuclear mitochondrial pseudogenes (numts) that are apt to induce incorrect inference for phylogenetic relationships (Song et al. 2008, 2014). We need further investigations with orthologous genes for elucidating the distinct phylogenetic position of taxa of interest.

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# Phenotypic plasticity in color without molt in adult grasshoppers of the genus *Sphingonotus* (Acrididae: Oedipodinae)

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## Abstract

Homochromy (i.e. that individuals have a similar color as their environment) is frequent in grasshoppers, and probably functions to reduce detection by potential predators. Nymphs of several soil-perching grasshopper species are known to show color changes during development that increase homochromy, with color being determined with each molt. While this is well documented for young individuals, the only color change in response to the environment that has been recorded for adult grasshoppers of these species is an overall darkening of the individual when exposed to dark surfaces. Whether grasshoppers can also adaptively change color hue is relevant for our understanding of the evolution of locally adapted crypsis. We therefore exposed two groups of adult grasshoppers to a bluish-gray substrate or a reddish-brown substrate, and recorded their color over time. Quantitative digital image analysis showed that adult soil-perching grasshoppers remained capable of adapting to changes in the color of their surroundings through a plastic response. Compared to nymphs, the changes are not as strong and much slower. We suggest that color change in adults occurs through the ongoing deposition of melanins, with eumelanin making individuals more bluish-gray and pheomelanin making individuals more reddish-brown. The fact that color change is possible but slow supports that other mechanisms, such as habitat choice or selective predation, may also play a role in adapting local populations to substrate color. In addition, the ability of these grasshoppers to produce different melanins in response to the environment supports a previous suggestion that they might be useful in the future development of animal models to study melanin-related diseases like melanoma and Parkinson's disease.

## Key words

homochromy, crypsis, color change, image analysis, habitat choice, pheomelanin, eumelanin

## Introduction

Crypsis is a well known anti-predation mechanism observed in a wide variety of species. It is common among plant- and ground-dwelling insects such as mantises, phasmids, grasshoppers and bush crickets. Adaptive phenotypic plasticity (the ability of a single genotype to change its phenotype in response to environment cues) is often key to optimizing crypsis in animals whose habitat is heterogeneous through space or time (Umbers et al. 2014,

Valverde and Schielzeth 2015, Kang et al. 2016). For instance, for grasshoppers four types of color change have been recorded: green-brown morph switching, color pattern changes, hue shifting and blackening (Rowell 1972). While color pattern has often been found to be determined mostly by genes and maternal effects (Karlsson et al. 2009, Forsman 2011, Karpesta et al. 2012b), hue variation is thought to be driven to a great extent by an adaptive plasticity response to environmental cues, as the new overall color tends to match that of the subject's surroundings (Ergene 1955, Yerushalmi and Pener 2001, Valverde and Schielzeth 2015). There is also evidence of blackening in response to temperature and exposure to solar radiation as well as dark substrates (Forsman 2011, Karpesta et al. 2012a, Valverde and Schielzeth 2015). Green-brown morph determination and switching remains the less understood of the four types of color change but there is positive evidence for both genetic and environmental influences (Rowell 1972, Valverde and Schielzeth 2015). The environmental cues that are used for adaptive plasticity to enhance crypsis are often unknown, but effects of temperature, humidity and/or visual input have been recorded (Rowell 1972, Umbers et al. 2014, Valverde and Schielzeth 2015).

The family Acrididae (which contains amongst others the band-winged grasshoppers and locusts) is the most studied group of grasshoppers. This is partly because it is the largest and most widespread family, can be responsible for tremendous agricultural losses, and is used in many countries for human consumption. They often show homochromy, and those species that perch on the ground tend to strikingly resemble the color of their local substrate. In his revision of this family's variable coloration, Rowell (1972) proposed three non-exclusive causes for this local adaptation in color: differential predation, color change, and habitat selection. He deemed the first one immediately acceptable, and additive effects of the other two seemed logical to attain an almost perfect color match. However, studies regarding the color change in Acrididae grasshoppers have normally been conducted with nymphs or, at most, recently metamorphosed adults (Valverde and Schielzeth 2015) while fully developed imagoes, though indeed cryptic, have not received as much attention.

Apart from neglect of plasticity in adults, another problem with most earlier studies is that assessments of color change have been

done rather subjectively, either assigning individuals to discrete arbitrary color levels or comparing the subject's color to standard charts which do not allow true quantitative analyses. To overcome these limitations, when possible, it is preferred to use objective digital image analysis as a less biased approach (Stevens et al. 2007).

We have used this approach to study the color matching of azure sand grasshoppers, *Sphingonotus azurescens* (Rambur) (Orthoptera: Acrididae: Oedipodinae) colonizing distinctly colored novel urban habitats in Seville, Spain. While this species naturally occurs on open sand or clay soils with little to no vegetation, we have recently found them to locally also use abandoned man-made surfaces (sidewalks, bicycle paths, asphalt roads) for perching, feeding, courtship, and reproduction. We found that individuals using these pavements were consistently more cryptic on their local pavement than on other, adjacent pavements. This shows that these grasshoppers are able to adapt their color to fine scale environmental variation, even when it involves novel materials (asphalt, bricks, tiles) that historically they have not interacted with (Edelaar et al. in preparation). Given the degree of daily movement of these grasshoppers (on average 12 meter/day, Edelaar et al. in preparation), this observed fine scale population differentiation in color among pavements should quickly cease to exist, unless something helps to maintain color divergence. Surprisingly, previous studies show that this appears to be mainly due to habitat choice, since selective predation and color change appeared to be too weak and too slow, respectively, to explain the observed patterns (Edelaar et al. in preparation). With respect to color change, this conclusion rests on the observation that the response to a black background is limited and slow, and virtually nonexistent when a white background is used (Edelaar et al. in preparation, see also Ergene 1953). However, the different pavements do not only differ in darkness but also in hue, and it has not yet been tested whether changes in hue in response to background color are possible in adult azure sand grasshoppers, nor in any other grasshopper species.

There is an additional reason why color plasticity in these adult grasshoppers deserves a closer look. The reddish color displayed by azure sand grasshoppers has recently been reported to be related to the presence of pheomelanin, a pigment formerly thought to be restricted to vertebrates only. Interestingly enough, this pigment's pathway features mixed-type melanins arising from both dopamine and DOPA, a process that in vertebrates has only been reported for neuromelanin (Galván et al. 2015). This is important because of the link between neuromelanin and Parkinson's disease. Grasshopper (and maybe other insect) species with this kind of biochemical pathway may therefore play a role in the future investigation of this pigment's link to this important disease (Galván et al. 2015). Being able to change the production of pheomelanin in adult grasshoppers by a change in background might therefore be very useful.

For this variety of reasons, our main objective in this study was therefore to test whether this species of acridid grasshopper is still capable of changing in hue in response to background color, even after reaching full maturity.

## Methods

*Study subject.*— The azure sand grasshopper is a ground dwelling species which predominantly inhabits little vegetated, xeric scrublands and grasslands and perches on the bare soil rather than on vegetation. They show a base coloration that varies from reddish-brown to bluish gray, and these colors can vary from being very



**Figure 1.** Example of an image taken for color measurement. The individual was held in place by the transparent plastic lid of the Petri dish to obtain a correct position. Brightness and hue were measured in the red diamond-shaped part of the thorax. The color of a small area of the background paper was also measured (red circle) as a reference gray standard to correct for lighting variation among images.

pale to very dark. Color variation is continuous and there is no known green-brown polymorphism in this species. Their base color generally resembles that of the substrate surrounding the individual. They also show a variable pattern of dark markings, and a pale band halfway down the anterior pair of wings and the hind legs which helps to disrupt their outline and therefore provides additional crypsis (Fig. 1).

Nineteen individuals were collected in late August and early September 2013 in the vicinity of Dos Hermanas (Seville, Spain) and kept as a group for two months in one of two transparent plastic boxes (30 × 40 cm). These boxes were each filled with either "blue" (fine bluish gray gravel) or "red" (red-brown earth) substrate, in order to test if adult grasshoppers would change their color accordingly. Individuals were either assigned to the treatment "blue" (10 individuals - 8 males and 2 females) or "red" (9 individuals - 5 males and 4 females). Individuals were marked by writing a number on their anterior wing tips with a water- and light-resistant marker pen. Food was a mixture of dried wheat bran (45%), dried mosquito larvae (45%), and powdered milk (10%) (the species is omnivorous). Bottled mineral water was available in the form of a gel (ReptiGel). Ambient light was provided by regular fluorescent ceiling lamps from 8:00 to 20:00 hours, and heat was provided from below using heating mats for terrariums in order to keep ambient temperature between 35 and 40 degrees Celsius.

*Image production and color measurement.*— At the beginning of the experiment and for the duration of the treatments, we regularly took photographs of each individual to subsequently measure its color (Fig. 1). The experiment was terminated after 49 days, when

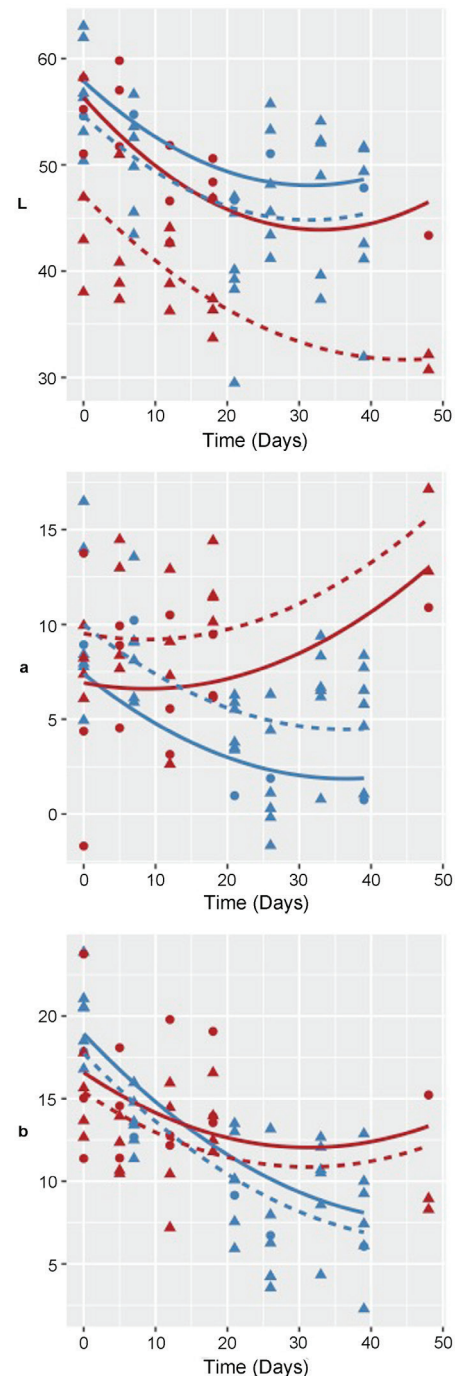
most individuals had died of old age. In order to create comparable images, all photographs were taken using the same setup: a Pentax K-r DSLR camera with a dual lamp Mecablitz 15MS-1 diffuser macro flash, mounted on an 18-55mm Pentax kit lens. The lens was always locked at 55mm and the camera set to shoot in RAW format with fixed manual settings ( $f/14$  aperture,  $1/50$  shutter speed, ISO 200). Each image featured a 90% reflectance gray paper background to control for white balance and flash intensity (Fig. 1). The grasshoppers were held in the same position (offering a full dorsal view, with the head pointing to the top of the image) and at the same distance from the lens by pressing them into a base of cotton wool with the transparent plastic lid of a Petri dish. A diamond-shaped zone from the posterior part of the grasshoppers' pronotum was used to measure color over time for each individual, as this area is flat and representative for overall individual color (Fig. 1).

Any color difference between grasshoppers from different treatments could also be due to soiling of the individuals with fine dust from the substrates. In fact, this happened in a first trial with finely mashed up red soil, so we restarted this treatment three weeks later with newly collected individuals from the same area using intact, natural pieces of red soil (which has caused a shift in the timing of sampling for each treatment and may account for differences in color between treatments at the beginning of the experiment, since older grasshoppers tend to be darker, see Fig. 2). We therefore thoroughly cleaned all subjects which were still alive when the experiment was finished and compared the color change between treatments ("Cleaned" dataset,  $N=5$ ). To clean the grasshoppers, individuals were first frozen to rapidly kill them, and then rubbed with a piece of cotton dipped in water with dishwashing detergent and cleared under running water. Images were taken when the cleaned individuals were dry again. Few grasshoppers reached this cleaning stage due to the mortality inherent to working with older individuals, and only live grasshoppers could be tested since color changes rapidly after natural death.

Color was objectively measured using the color analysis features of the Mica Toolbox plugin (Troscianko and Stevens 2015, we used version 1.12 Mac) for imageJ (Schneider et al. 2012). Briefly, camera RAW image files were transformed into multispectral images using a Human cone-catch model. We used this model since measures with a photospectrometer showed that neither the grasshoppers nor the natural and urban substrates reflect UV radiation. The XYZ values obtained from these images were converted to the CIE- $L^*a^*b^*$  color space for easier interpretation of luminosity and hue variation. CIE- $L^*a^*b^*$  defines colors using 3 independent axes: L for lightness, a for green-to-red hue and b for blue-to-yellow hue. Higher L, a and b values describe a lighter, redder, and yellower color, respectively. Preliminary experiments indicated that color values were highly repeatable among images taken for the same individual on the same day, so in this study only one image was taken per individual per day.

*Statistical analysis.*— The statistical analysis software R (R Core Team 2015) and the packages lme4 (Bates et al. 2015), ggplot2 (Wickham 2016) and gridExtra (Auguie 2016) were utilized to visualize data, test our hypotheses and plot results.

To test for a differential change in color between individuals exposed to the distinctly colored soils, a linear mixed model was fitted to the "Change over time" measurements on each CIE- $L^*a^*b^*$  axis. This model contained Substrate (red or blue), Sex,



**Figure 2.** Average changes in color over time (days since the start of each treatment) for adult azure sand grasshoppers kept on bluish-gray stones (blue symbols and lines) or reddish-brown soil (red symbols and lines). Color is expressed in the three independent dimensions of the CIE- $L^*a^*b^*$  space (see text). Lines show the predicted values for each CIE- $L^*a^*b^*$  dimension according to a fitted model containing Time, Substrate, Sex, Time:Substrate and sqTime; we excluded the subject random effect for better visualization. Continuous lines and circles are for females while dashed lines and triangles are for males. The "Red Earth" treatment had to be restarted using grasshoppers from the same field location but captured later in time. As a result, the timing of data collection is out of phase between treatments, and L is initially slightly lower for "Red Earth" individuals (since the species gets darker with age).

Time (since the beginning of the experiment, in days), Squared time (to fit non-linear change) and the Time:Substrate interaction as fixed effects. This interaction tests our main hypothesis that any color change over time depends on the substrate. As a random effect, we added Subject (individual identity) to correct for the repeated nature of the data.

To check that any differential color changes were not due to soiling, we also fitted similar linear mixed models to the "Cleaned" dataset. This model containing Substrate (the treatment), Status (living at the beginning versus dead + cleaned at the end), sex and Substrate:Status interaction as fixed effects and Subject again as a random effect.

The statistical support for any effects on color was determined by comparing the AIC value of our full model to that of a similar model lacking the studied effect. Given a set of statistical models that try to explain the same data, the AIC (Akaike Information Criterion) gives an estimation of the relative quality of each one of them, based on likelihood and with a penalty for including more parameters. The lower the AIC value of a model, the stronger the support for that particular model. For comparison with more traditional ways of testing for statistical significance, the AIC difference is presented with a p-value obtained by a loglikelihood-ratio test that compared the same two models as described above.

## Results

Regarding the color change of live grasshoppers over time, substrate: color showed a non-significant effect on grasshopper lightness (DAIC=0.04,  $p=0.16$ ,  $c^2=1.96$ ,  $df=1$ ), and no differential change in lightness over time (Fig. 2). In contrast, hue values were influenced by the substrate on which individuals were kept (Fig. 2): the Time:Substrate interaction received considerable statistical support for both the green-red axis a (DAIC=-11.9,  $p<0.001$ ,  $c^2=13.9$ ,  $df=1$ ) and the blue-yellow axis b (DAIC=-8.4,  $p=0.001$ ,  $c^2=10.4$ ,  $df=1$ ). Individuals kept on bluish stones turned to a duller, more desaturated color while the ones kept on the red earth became relatively more red and yellow colored.

Regarding the color change of grasshoppers after freezing and cleaning, we also found strong statistical supports that substrate color influenced adult grasshopper color, although the patterns were somewhat different to those for live grasshoppers (Fig. 3). The interaction Substrate:Status was strongly supported for L (DAIC=-8.02,  $p=0.002$ ,  $c^2=10.02$ ,  $df=1$ ), for a (DAIC=-16.08,  $p<0.001$ ,  $c^2=18.08$ ,  $df=1$ ) and for b (DAIC=-17.33,  $p<0.001$ ,  $c^2=19.34$ ,  $df=1$ ). Compared to the start of the experiment, and after being frozen, grasshoppers kept on red earth turned darker, a bit redder and a bit less yellow, a pattern that is very similar to the live grasshoppers. In contrast, grasshoppers kept on blue stones became much darker, redder, and more blue, a pattern that is quite different from the live grasshoppers. This conforms to our visual assessments (see Fig. 4): they showed a much darker, purplish color after being frozen and cleaned.

## Discussion

Our results show that color change in response to the color of the substrate is not restricted to nymphs but also occurs in fully developed *S. azurescens* imagoes (Figs 2-4). These changes are similar in appearance to those we have seen in nymphs of this species (Edelaar et al. 2017, pers. obs.), as well as those reported for nymphs in other Acrididae species (Rowell 1972). Individuals changed their color in such a way that they increased resemblance

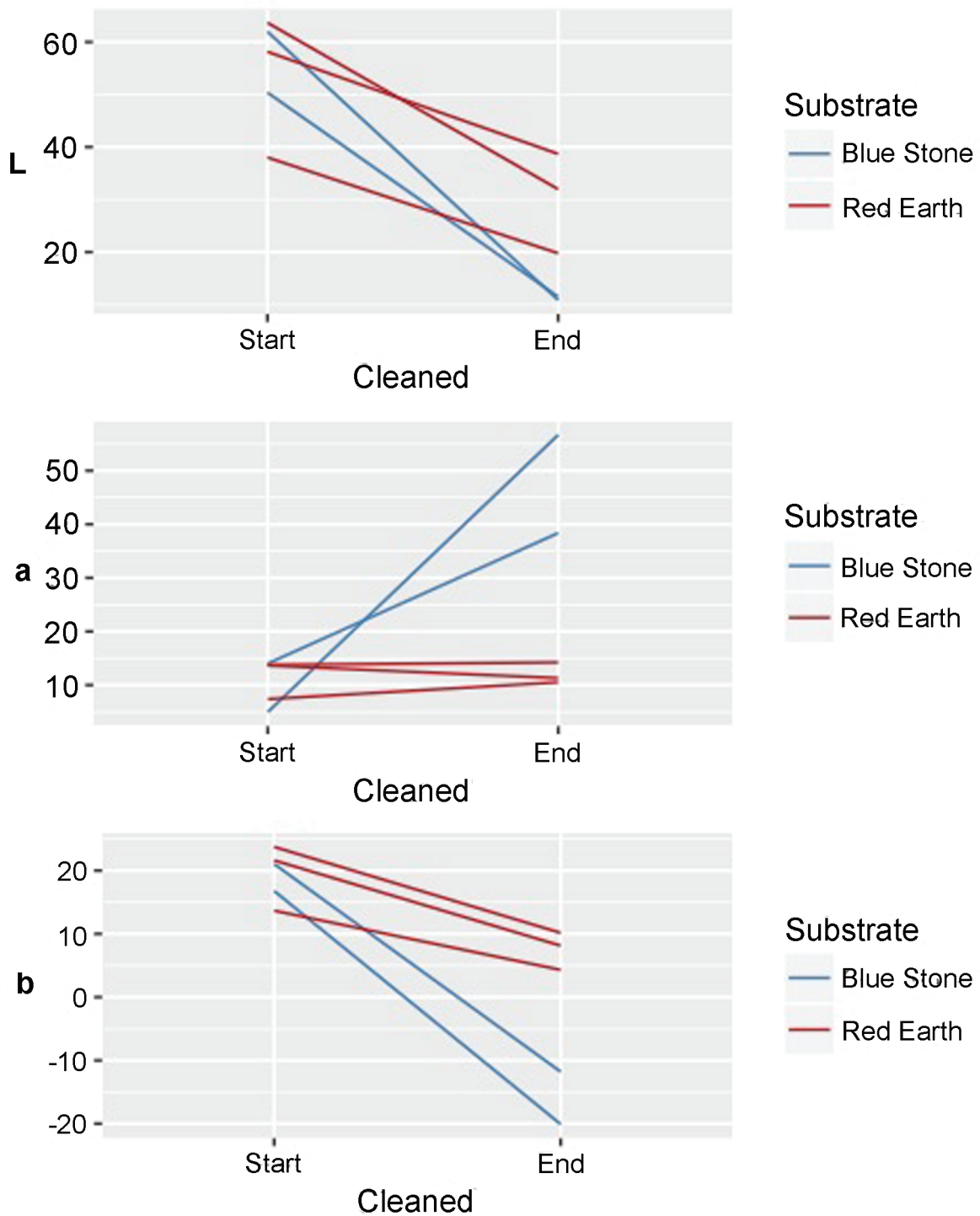
(at least to the human eye) to that of the substrate on which they were kept. It is therefore likely that the observed color change is due to direct visual input, and functions to increase crypsis and reduce predation rate under field conditions, as suggested before (Rowell 1972, Cox and Cox 1974, Edelaar et al. 2017). As far as we know, such adaptive changes in color hue have not been recorded before for adult grasshoppers, only for nymphs.

Apart from changes in hue, we also observed changes in lightness, with individuals in both treatments becoming darker over time. This is in agreement with previous experiments we have performed with this species (Edelaar et al. 2017, pers. obs.). These have shown that nymphs are capable of adaptive plasticity in darkness and can become darker or paler, depending on the rearing substrate (and even more so when exposed to simulated predation risk: Edelaar et al. 2017), while adults generally turn darker over time, but more so when kept on a dark substrate. Whether the observed darkening in this experiment is due to aging or due to substrate matching is not known, but it is clear that adults can change color over time.

Exactly how this is done cannot be addressed with our data, but a likely explanation given the combined results and observations is that pigments can still be deposited in the cuticle of adults, but cannot be removed afterwards. This would explain why a medium gray adult placed on a white background will virtually stop darkening over time, but it will not become paler (i.e. a poor cryptic coloration persists), whereas nymphs can become paler when they molt. The color changes we observed in response to the different substrates are then likely due to the deposition of different pigments: more brownish ones when kept on brown soil, and more gray ones when kept on gray stones. In both treatments, such deposition of additional pigments to change an individual's hue is in line with the observed general darkening. That several pigments are involved is also hinted at by the distinct color differences observed after freezing and cleaning: apparently the effects of freezing differed between the individuals from the different treatments, because the procedure was identical for both groups and the color change for individuals kept on gray stones from darkish gray towards more violet would not be expected if the color difference was simply due to external pollution. Moreover, the individuals from this same experiment were subsequently used for pigment analysis (Galván et al. 2015). That analysis not only reported one of the first demonstrations of pheomelanin (the brown version of melanin) in non-vertebrates, but it also found that the grasshoppers kept on brown soil were rich in pheomelanin, while the grasshoppers kept on gray stones were rich in eumelanin (the black version of melanin) (see Galván et al. 2015, online Supporting Information). Hence, at least part of the changes in color we saw in response to manipulated substrate color can be explained by the presence (and probably *de novo* deposition) of the different chemical forms of melanin.

A noteworthy observation is that the color changes seem to become slower as time passes (Fig. 2). This might be because grasshoppers were reaching the coloration they aimed for, but alternatively the ability to adaptively change color decreases with age or with the amount of pigment already deposited. Our experience with this species is that, although similar in direction and overall appearance, the adaptive color change is remarkably slower in adults than it is in nymphs. This has behavioral and ecological implications. Given that adults are around for a longer time than nymphs, and that (larger, flying) adults are more mobile than nymphs, one would expect that adults come into contact with a greater range of differently colored substrates.

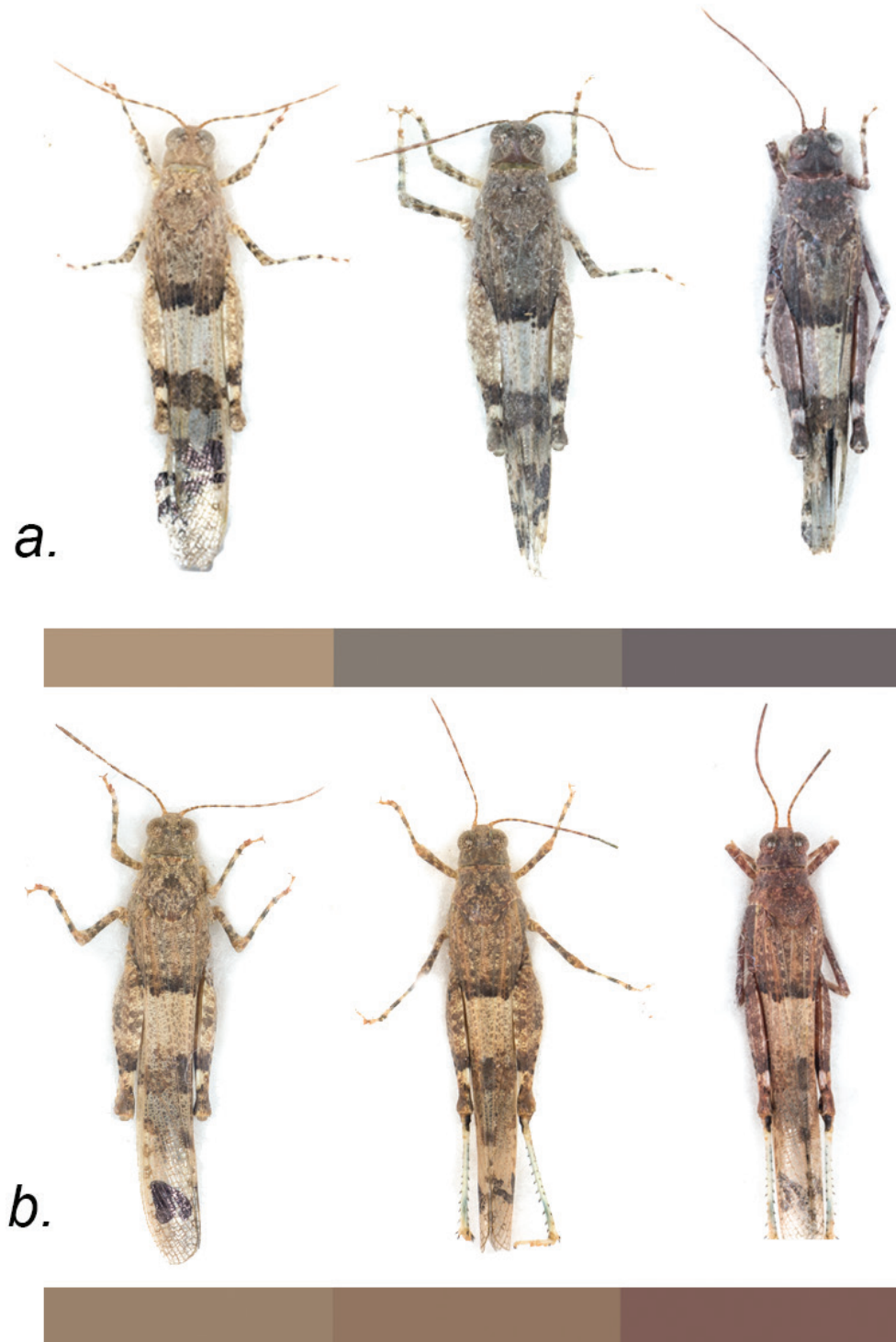




**Figure 3.** Individual changes in color in the final surviving azure sand grasshoppers when kept on bluish-gray stones (blue lines, N = 2) or reddish-brown soil (red lines, N = 3). The comparison is between the same individuals at the beginning of the experiment and once frozen and cleaned at the end. Color is expressed in the three independent dimensions of the CIE-L\*a\*b\* space (see text).

Yet local populations of adult grasshoppers are typically quite well matched in color to their local environments, even if these environments are very close to each other in space. Edelaar et al. (in preparation) describe a situation in which grasshoppers

living on differently colored, adjacent pavements (e.g. dark asphalt roads compared to pale sidewalks) are locally adapted in color, despite the fact that the observed degree of movement (on average 12 meter/day) would predict a homogenization of



**Figure 4.** Change in color over time for two example individuals. From left to right: at the start of the experiment, 7 weeks later, and after freezing and cleaning at the end of the experiment. Individual **a.** was kept on blue-gray substrate and individual **b.** was kept on reddish-brown substrate. For visualization, the bars under the images show the average dorsal color as captured by the CIE-L\*a\*b\* values measured for each individual at each point in time.

color across pavements in one day. The fact that the plastic color change as reported here is adaptive but relatively slow supports the conclusion that this local adaptation is in fact mostly driven by the grasshoppers' selection of the environment, with

individuals choosing their perching pavement as a function of their own color in order to increase crypsis.

The plasticity in the production of pheomelanin in grasshoppers (both nymphs and adults) might be relevant for future applied studies,

because in humans pheomelanin is associated with increased risk of melanoma in the epidermis (Mitra et al. 2012) and to Parkinson's disease in the brain (Spencer et al. 1998). Finding pheomelanin and understanding its chemical pathways in invertebrates may allow the development of new animal models for these diseases (Galván et al. 2015). Having a simple manipulation technique available to vary the production of pheomelanin (placing individuals on brown soil) would add to the versatility of such a model.

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# Effects of different mowing regimes on orthopterans of Central-European mesic hay meadows

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## Abstract

Method, frequency and date of mowing influence the presence and population size of Orthoptera species, which show strong dependence on the vertical structure of grasslands. Responses of orthopteran assemblages to the effects of various mowing regimes applied to different parts of the same habitat are still not fully understood. In this study, we asked how different mowing regimes (mowing in May; mowing in September; mowing in May and September; abandonment of mowing) influence species richness, Shannon diversity and density of local orthopteran assemblages on a small spatial scale in Central European mesic hay meadows. Furthermore, the study aimed to determine the type of meadow management that is most suitable for preserving local orthopteran assemblages. The date of mowing had no significant overall effect on species richness, density or diversity of grasshoppers. However, grasshopper species richness and Shannon diversity were reduced immediately after mowing (in June sampling of sites mown in May), and rose later in the season. Grasshopper density was low on abandoned sites which were not mowed in the last ten years and there was a negative correlation between orthopteran density and vegetation height. Nymphs, on the other hand, showed elevated density just after mowing which was reduced later in the season. Life forms of the orthopteran assemblages showed dominance of pratinicol species. Silvicol species were found only in abandoned habitats, while arbusticol species were found only on abandoned patches and patches mown in September. Results showed that the long-term preservation of natural orthopteran assemblages in mesic hay meadows would benefit from landuse practices which are diversified spatially and temporally, as practiced in traditional extensive management regimes.

## Key words

density, vegetation height, grassland management, Hungary, biodiversity conservation

## Introduction

Orthoptera (grasshoppers, crickets and katydids) are considered one of the best taxa for the ecological evaluation of the habitat quality and management of grasslands (Kruess and Tschamtké 2002, Batáry et al. 2007, Fartmann et al. 2012). Due to their utility as bioindicators, special attention is paid to the group in practi-

cal nature protection (Noss 1990, Pearson 1994, Déri et al. 2007, Bazelet and Samways 2011). Mowing changes the vertical structure of vegetation, which plays a decisive role in the organization of orthopteran assemblages (Joern 1979, Guido and Chemini 2000). Cutting of the vegetation triggers a marked change in its nutritional value (Smith and Capinera 2005) and microclimatic conditions (Stebaev and Nikitina 1976), affecting the habitat potential for orthopterans. In addition, mowing significantly increases the exposure of individuals to predation (Belovsky and Slade 1993, Braschler et al. 2009). Mowing changes the conditions in a direct way for those species that lay their eggs on the vegetation (Gardiner and Hassall 2009). Indirect effects include microclimatic conditions becoming unfavourable, particularly for those species that lay their eggs in the ground (Stoutjesdijk and Barkman 1992, Wingerden et al. 1992).

It is clear therefore that the method, frequency and date of mowing are all basic factors influencing the presence and the current population size of orthopteran species, as well as the development of the structure of orthopteran assemblages, both in the short and the long-term (Buri et al. 2013). Previous studies found that the mowing operation of grasslands itself has a temporary negative impact on orthopterans as a result of mortality and the loss of plant production (Gardiner and Hill 2006, Gardiner and Hassall 2009, Humbert et al. 2010, Rada et al. 2014). In the medium and the long term, however, species richness, diversity and density of assemblages is highest if regular mowing is applied (Marini et al. 2009), and lowest if the meadow is abandoned (Nagy and Kisfali 2007). Extreme and frequent mowing has a negative effect on both the abundance and the species richness of orthopterans (Marini et al. 2008, Buri et al. 2013).

According to the results by Chambers and Samways (1998), species richness and abundance of orthopterans increases from single mowing per year towards mowing three times per year. In wet and semi-dry grasslands, mowing twice a year usually results in a richer, more structured vegetation of higher-yielding biomass than single mowing, which determines the occurrence of orthopterans (Jutila and Grace 2002). One of the main underlying factors for this relationship lies in the fact that the repeated mowing of the vegetation provides good germination and growth conditions even for less competitive plant species (Parr and Way 1988, Bak-

ker et al. 2002, Bissels et al. 2006). Buri et al. (2013) revealed that small changes in grassland management (e.g. delaying mowing, leaving uncut grass patches) could result in significant positive changes in density and species richness of orthopterans.

Órség National Park is situated at the western border of Hungary and belongs to IUCN category V of protected areas. Plant species rich mesic hay meadows of the national park developed through centuries of human impact. These grasslands were managed traditionally by mowing twice a year (May-June and August-September). Since 1990, due to economic considerations, once-a-year (May-June) mowing became the typical way of management and abandonment became widespread as well (Babai et al. 2015). Around 2000, mowing once-a-year in August-September was introduced on some valuable grasslands due to the prescriptions of the national park administration to protect butterfly species, such as *Maculinea* species (Körösi et al. 2014). To understand the effect of the above listed management regimes on the plant and animal communities of meadows, Órség National Park Directorate launched an experiment on four of its own meadows in 2007. Patches within the same habitat types were subjected to different mowing regimes: mowing once a year at the end of May, mowing once a year in the beginning of September; mowing twice a year both at the end of May and beginning September; abandoned without any management. Study areas were managed just in the above mentioned way: no other management practices were included (e.g. grazing). The abandoned areas were last mowed before 2007. Trees and scrubs were removed, and at the time of fieldwork, abandoned sites consisted of tall-grass vegetation only. Vegetation results of this experiment were gathered by Szépligeti et al. (2016). In 2014, seven years after establishment of the experiment, results showed that the species richness and diversity of the vegetation were the highest at patches of double mowing each year, while the abandoned areas had the lowest values. Areas receiving once-a-year, i.e. spring or autumn mowing occupied an intermediate position in terms of the above parameters (Szépligeti et al. 2016). A keystone species of nature conservation, *Phengaris teleius* (Bergsträsser) butterfly and its host plant, *Sanguisorba officinalis*, showed the highest density on the patch mown once a year in autumn (Körösi et al. 2014).

We aimed to answer the following main question in this paper: how do different mowing regimes influence species richness, diversity and density of orthopteran assemblages of some typical natural Central-European mesic hay meadows? We hypothesised that autumn mowing once a year could result in the highest species richness, diversity and density in local orthopteran assemblages. Furthermore, we make recommendations for mowing strategies to preserve the orthopteran assemblages in these local mesic hay meadows.

## Methods

**Study sites.**— Vegetation of the four study sites were identified as mesophilic hay meadows [*Alopecuro-Arrhenatheretum* (Mathé and Kovács 1960) Soó 1971]. Site I and II (geographical centres: Site I: N46.768, E16.329 / Site II: N46.766, E16.334) were separated by 200 m from each other, while Site III and IV (geographical centres: Site III: N46.737, E16.374 / Site IV: N46.736, E16.377) were located 5 km further downstream in the valley of Szentgyörgyvölgyi stream and also 200 m from each other (Fig. 1). Sites were situated at 210–230 m above sea level and were characterized by alluvial soils. Groundwater was usually close to the surface. The annual mean temperature was 9.5 °C, the annual mean rainfall was around 800 mm (Dövényi 2010).

Within the four sampling sites (Site I–IV) adjacent quadrats of 20 m × 20 m were designated (16 quadrats in Site I and II, 12 quadrats in Site III and IV, see Fig. 1). Quadrats were assigned to four different treatments, and each treatment was applied consistently each year beginning in 2007: (a) mowing once a year at the end of May (M); (b) mowing once a year at the beginning of September (S); (c) mowing twice a year both at the end of May and the beginning of September (MS); (d) abandoned without management (C). Proportion of the treatments was similar on each site: in sites I and II there were four quadrats of each type of treatment (n = 16 quadrats in total per site); and in sites III and IV there were three quadrats of each treatment (n = 12 quadrats in total per site). Mowing was carried out by a tractor powered RK-165 type drum mower.

**Data collection.**— Data were collected three times at each study site in 2015 (June, July, August). The collection of orthopterans was carried out by sweep netting, using 300 sweeps within each quadrat in each meadow. Every sampling of 300 sweeps covered in Site I and II four 20 m × 20 m quadrats (altogether 1,600 square metres) and in Site III and IV three 20 m × 20 m quadrats (altogether 1,200 square metres). Specimens collected per treatment type in each meadow were considered as one sample. To the samples collected by sweep netting we added a simple count of the number of adult specimens which were detected by direct observation/collection. Sweep netted samples were identified to species level (excluding *Chorthippus* nymphs).

Nomenclature of orthopteran species followed the work of Cigliano et al. (2017). Categories of Uvarov (1977) and Ingrisch and Köhler (1998) were used for classification of life forms (arboricol: species found in habitats ruled by tree-sized elements; arbusticol: species found in habitats ruled of shrub-sized items; silvicol: species found in forest habitats with a grass understory; pratinicol: species found in grasslands of tall grass; graminicol: species found in grasslands of short grass).

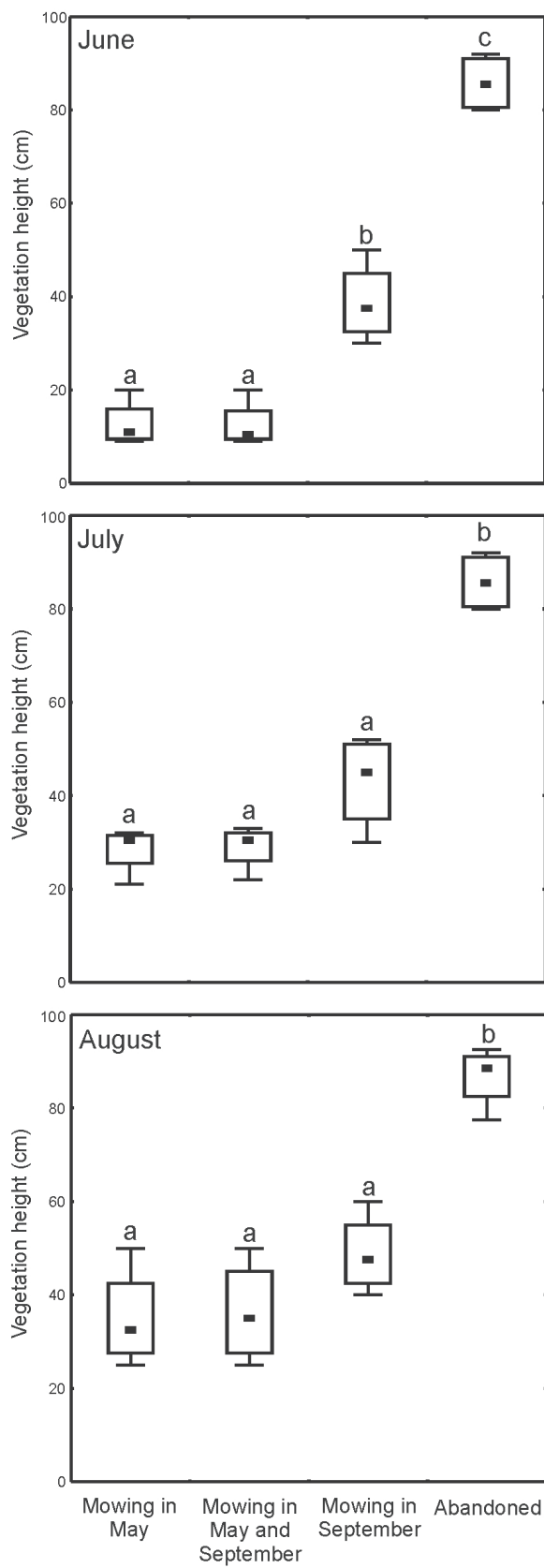
Characterization of climatic requirements of the species as thermophilic, moderately-thermophilic, mesophilic, moderately-hygrophilic, and hygrophilic were assigned based on works of Varga (1997), Rácz (1998) and Ingrisch and Köhler (1998).

**Covariables.**— Microclimate and habitat data (average height and cover of the vegetation) were collected at 2–3 pseudo-randomly selected spots in each orthopteran sampling area. Microclimate was measured by TESTO 625 equipment (air temperature and humidity at the surface of the soil, and at 10, 20, 30, and 120 cm height). Height of the vegetation was measured in cm with the use of a 30 cm wide and 100 cm high white card. Total cover of the vegetation was measured in a square metre quadrat occurring around the spot. Related to each orthopteran sampling, percentage cover of each plant species was estimated. Average values of the data measured in the same orthopteran sampling area were used.

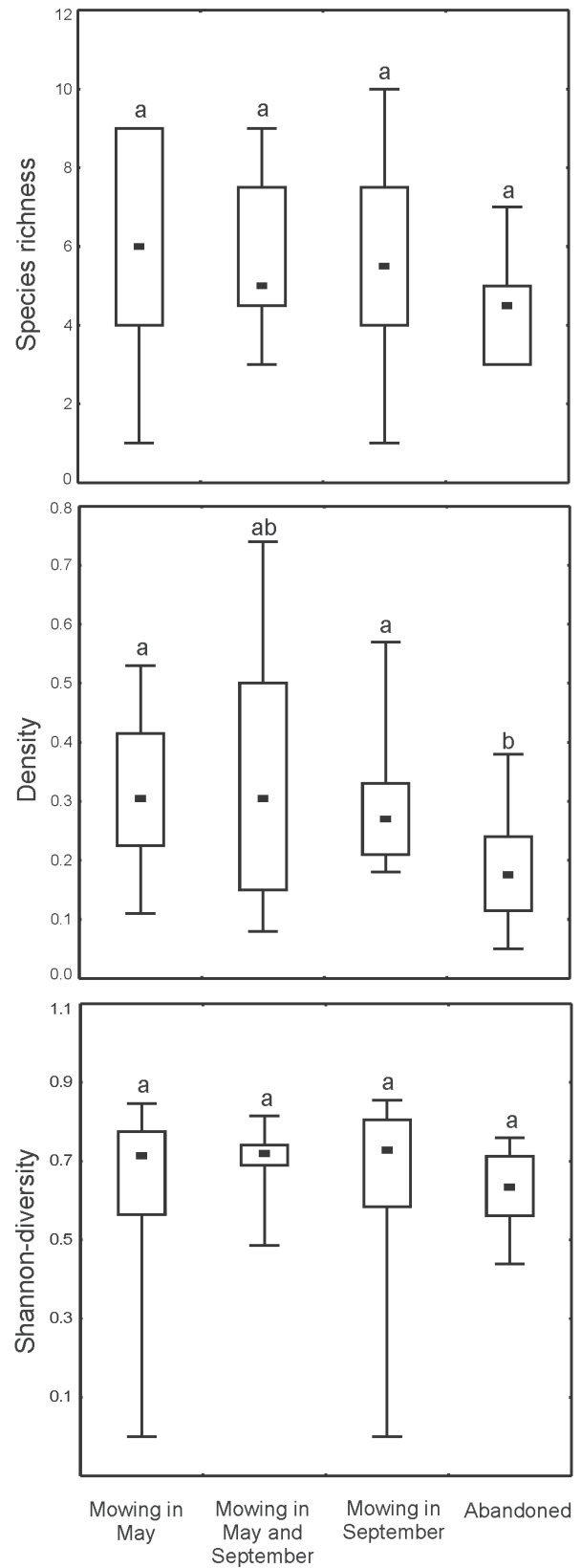
**Data processing and analyses.**— We derived the following variables from field data on orthopterans: (a) species richness; (b) total density of orthopterans (specimens/m<sup>2</sup>); (c) total density of nymphs (specimens/m<sup>2</sup>); (d) Shannon diversity; (e) life-form spectra; (f) ecotype spectra. All samples from the same treatment, site and season were clumped.

We determined the relative values of air temperature and humidity data: data measured at the soil surface/10/20/30 cm height minus data measured at 120 cm height. According to our previous results (Bauer and Kenyeres 2006), the impact of weather condi-



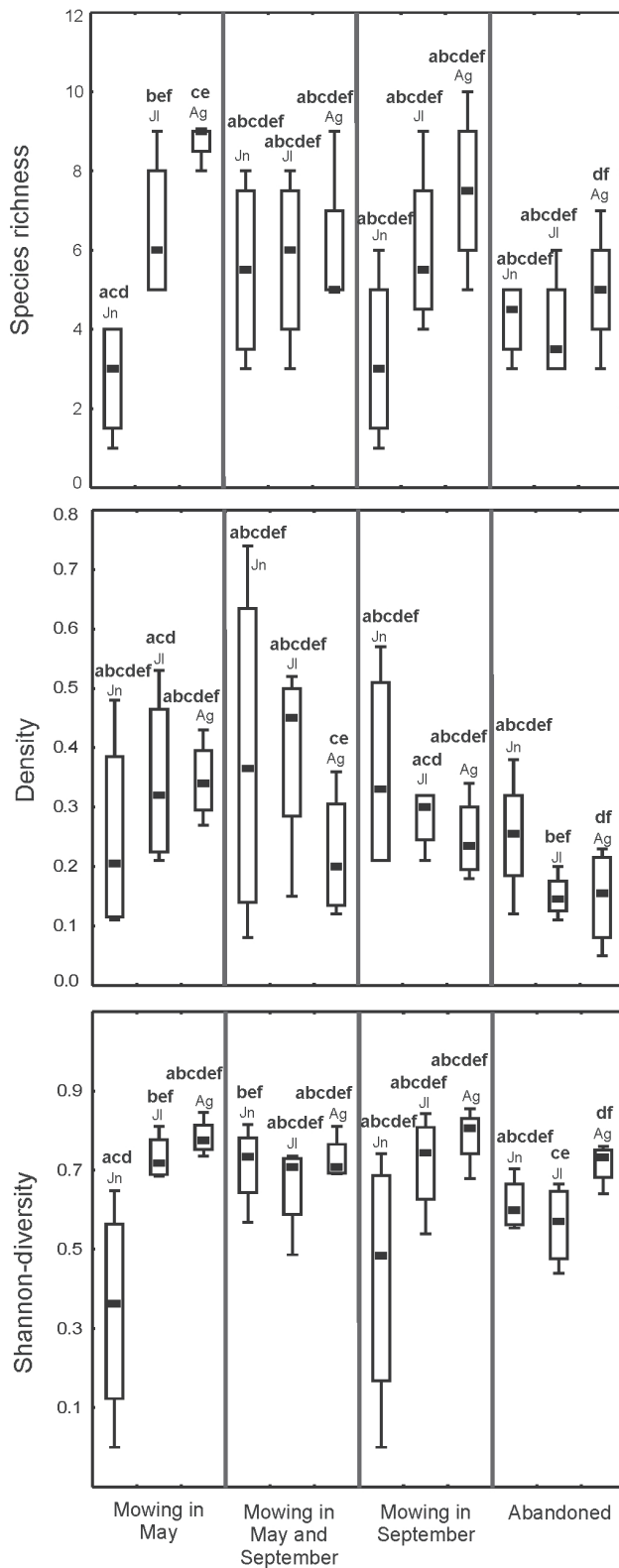


**Figure 2.** Box-plots (median values with minimum, maximum and  $\pm$ SE) of vegetation height in four treatment types. Significant ( $p < 0.05$ ) differences detected by Mann-Whitney U test are indicated by different letters.



**Figure 3.** Box-plots (median values with minimum, maximum and  $\pm$ SE) of species richness, density (specimen/m<sup>2</sup>) and Shannon-diversity of orthopteran in four treatment types of sites I-IV. Significant ( $p < 0.05$ ) differences detected by Mann-Whitney U test are indicated by different letters.





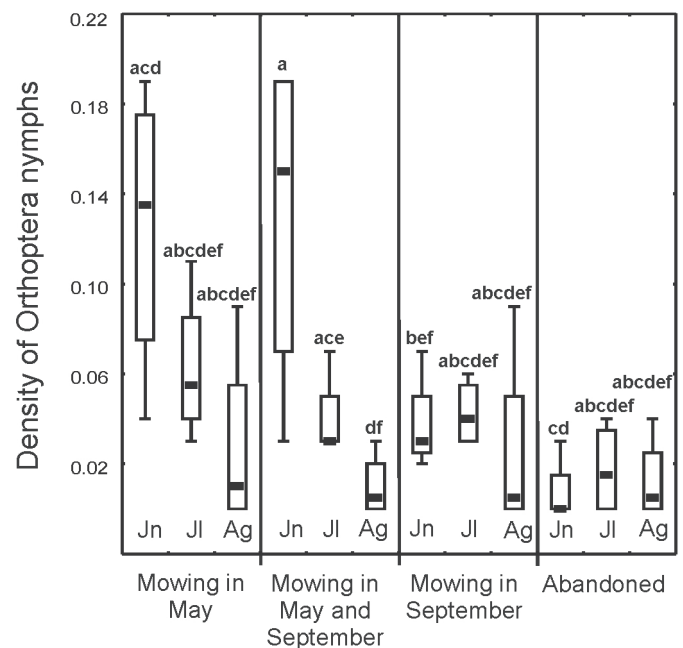
**Figure 4.** Box-plots (median values with minimum, maximum and  $\pm$ SE) of species richness, adult density (specimen/m<sup>2</sup>) and Shannon diversity of orthopterans in four treatment types (mowing once a year in May; mowing twice a year in May and September; mowing once a year in September; abandoned) in June (Jn), July (Jl) and August (Ag). Significant ( $p < 0.05$ ) differences detected by Mann-Whitney U test are indicated by different letters.

Shannon diversity in June was significantly higher on patches mown in May and September (MS) than on patches mown in May ( $U_{MJn-MS-Jn}=1$ ,  $p=0.03$ ) (Fig. 4). In July Shannon diversity increased significantly on patches mown in May (M) ( $U_{MJn-MJl}=0$ ,  $p=0.026$ ). Shannon diversity in July and August was similar on treated patches, but in July was significantly lower on abandoned patches than on patches mown in May ( $U_{MJl-CJl}=1$ ,  $p=0.002$ ).

Density of nymphs (Fig. 5) in June was significantly higher on patches mown in May (M) than on patches mown in September (S) or abandoned (C) ( $U_{MJn-SJn}=0$ ,  $p=0.04$ ;  $U_{MJn-CJn}=1$ ,  $p=0.02$ ).

Based on the results of PCA carried out on the pooled samples, orthopteran assemblages of different treatment types (M, S, MS, C) could not be clearly distinguished. At community level, only individual assemblage composition of the abandoned area showed a low level of independence (Fig. 6). The latter result was also visible in the life form and ecotype data. Silvicol species were found only on abandoned (C) patches. Arbusticol species were found on abandoned (C) patches and those maintained by September mowing (S). Graminicol species were found in grasslands mown in May (M), and abandoned ones. Pratinicol species dominated all examined patches (see Appendix). On the ecotype spectrum, a high proportion of hygrophilic species was seen in areas mown in May (M) and mown in May and September (MS), while mesophilic species reached high abundances in the patches treated by September mowing (S).

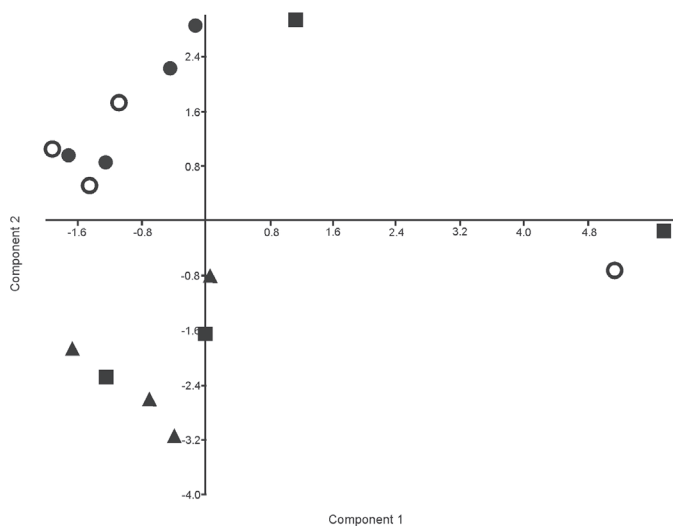
Generalized linear model (with Poisson distribution, including all sites and treatment types) showed significant negative relations between the vegetation height and density of orthopterans (Table 1). In addition, several significant positive correlations were found between the density of orthopterans and ambient temperature values. Significant negative correlations were found



**Figure 5.** Box-plots (median values with minimum, maximum and  $\pm$ SE) of nymphal density (specimen/m<sup>2</sup>) of orthopterans in four treatment types (mowing once a year in May; mowing twice a year in May and September; mowing once a year in September; abandoned) in June (Jn), July (Jl) and August (Ag). Significant ( $p < 0.05$ ) differences detected by Mann-Whitney U test are indicated by different letters.

**Table 1.** Results of generalized linear model (Poisson distribution) of species richness, density and Shannon diversity of orthopterans in relation to vegetation height, relative temperature in the vegetation (June, July, August)(significant values in bold).

		Vegetation		Orthopterans					
		Vegetation height		Species richness		Density		Shannon diversity	
		Estimate	p	Estimate	p	Estimate	p	Estimate	p
Orthopterans	Species richness	-0.0181	0.191						
	Density	<b>-0.2178</b>	<b>&lt;0.001</b>	<b>4.1727</b>	<b>&lt;0.001</b>				
	Shannon diversity	0.0005	0.904	0.0708	0.210	0.0052	0.519		
Relative temperature in the grass	Ground surface	<b>-6.9572</b>	<b>&lt;0.001</b>	0.2617	0.214	<b>4.8088</b>	<b>&lt;0.001</b>	0.0025	0.971
	10 cm height	<b>-5.8852</b>	<b>&lt;0.001</b>	0.1209	0.572	<b>4.4912</b>	<b>&lt;0.001</b>	-0.0066	0.927
	20 cm height	<b>-4.6333</b>	<b>&lt;0.001</b>	0.1526	0.501	<b>4.4454</b>	<b>&lt;0.001</b>	-0.0039	0.960
	30 cm height	0.1302	0.867	0.1317	0.556	<b>2.0167</b>	<b>&lt;0.001</b>	0.0049	0.950
	Mean	<b>-5.3531</b>	<b>&lt;0.001</b>	0.1960	0.407	<b>4.8699</b>	<b>&lt;0.001</b>	-0.0009	0.990



**Figure 6.** PCA based on the orthopteran samples of four study sites (sites I–IV) (black circle: mowing once a year in May; black square: mowing twice a year in May and September; empty circle: mowing once a year in September; black triangle: abandoned).

between the height of the vegetation and the temperature of soil surface and most of the regions of the sward.

## Discussion

Our study showed that adult orthopterans were present with lower density in abandoned areas than in areas which had been mown, regardless of when or how often the mowing took place. However, density of nymphs was highest in areas which had recently been mown. Nymph densities were highest in June on sites which had been mown in May, regardless of whether the site was mowed again in September or not. The nymphs which accounted for these high densities were mostly *Pseudochorthippus* and *Chorthippus* spp.

The negative correlation between density of orthopterans (including both nymphs and adults) and vegetation height may be related to the fact that cutting of vegetation in May resulted in shorter but thicker sward structure (Jutila and Grace 2002) rich in leaves of more favourable plant species [mesophytic plants, with medium (4–6) Water balance-value, see Borhidi 1995]. Further,

the short, thick sward structure may offer better conditions for the vulnerable, less mobile nymphs in terms of mobility, and hiding from predators (Braschler et al. 2009).

It is well known that impacts of mowing and removal of the harvest can lead to 70% mortality of orthopterans (Humbert et al. 2009). Our results did not provide support for this phenomenon as high orthopteran densities were detected on patches mowed just a few weeks before Orthoptera sampling. This discrepancy was probably caused by the fact that mown patches were situated close to uncut refuges (Humbert et al. 2012), and cutting height, influencing maintenance of ground-dwelling fauna (Humbert et al. 2009), was higher than 10 cm.

Orthopteran assemblages are linked to vegetation units of higher taxonomical level rather than to plant species (Kemp et al. 1990, Bauer et al. 2004). The scale of the different treatments could have enabled small scale migration of orthopterans to and from the uncut plots (Humbert et al. 2012). This explains why areas mown using different regimes (M, S, MS) did not have different grasshopper assemblages.

Species that were found to be dominant in our study (*Pseudochorthippus parallelus*, *Roeseliana roeselii*) were also found most characteristic in humid, intensely mown meadows by other authors in nearby regions of Europe (Gardiner et al. 2002, Marini et al. 2008, Poniatowski and Fartmann 2008). Our results confirmed that abundances of orthopteran assemblages are highly influenced by land use (Guido and Chemini 2000, Kruess and Tscharrntke 2002, Knop et al. 2006, Kenyeres and Cservenka 2014). This can be deduced clearly from the fact that the choice of habitat by orthopterans is mainly influenced by vegetation structure (O'Neill et al. 2003).

Based on our results, abandonment management had a negative impact on the grasshopper density but did not significantly affect species richness or Shannon diversity of orthopterans of hay meadows. This result is entirely consistent with the findings of the botanical studies of Szépligeti et al. (2016) and orthopterological investigations in other study areas (Nagy and Kisfali 2007). The uncut patches could balance the temporal negative effect of mowing. Therefore providing refuges during each mowing session may be an alternative to subjecting different parts of the meadow to different mowing regimes. Considering conservation of insects with low dispersal ability, a maximum of 30 m distance between two refuges would be optimal (Hossain et al. 2002). It is important to note that the margins (Marshall 2002) and refuges (Humbert et al. 2009) should be located in rotation in different parts of the mown fields (Buri et al. 2013) and should be mown at the next

haymaking event (see results of abandonment, e.g. decreasing of plant diversity, invasions of *Solidago gigantea*) (Szépligeti et al. 2016).

Although our study did not reveal it, mortality caused by mowing could still be assumed (Gardiner and Hill 2006). Therefore, we recommend that grasslands should be maintained by bar mowers, which cause 50% lower mortality than rotary mowers (Humbert et al. 2009). To benefit conservation of orthopteran species that lay their eggs in or near the soil, cutting height should be higher than 10 cm (Gardiner and Hassall 2009, Humbert et al. 2012). Considering yearly differences in mean temperature and rainfall, the mowing regime timing should depend on weather conditions of the given year (Gardiner and Hassall 2009, Buri et al. 2013) in order to provide vegetation structure and microclimate required by orthopterans of mesic hay meadows.

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## Appendix 1

Species composition and quantity of the pooled samples of different mowing regimes (LF: life form; EF: ecotype form; M: mowing once a year in May; MS: mowing twice a year in May and September; S: mowing once a year in September; C: abandoned without management; arbu: arbusticol; gra: graminicol; pra: pratinicol; sil: silvicol; hyg: hygrophilic; mes: mesophilic; m-hyg: moderately-hygrophilic; m-ther: moderately-thermophilic; ther: thermophilic).

Taxon	LF	EF	M	MS	S	C
Caelifera						
Acridoidea						
Acridomorpha						
Acrididae						
Gomphocerinae						
<i>Chrysochraon dispar</i> (Germar, 1834)	pra	m-hyg	3	37	13	43
<i>Euchorthippus declivus</i> (Brisout de Barneville, 1848)	gra	ther	1		2	
<i>Euthystira brachyptera</i> (Ocskay, 1826)	pra	mes	28	28	29	14
<i>Chorthippus biguttulus</i> (Linnaeus, 1758)	pra	m-ther	4		8	
<i>Chorthippus brunneus</i> (Thunberg, 1815)	pra	m-ther	6	10	8	1
<i>Chorthippus dorsatus</i> (Zetterstedt, 1821)	pra	mes	24	6	16	2
<i>Chorthippus oschei</i> Helversen, 1986	pra	mes	3	3	1	1
<i>Chorthippus</i> sp. (nymphs)			86	37	75	15
<i>Gomphocerippus rufus</i> (Linnaeus, 1758)	sil	mes			1	
<i>Pseudochorthippus parallelus</i> (Zetterstedt, 1821)	pra	mes	48	62	62	34
<i>Omocestus haemorrhoidalis</i> (Charpentier, 1825)	pra	ther		1		
<i>Omocestus viridulus</i> Linnaeus, 1758	pra	mes		1		
<i>Stenobothrus lineatus</i> (Panzer, 1796)	pra	m-ther				2
Melanoplinae						
<i>Odontopodisma schmidtii</i> (Fieber, 1853)	pra	mes		2		1
Oedipodinae						
<i>Mecostethus parapleurus</i> (Hagenbach, 1822)	pra	hyg	122	111	79	39
<i>Stethophyma grossum</i> (Linnaeus, 1758)	pra	hyg	5	5	1	1
Pezotettiginae						
<i>Pezotettix giornaie</i> (Rossi, 1794)	gra	ther	3		5	
Tetragoidea						
Tetrigidae						
Tetriginae						
<i>Tetrix bipunctata</i> (Linnaeus, 1758)	sil	m-ther			1	
Ensifera						
Tettigonioidea						
Tettigoniidae						
Conocephalinae						
<i>Conocephalus discolor</i> Thunberg, 1815	pra	hyg	7	2	3	3
<i>Ruspolia nitidula</i> (Scopoli, 1786)	pra	m-hyg	15	8	7	10
Tettigoniinae						
<i>Decticus verrucivorus</i> (Linnaeus, 1785)	pra	mes	1	6	3	1
<i>Roeseliana roeselii</i> (Hagenbach, 1822)	pra	m-hyg	17	65	28	50
<i>Tettigonia viridissima</i> Linnaeus, 1758	arbu	mes		1		2
Phaneropteridae						
Phaneropterinae						
<i>Leptophyes albovittata</i> (Kollar, 1833)	arbu	ther		4	5	
<i>Phaneroptera falcata</i> (Poda, 1761)	arbu	ther	3	11	9	1



# The genus *Ectadia* (Orthoptera: Phaneropteridae: Phaneropterinae) in East Asia: description of a new species, comparison of its complex song and duetting behavior with that of *E. fulva* and notes on the biology of *E. fulva*

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## Abstract

The genus *Ectadia* is an East Asian genus of the tribe Elimaeni (Phaneropterinae) and is known for the complex stridulatory files of its members. The calling song was so far known from only one species, the relatively widespread *Ectadia fulva*. Here a new species from Yunnan, China, is described, which is morphologically similar to *E. fulva* except for the stridulatory file. In this character *E. fulva* deviates strongly from the new species and from all other *Ectadia* species by its high tooth number. The long lasting song (duration 30 to 60 seconds at 25 °C) of the new species is very complex and differs widely from the much shorter song of *E. fulva*. Its spectral composition changes during stridulation. In the new species the females respond acoustically during gaps in the final part of the male song, whereas in *E. fulva* they answer typically after the male song. During mating *Ectadia fulva* males transfer small spermatophores without spermatophylax. All of its six nymphal stages are cryptically colored. In karyotype the new species being the first studied *Ectadia* species is similar to the related genera *Elimaea* and *Ducetia* (all 2n=29 chromosomes in the male).

## Key words

calling song, stridulatory file, male-female-duet, carrier frequency, Yunnan

## Introduction

In nearly all species of the subfamily Phaneropterinae, males and females communicate by duetting to find a mate (see Heller et al. 2015 for a review). The male seems always to initiate the duet, but the structure of its songs varies widely. Many species have simple songs; however, there are several genera with quite complicated male calling song patterns. The genus *Ectadia* Brunner von Wattenwyl, 1878 belongs to the second group. *Ectadia* is a mainly (South) East Asian genus with at present eight species. Males of those species do not have titillators in their genitalic organs and the cerci are quite similar, but they differ widely in their stridulatory organs that are known from all species except of the Indian *E. pilosa* (see Liu et al. 2004, Gorochov 2009).

Six species are endemic to South China (Yunnan) and northern Vietnam (Liu et al. 2004, Gorochov 2009), one species (*E. fulva* Brunner von Wattenwyl, 1893) is more widespread in South East Asia (Myanmar, Thailand, Vietnam, China: Yunnan, Guanxi, Fujian; Liu et al. 2004, Kang et al. 2014; Fig. 1), but the type species of the genus (*E. pilosa* Brunner von Wattenwyl, 1878) is only known from one male collected in Kashmir (India). Thus clearly the centre of its distribution is the Chinese province Yunnan where five species have been found. It may not be so surprising to find a high number of species in this relatively small area, since Yunnan is one of the global biodiversity hotspots, well known for plant diversity (Li et al. 2015, Tang 2015), although not yet so much for insects (but see e.g. Morgan et al. 2011). Therefore, when we heard an unknown calling song of an *Ectadia* species during a joint excursion (CXL and KGH) in Yunnan, we expected to find one of these species. Surprisingly, however, our species differed morphologically from all other known species. Its song was even more complicated (some details mentioned already in Heller et al. 2015 as *Ectadia* sp.) than that of *E. fulva*, the morphologically most similar species and the only one with known song (Ingrisch 1998, Heller et al. 2015).

In this paper we will describe the species as new and as the sixth member of the genus in Yunnan. In addition, we will present some more data of song and behaviour of *E. fulva* in comparison.

## Material and methods

**Measurements.**— Total body length, lateral aspect, refers to the midline length of the insect from fastigium verticis to tip of abdomen including the subgenital plate. In females, the ovipositor is not included in the measurement of the body length. Measurements of ovipositors are taken laterally from tip to base not regarding the curvature.

To obtain the mass data, living animals and spermatophores were weighed to the nearest mg (balance Mettler PM 640).

**Depositories.**—IZCAS Insect Collection of Institute of Zoology, Chinese Academy of Sciences, Beijing, China. CH Collectio Heller.

**Acoustics.**— The male calling song of *E. diuturna* sp. n. was recorded in the field and in the laboratory using a digital bat detector (Pettersson D1000X) with sampling rates between 100 and 300 kHz. Duets were recorded in stereo using a Sony ECM-121 microphone (frequency response relatively flat up to 30 kHz according to own tests) and an Uher M645 microphone connected to a personal computer through an external soundcard (Transit USB, "M-Audio"; 64-kHz sampling rate). The recordings of *Ectadia fulva* were made in the laboratory with a Racal store 4-D tape recorder using microphones Brüel and Kjær 4133 and 4135 (frequency response flat up to 40 and 70 kHz respectively)(KGH) and with Kenwood KX880HX and Sony D7 tape recorders using Sennheiser BlackFire541, Sennheiser stereo and AIWA CM-S1 stereo microphones (SI) and digitised years later.

The stridulatory movements of *Ectadia fulva* were registered by an opto-electronic device (Helversen and Elsner 1977, modified as in Heller 1988).

Song measurements and spectrograms were obtained using Amadeus II and Amadeus Pro (Martin Hairer; <http://www.hairersoft.com>). Oscillograms of the songs were prepared using Turbolab (Bressner Technology, Germany). All recordings were made at temperatures between 18 and 25 °C. The singers were caged in plastic tubes or gauze cages with microphone fixed or hand-held at distances between 5 (duet) and 80 cm.

For measurements of the stridulatory files, replicas were made using the cellulose nitrate technique described by Ragge (1969). For tooth spacing, each point in the figures represents the mean of ten consecutive intervals.

**Acoustical terminology.**— Tettigonioids produce their songs by repeated opening and closing movements of their tegmina. The sound resulting during one cycle of movements is called a syllable, often separable in opening and closing hemisyllable (Ragge and Reynolds 1998). While most species open and close their wings with a more or less uniform amplitude, some species use more complicated patterns, where the term syllable is difficult to apply, if the movement pattern can be reconstructed at all. For the description of the songs of *Ectadia* we will use the terms micro- and macrosyllables. Movement recordings in *E. fulva* have demonstrated (Heller et al. 2015), that the short impulse (see below) groups (microsyllables) are produced during contact of the large file teeth, the longer lasting impulse groups (macrosyllables) during contact of the small file teeth with the scraper. For the new species we assume the same basic pattern. Syllable duration: time period measured from the first impulse to the last; syllable period: time period measured from the first impulse to the first impulse of the next syllable; impulse: a simple, undivided, transient train of sound waves (here: the damped sound impulse arising as the effect of one tooth of the stridulatory file).

In *Ectadia* (as in other species), after some time the same or a similar pattern of syllables and pauses is repeated. This grouping, often separated from the next by a silent interval, is called a song unit.

**Chromosomal analysis.**— The sample of a male paratype of *Ectadia diuturna* sp. n. (CH7661) was used for cytotoxic analyses. Gastric caeca were incubated in a hypotonic solution (0.9% sodium citrate), fixed in ethanol - acetic acid (3:1, v/v) and squashed in 45% acetic acid. Cover slips were removed by the dry ice procedure and then preparations were air dried. Constitutive heterochromatin was revealed by the C-banding technique as described by Sumner (1972). Fluorescence *in situ* hybridization (FISH) with ribosomal 18S rDNA (rDNA) genes was performed exactly as described in Grzywacz et al. (2011).

## Results

### *Ectadia diuturna* Heller & Liu, sp. n.

<http://lsid.speciesfile.org/urn:lsid:Orthoptera.speciesfile.org:>

TaxonName:498339

<http://zoobank.org/F95FF011-8A43-4959-AB8D-692D5968D562>

Figs 3–9 morphology, Figs 10–13 song, Fig. 14 chromosomes

*Ectadia* sp. in Heller et al. 2015, p. 3, 6. fig. 5

**Material examined.**— Holotype male, China, Yunnan, Honghe, Pingbian, Daweishan Forest Reserve, 22°56.55'N, 103°41.43'E, 1700–2100 m a.s.l., 15–17 viii 2013, leg. Liu Chunxiang, #CH7660, in ethanol. Depository IZCAS.

Paratypes 11 males (#CH7659, 61–62), 6 females (#CH7663–5), dried/in ethanol, same data as holotype, all in IZCAS except #CH7659, 61, 64 (in CH). In addition sound recordings of two males, not collected.

**Diagnosis.**— Differs from all species of the genus in proportions of anal (1/3) and basal (2/3) part of the stridulatory file and number (about 100; Table 1) and distribution of teeth on the stridulatory file (Figs 2, 3). From *E. apicalis*, which has a similar stridulatory file, it differs by larger size and the hind wings being clearly longer than the tegmina while slightly shorter in *E. apicalis*. In general habitus it is most similar to *E. fulva*, but has a much lower number of teeth on stridulatory file (see Fig. 3) and narrower tegmina (Fig. 4).

**Description.**— Male. General habitus see Fig. 5.

Fastigium verticis narrower than first segment of antennae, sulcate dorsally, not contacting fastigium frontis. Complex eyes approximately round. Pronotum with disc nearly flat, only last quarter elevated; anterior margin slightly concave, posterior margin truncate with small notch in middle; medial carina inconspicuous; transverse sulcus V-shaped; lateral carina weakly developed; lateral lobe of pronotum much longer than high, anterior margin straight, posterior margin obtuse-rounded, ventral margin oblique downward posteriorly, humeral sinus present but inconspicuous.

Prothoracic spiracle large, but mostly covered by a posterior extension of paranotum.

Tegmen shorter than hind wing, posteriorly with long parallel part (Figs 4A, 5A); venation see Fig. 4. Mirror in stridulatory area of right tegmen with a more or less circular deepening, similar to that in *E. fulva* (Fig. 6). The vein demarcating the distal end of the stridulatory area curved smoothly, not with an edge as in *E. fulva* (Fig. 6). Stridulatory file comparatively straight but slightly thinner in middle, with circa 90 densely spaced fine teeth in basal area and about 10 widely spaced large teeth in distal (=apical or anal) area (Fig. 3). Fore coxae without spine, fore tibiae dorsally sulcate and spinuliferous. All femora ventrally spinulose; mid and hind femur with 5–6 spines on outer ventral margins; hind tibiae with many spines on both dorsal margins. Tenth abdominal tergite prolonged backwards, hind margin emarginate; cerci stoutest at base, basal quarter sharply acuminate, then first slightly widening, later gradually narrowed till apex, dorso-ventrally flattened; subgenital plate split for almost apical two thirds, up-curved (Figs 7, 8). Internal genitalia membranous, not sclerotized titillator.

**Color.**— Body and tegmina green, parts of legs, dorsal side of pronotum and dorsal field of tegmina brown, tegmina with some small brown dots (Figs 4, 5).



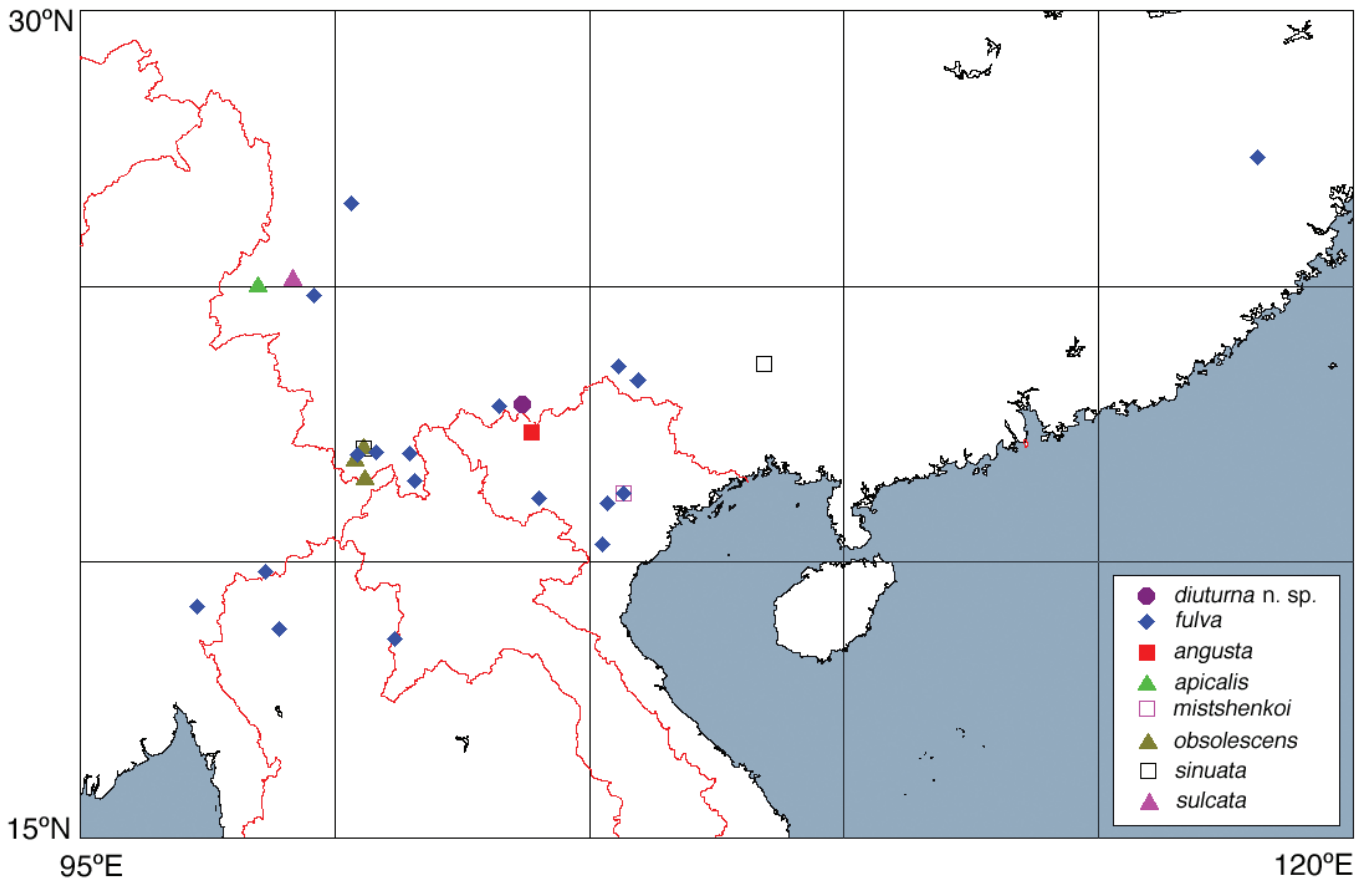


Figure 1. Distribution map of genus *Ectadia* (except *E. pilosa*, Kashmir; based on data of Brunner von Wattenwyl 1893, Liu et al. 2004, Gorochov 2009, Bey-Bienko 1962, Ingrisch 1998, Bai and Shi 2014).

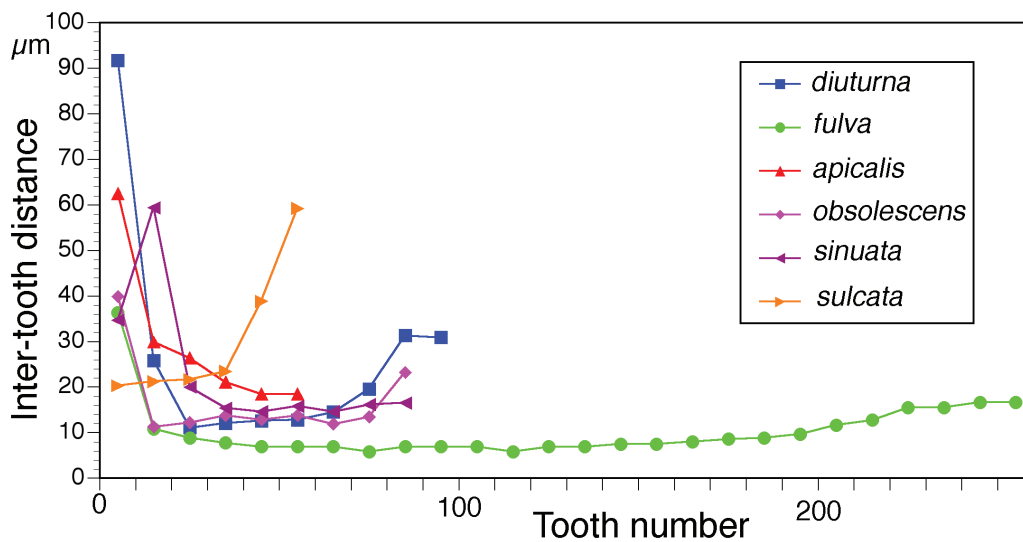
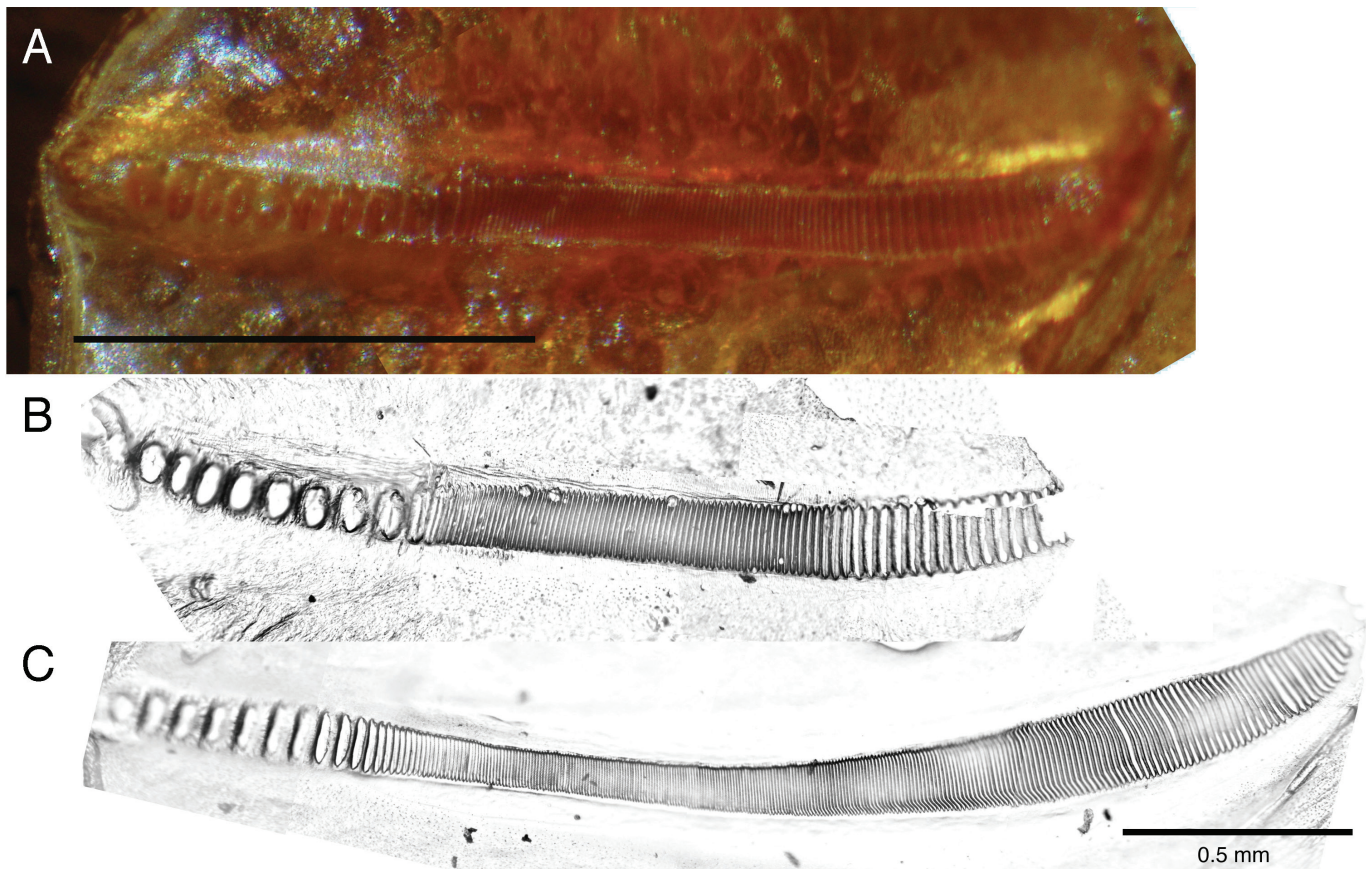


Figure 2. Inter-tooth spacing in male stridulatory files of *Ectadia* species.

*Female*.— Mostly similar to male except abdominal apex and tegmina (stridulatory organs).

Pronotum with disc nearly flat; ventral and dorsal edge of paranotum parallel.

Right tegmen at posterior edge in basal part with about 9 short cross veins carrying stout spines used to produce the female acoustic response (similar as shown in Heller et al. 1997); more distally small cross veins and even longitudinal veins with many



**Figure 3.** Stridulatory file of *E. diuturna* sp. n. and *E. fulva* (articulation of tegmen to the right). A. File of *E. diuturna* sp. n. on the underside of left male tegmen. Scale 1 mm. B–C. Replica of the file of B. *E. diuturna* sp. n. in comparison to that of C. *E. fulva*.

**Table 1.** Proportions of distal and basal part and number of teeth in stridulatory file in *Ectadia* species.

Species	Length of distal part of file	Number of teeth in distal part	Length of basal part of file	Approximate number of teeth in basal part	
<i>diuturna</i>	1/3	10	2/3	90	this paper
<i>angusta</i>	1/4	2*	3/4	>80*	Gorochov 2009
<i>apicalis</i>	1/2	9	1/2	60	Liu et al. 2004
<i>fulva</i>	1/6	10	5/6	250	this paper
<i>mistshenkoi</i>	1/5	2*(8)	4/5	> 110*	Gorochov 2009
<i>obsolescens</i>	1/4	1–4	3/4	120–125	Liu et al. 2004
<i>sinuata</i>	1/2	20–25	1/2	many	Liu et al. 2004
<i>sulcata</i>	1/2	60	1/2	20	Liu et al. 2004

\* additional information by Gorochov in litt.

slender spines, probably used to prevent accidental wing opening. In left tegmen, posterior edge in basal part scraper-like in-curved. Supra-anal plate triangular; cerci rather short, conical. Ovipositor fairly broad and short, upcurved, both margins at tip denticulate (Fig. 9). Subgenital plate triangular, apex obtusely rounded.

**Color.**— Green except a white mid-line on pronotum and dorsal edges of tegmina (Fig. 5B).

**Egg.**— Thin, flattened, ovoid, typical phaneropterine shape.

**Measurements** (length in mm).— Body: male 14–20, female 17–22; pronotum: male 3.7–4.8, female 3.9–4.2; tegmen: male 26.7–31,

female 22–25.3; tegmen width (greatest): male 5.1–6.5, female 4.9–5.1; hind wing: male 33–38; female 26–29.4; post femur: male 20.9–24, female 20.5–23.4; length of ovipositor: 6.5–8.1; width of ovipositor 1.9–2.1, length of egg 4.7–4.8; width of egg 1.6–1.8 (n=12 males, 6 females, partly dried, partly in ethanol).

**Song.**— Time-amplitude-pattern. In *Ectadia diuturna* sp. n. one song unit typically lasted about 30 to 60 s (all data for 25°C except otherwise mentioned; up to 80 s in the field at 18°C). It was followed by the next unit after a silent interval as short as some seconds, but also as long as some minutes. The shortest intervals were observed when males and females were in acoustical contact. Each unit contained four phrases each of which consisted of



Figure 4. Transmitted light scan of left tegmina. A. *Ectadia diuturna* sp. n., B. *E. fulva* (the fold in the basal part is an artifact resulting from the strong curvature of the tegmen). Scale 10 mm.

a different combination of micro- and macrosyllables. A simple and clear example is shown in Fig. 10A. However, often the different phrases were connected by transitional phrases. A song unit started with a series of tick-like macrosyllables, separated by intervals of about 150 ms (phrase A; see Fig. 11 for details). Sometimes the intervals between the ticks were larger and more irregular. Between these loud syllables (occasionally?) short and soft sounds were observed. At the end of a phrase typically two tick sounds followed each other quite closely (10 ms interval) a few times. Such phrases were also heard isolated outside complete song units, possibly representing unsuccessful attempts to start a song unit. After this phrase a series of macrosyllables followed (phrase B), in which always two types of impulse groups alternated, a long series (ca. 70 impulses; duration 120 ms), sometimes split into two or more by one or several unusually large intervals, and a short series (ca. 55 densely packed impulses; duration 30-40 ms). Both groups differed distinctly in carrier frequency (see below). In amplitude, this phrase was always lower than the loud phrase A. Its duration was the most variable of all phrases, from nearly missing up to ten seconds. Phrase C consisted of a long series of stereotypic elements (period about 500 ms), each containing two pairs of impulse groups as seen in phrase B. These pairs were separated from the next pair by an interval of about 200 ms. During this interval one (or two) loud macrosyllables were produced and additionally a series of softer isolated impulses. While in Fig. 10 an abrupt change from phrase B to C can be seen, in many other recordings there is a long transition with phrase C elements appearing more and more frequently (see e.g. Fig. 12). Phrase D finally consisted of several repetitions of the impulse series from phrase C (period about 400 ms) without macrosyllables.

The females responded near the end of a song unit (Fig. 12). They answered mostly not only once, but several to many times (1-25 responses per song unit). The responses occurred nearly always during phrase D, and often also additionally at the end of phrase C. Very rarely they were heard during phrase A, in this case the females possibly assuming a late phrase D from the previous song unit. In many phaneropterine species, the females respond with a species-specific delay after the male song or some parts of it (e.g. Heller and Helversen 1986). This is probably also the case in *E. diuturna* sp. n., but the response delay is difficult to measure exactly, because often several possible trigger events are closely together, and the response consists of several loud impulses distributed over more than 100 ms. The delay is obvious in the range of 150-250 ms. In any case, often very soft impulses (more than 20

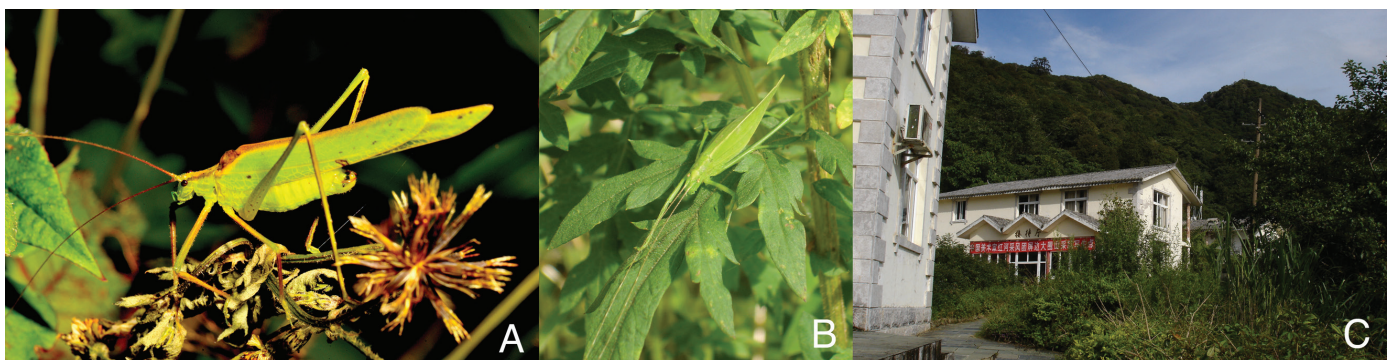
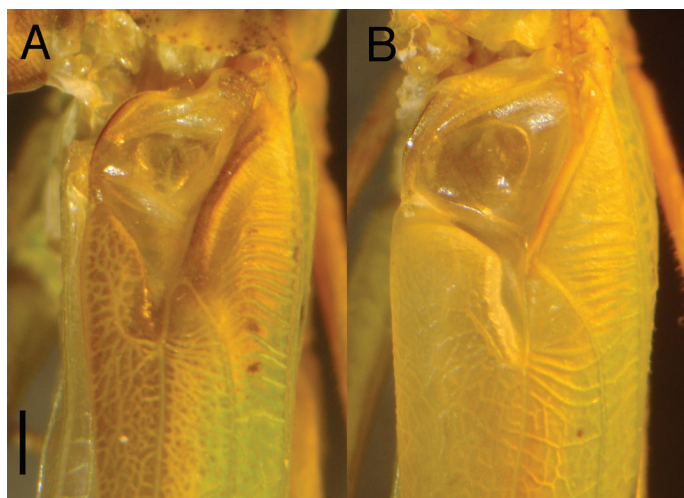
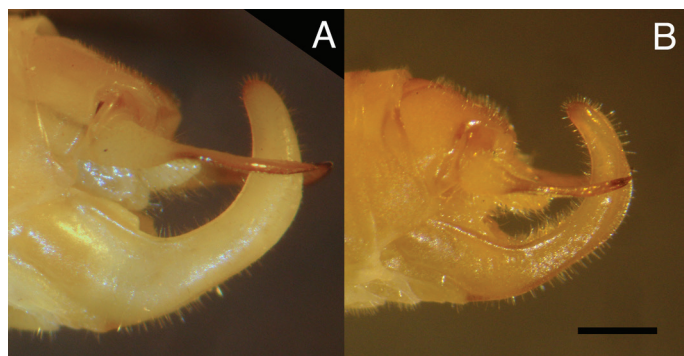


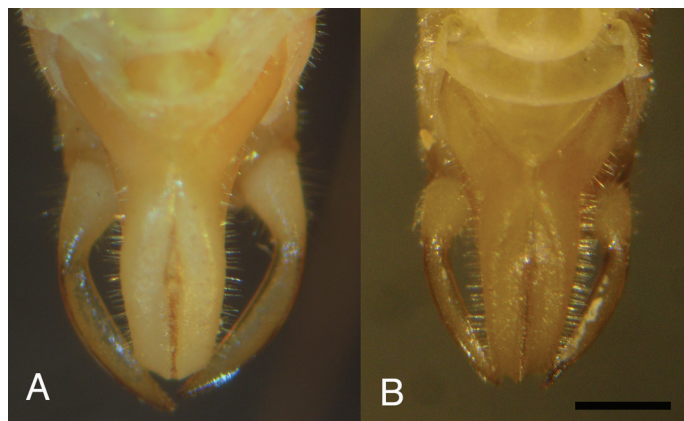
Figure 5. Habitus and habitat of *E. diuturna* sp. n. A. male (photo by Guoqing Ma), B. female, C. habitat at type locality. The photo of the male is shown under *E. fulva* in Kang et al. (2014).



**Figure 6.** Stridulatory area with mirror at base of right tegmen. A. *E. diuturna* sp. n., B. *E. fulva*. Scale 1 mm.



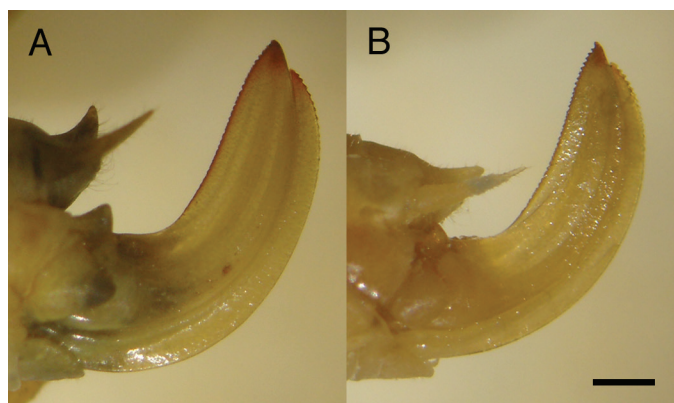
**Figure 7.** Lateral view of male subgenital plate. A. *E. diuturna* sp. n., B. *E. fulva*. Scale 1 mm.



**Figure 8.** Ventral view of male subgenital plate. A. *E. diuturna* sp. n., B. *E. fulva*. Scale 1 mm.

dB softer than the main response) were observed that occurred much faster (70–80 ms) than the loud impulses.

**Carrier frequency.**— During one song unit, parts with quite different spectral composition were observed. The short, tick-like elements



**Figure 9.** Lateral view of female ovipositor. A. *E. diuturna* sp. n., B. *E. fulva*. Scale 1 mm.

of which phase A and D consisted and which occurred also in phase C, had always a quite similar spectrum. Its peak was situated at about 20 kHz, in the lower half with strong components starting abruptly at about 5 kHz, in the higher half continuously decreasing in power until 40 kHz (Fig. 13). Also the softer and shorter impulse groups found in phase B and C were similar to this pattern presenting a relatively narrow peak at 20 kHz and a side-peak at 40 kHz. However, the longer and often louder impulse groups from phase B and C differed distinctly. Their spectrum contained two clearly separated peaks. Besides a high and narrow peak at 10 kHz it showed a broad maximum at 60 kHz (Fig. 13). These impulse groups alternated with the shorter and softer impulse groups (see fig. 5 in Heller et al. 2015). The female response had its maximum between 10 and 20 kHz (recorded in audible range only).

**Chromosomes.**— The analyzed species is characterized by a male chromosome number  $2n=29$  and sex determination system X0. All chromosomes are acrocentric and the X chromosome is the largest element in the set. C-banding of mitotic metaphase revealed constitutive heterochromatin blocks (thin C-bands) in the paracentromeric region in most chromosomal elements, with the exception of one small pair of autosomes (thick C-bands). Constitutive heterochromatin polymorphism involving telomeric C-bands was located in the first-sized pair (Fig. 14A). The observed rDNA-FISH signal on this largest pair, similar to C-bands, varied in size, suggesting the occurrence of polymorphism in copy number of rDNA sequences (Fig. 14B).

**Habitat.**— Common in low bushes around buildings (Fig. 5C).

**Etymology.**— The name of the new species refers to its long lasting song: diuturna. Latin adjective, meaning long lasting

**Distribution.**— Known only from Yunnan, China.

***Ectadia fulva* Brunner von Wattenwyl, 1893**

Fig. 15 morphology, Figs 16–17 song

**Material examined.**—For the behavioral studies four males and three females (out of series of nine each) were used, all offspring from animals from Thailand, Chiang Mai, Doi Suthep-Pui, 18°48'N, 98°55'E, 1100–1150 m a.s.l., 13.04.1995, ex ovo, bred in lab., leg. S. Ingrisch. Stridulatory files in six males,

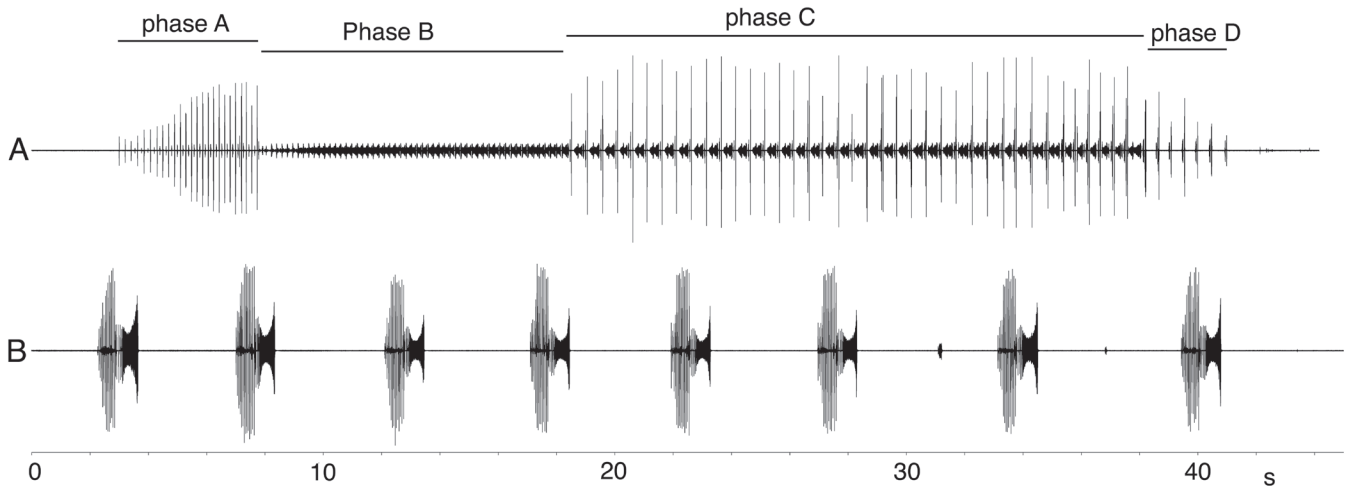


Figure 10. Oscillograms of male calling songs, comparative overview. A. *E. diuturna* sp. n., B. *E. fulva*, In A, the different phases of one song unit are indicated.

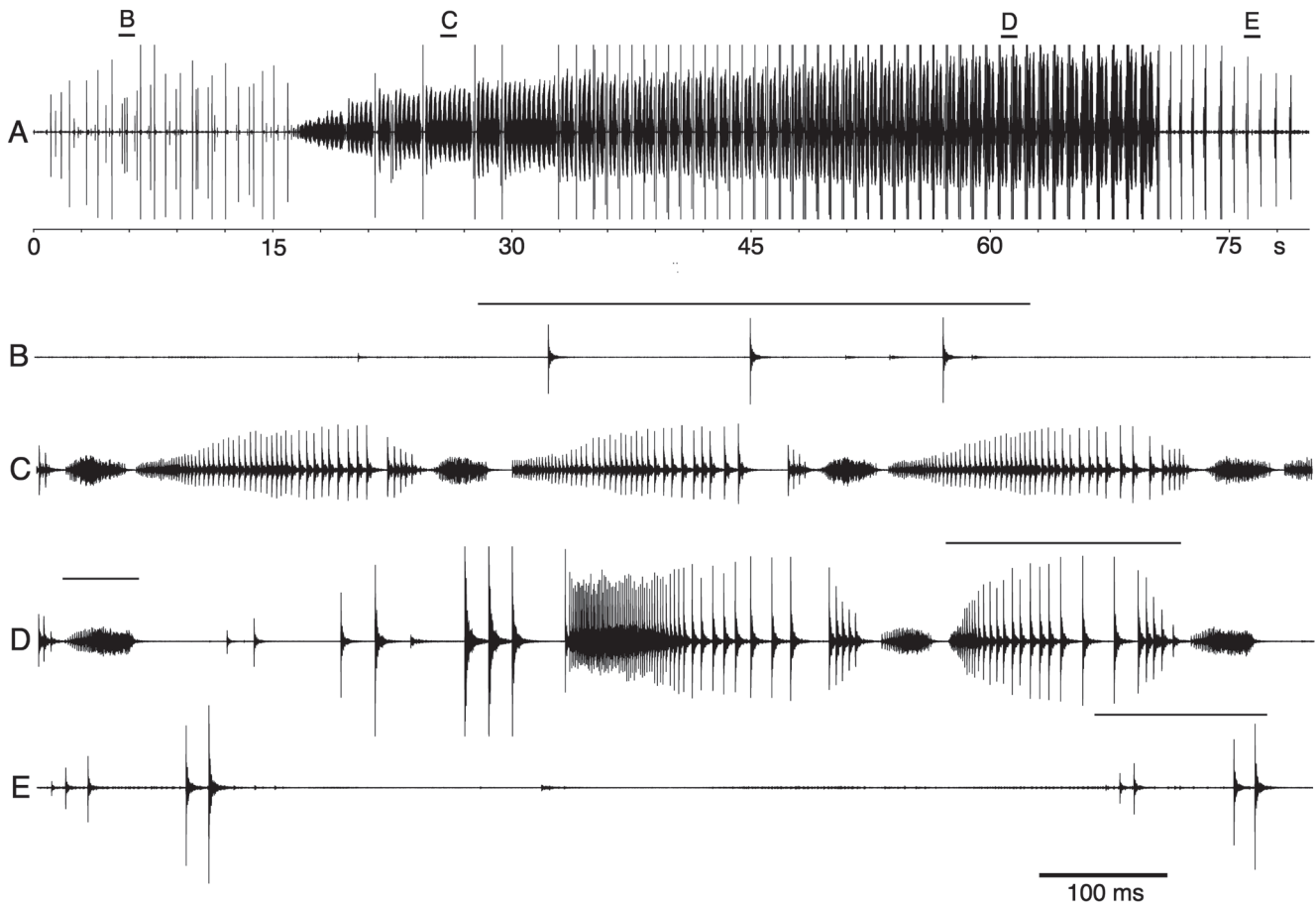
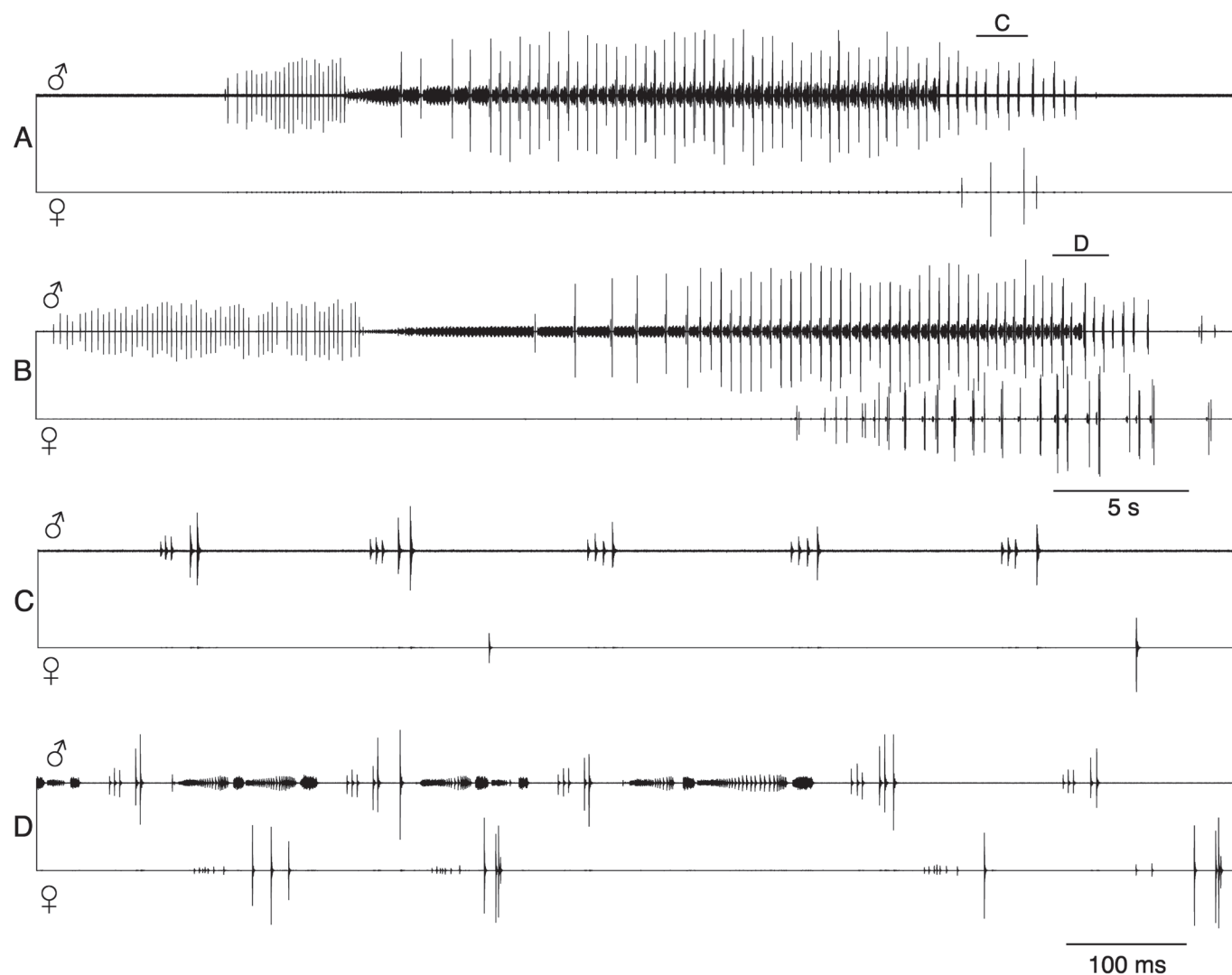


Figure 11. Oscillograms of male calling song of *E. diuturna* sp. n. A. Overview with indication of the origin of the details (B–E). In B, D and E, the origin of the spectra shown in Fig. 12 is indicated.

China, Yunnan Province, Jinping County, Fenshuiling National Nature Reserve, 22.88178768°E, 103.23377388°N, August 2012.

*Description.*— The species has been sufficiently redescribed by Liu et al. 2004. It may be added that the fore wings of the male are vaulted, bulging laterally in proximal half of tegmen



**Figure 12.** Oscillograms of male calling song and female response of *E. diuturna* sp. n., recorded simultaneously. A, B. Overviews with indication of the origin of the details (C–D).

length, flat with rounded tip thereafter; those of the female are shortened with acute tip. In both sexes the hind wings surpass the fore wings. The species shows a green-brown color polymorphism in both sexes. Of the specimens collected in the field, two males and two females were green and two males and one female brown. The brown color is of lighter shade in females (more ochreous) than in males (medium brown with dark pattern). In the offspring of a green female bred in lab, 19 males and 12 females became green, four males and nine females brown. Body, legs, fore wings and the projecting part of the hind wings were either all green or all brown, in two females pale yellowish brown. The broad dorsal field of the male tegmen including the stridulatory area was always dark brown in both color morphs while the narrow dorsal field of the female tegmen was yellow in green females or pale brown bordered by a dark brown line in brown females. In resting position, *E. fulva* sits with the antennae and the fore

legs stretched anteriorly in the body axis, the mid and the hind legs are stretched oblique posteriorly, while the abdomen and the wings are pointing dorso-posteriorly. That behavior resembles the situation in *Ducetia* (e.g. *D. japonica*) or *Elimaea* species.

**Song.**— Time-amplitude-pattern. The male calling song consisted of song units each lasting about 1.5 s repeated after an interval of about 4 s (Fig. 16A). Each was made of a series of microsyllables (about 15), repeated with 20 Hz, followed by a long series of impulses with decreasing intervals. This song unit is produced by opening the tegmina very widely, then closing and opening them several times only in part, resulting in the microsyllables. The microsyllables are not tick-like sounds as in *E. diuturna* sp. n., but consist of compact series each with a few impulses. Then the male closed the tegmina completely and very slowly. The contact of the scraper with the fine basal part of the file produced a long series of heavily damped impulses (Fig. 16; see also fig. 7 in Heller et al. 2014).

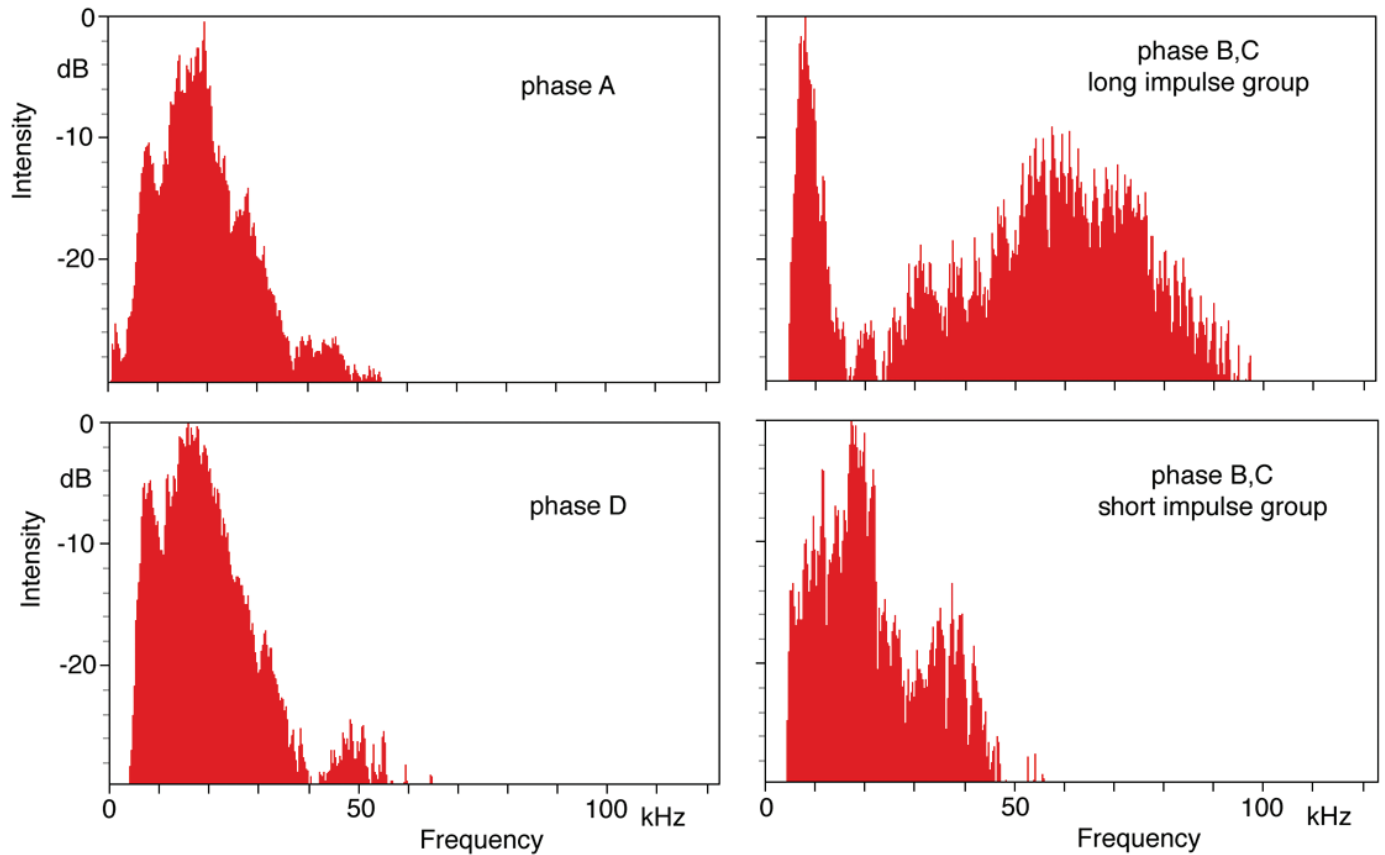


Figure 13. Power spectra of different parts of the calling song of *E. diuturna* sp. n.

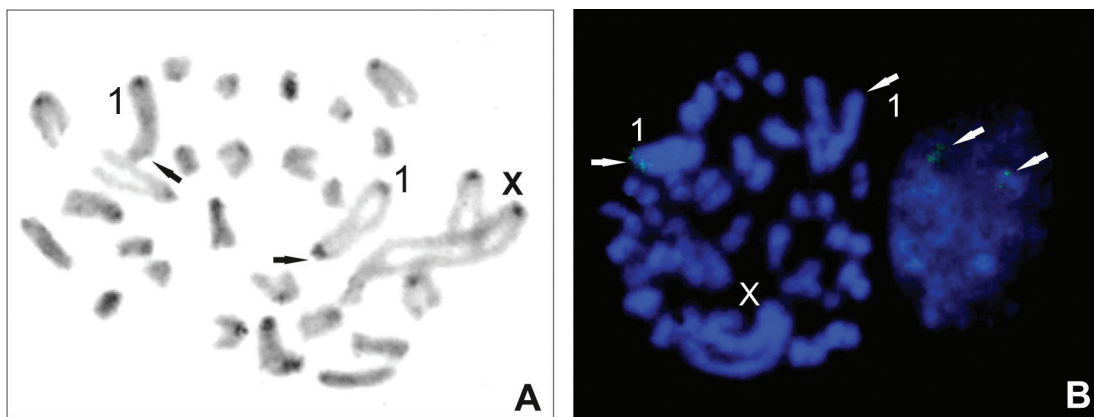


Figure 14. Male chromosomes of *Ectadia diuturna* sp. n. A. C-banded metaphase; B. FISH with 18S rDNA (green) probe in metaphase/anaphase (A – the left) and prometaphase (B – the right). Arrows indicate telomeric located C-bands (A) and cluster of 18S rDNA (B) in the heteromorphic large-sized chromosomes (marked with "1"); X, sex chromosome.

The female responded immediately after the end of the male impulse series (Fig. 16), typically with one or a few impulses, occasionally with one or a small series up to a few hundred ms later in addition. Sometimes responses were observed even before the male series had ended.

*Carrier frequency.*— Both parts of the song unit had a quite similar spectral composition with two peaks (Fig. 17). Besides a narrow low-frequent peak around 10 kHz there was a broad maximum at about 50-60 kHz. The female response showed a peak at 20 kHz, in width and placement intermediate between the two male peaks.

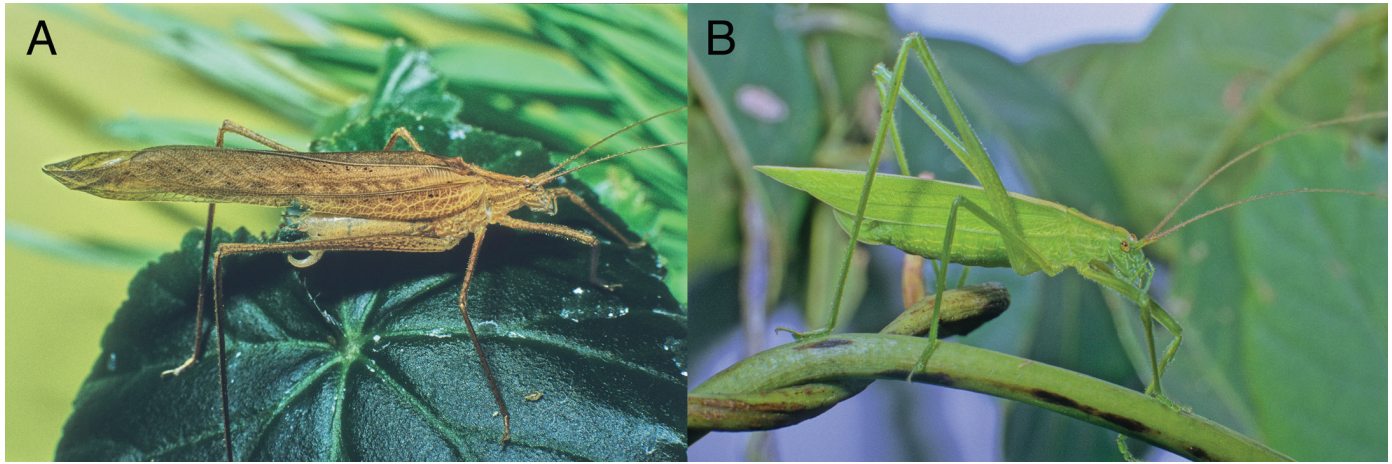


Figure 15. Habitus of *Ectadia fulva*. A. male, B. female.

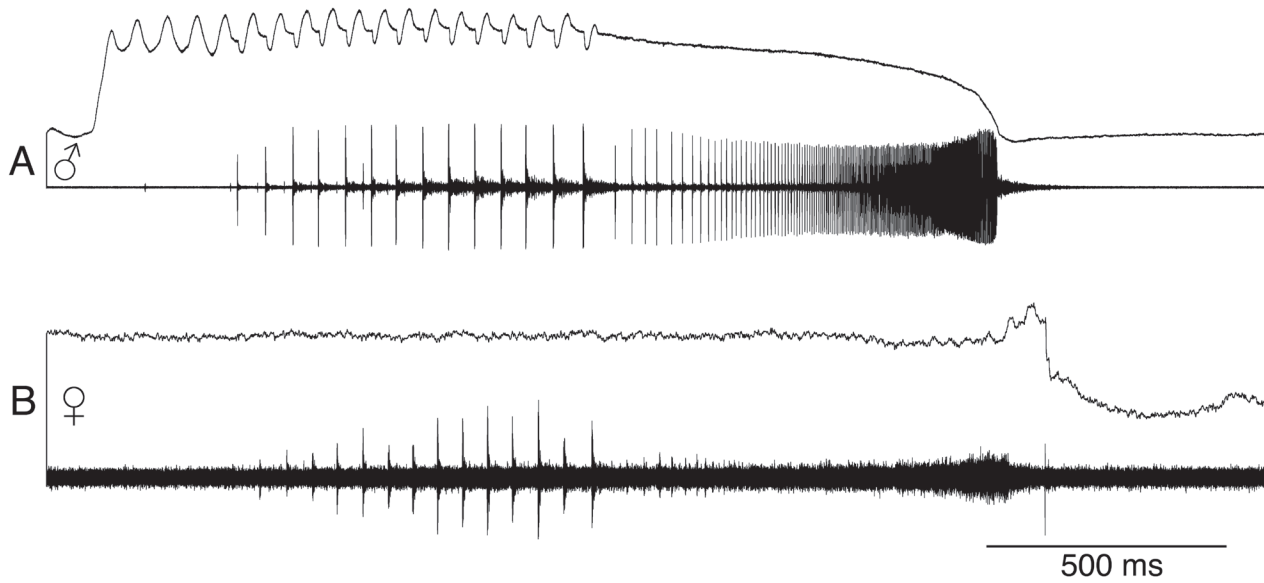


Figure 16. Male calling song A. and female response B. of *Ectadia fulva*. Oscillograms of stridulatory movement and song [synchronous registration of left tegmen movement and sound (upper line: upward deflection represents opening, downward closing; lower line: sound)]. Male and female were recorded separately.

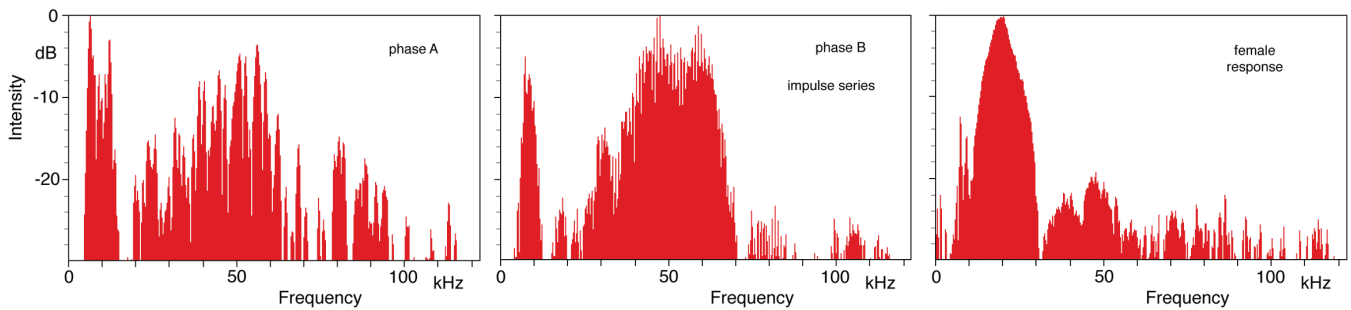


Figure 17. Power spectra of different parts of the male calling song and of the female response of *E. fulva*.





Figure 18. Female *Ectadia fulva* with spermatophore. A. overview, B. detail.

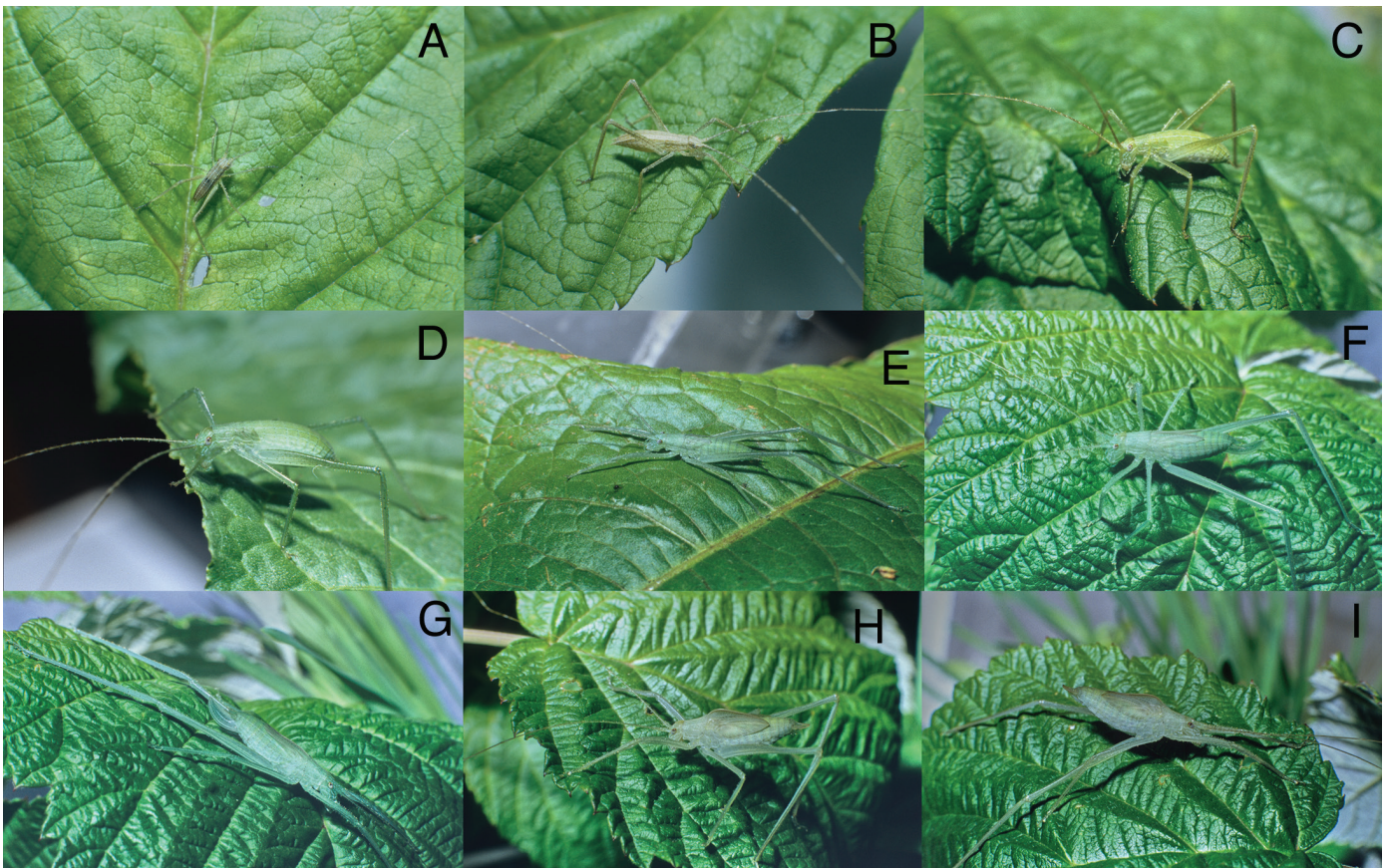


Figure 19. Nymphs of *Ectadia fulva*. A. First stage, B. second stage, C. third stage, D. 4<sup>th</sup> stage, E. 5<sup>th</sup> stage, male, F. 5<sup>th</sup> stage, female, G. 6<sup>th</sup> stage, female, H, I. 6<sup>th</sup> stage, male.

Besides the acoustical signals soundless vibratory body movements were observed in both sexes.

*Mating.*— In eight tests, a male (mean body mass  $199 \pm 16$  mg;  $n=7$ ) and a female (mean body mass  $626 \pm 71$  mg;  $n = 6$ ) were placed together for mating. Four couples mated with the males transferring

only very small spermatophores ( $1.3 \pm 0.6$  mg; no data on mating duration available; Fig. 18). Obviously the spermatophores consisted only from a pair of ampullas without spermatophylax.

*Nymphs.*— Postembryonic development occurred over six nymphal instars as in many other Phaneropterinae (Fig. 19). Development

from hatching to adult moult took 52–68 days at 20–23°C (Ingrisch 1998). Nymphs can be green or light brown. First instars appear green colored.

*Distribution.*— See Fig. 1.

## Discussion

Since the revision by Liu et al. (2004) the genus *Ectadia* is known for its diversity in male stridulatory files. Adding two more species, Gorochov (2009) again emphasizes characters connected to sound production, situated in the mirror area and the stridulatory file. However, until now only the song of *E. fulva* was known (Ingrisch 1998, Heller et al. 2015). *E. diuturna* sp. n. presents a song quite different in structure to that of *E. fulva*. The song of *E. fulva* consists of complicated but relatively short song units (less than 2 s) produced during opening and closing of the tegmina once completely, while the song units of *E. diuturna* sp. n. are very long lasting (more than 30 s) made by many movement cycles of the tegmina. The song of *E. diuturna* sp. n. thus resembles that of *Ducetia japonica* (Heller et al. 2017). Also the stridulatory files both in the *Ducetia japonica* group and *Ectadia* contain two parts with different structures. The genus *Ducetia* is phylogenetically probably close to *Elimaea* where *Ectadia* also belongs to (Gorochov 2009, Liu in prep.). However, *Ectadia diuturna* sp. n. and *Ducetia japonica* differ in a bioacoustically important point. During the long and complicated songs of *Ducetia*, no significant changes in spectrum were observed (Heller et al. 2017). While this is also true for *E. fulva*, in the song of *E. diuturna* sp. n. elements with three different spectra were recorded.

How may these different spectra be produced? From the spectral similarity between *E. fulva* and the long impulse group in *E. diuturna* sp. n., one can assume that this two-peak spectrum represents the typical vibration mode of the tegmina when excited during closing. A derived spectrum may result if the teeth are contacted during the opening movement. In several tettigonioid species the sounds produced during opening of the wings have different spectra than during closing (e.g. *Metrioptera* spp. Heller 1988, Skejo et al. 2015; *Lithodusa helverseni* Heller and Korsunovskaja 2009; *Saga* spp. Lemonnier-Darcemont et al. 2016, and with good reason assumed also for *Xiphelimum amplipennis* by Morris et al. 2016). This argument may apply for the short impulse group in *E. diuturna* sp. n. The different spectrum of the tick-like sounds may possibly result from the contact of large teeth situated anally near to the outer margin of the tegmen.

Do the different frequencies have any biologically important function? There is no simple answer. The argument that broad-banded songs or songs with different frequencies can give some information about the distance to a signaller (see Morris et al. 2016) is certainly correct. But since the spectrum of the female response – assumed to be similar in *E. diuturna* sp. n. to that of *E. fulva* – also has its peak at 20 kHz like the tick-like sounds and the short impulse group, one has to analyse carefully what happens if rivals hear a male song together with a female response. Communication by duetting is always susceptible to eavesdropping. Possibly the mixture of signals with different spectra make this kind of signal exploitation more difficult. Males of the African phaneropterine *Gonatoxia helleri* seem even to imitate the spectrum of the female response to disturb eavesdropping rivals (Heller and Hemp in prep.).

Surprisingly, the species which deviates in file morphology most widely from all others – *Ectadia fulva* – has by far the largest geographic range. One reason could be that this species seems to

be adapted to relatively low altitudes. It was found between 250 and 1300 m a.s.l. (Ingrisch 1998, Liu et al. 2004, Gorochov 2009), while *E. sinuata*, *E. angusta* and *E. diuturna* occur at 1600 m a.s.l. and much above (Liu et al. 2004, Gorochov 2009, this paper). Only in Vietnamese Tam Dao at 800–1000 m a.s.l. – relatively high for *E. fulva* – does this species occur together with *Ectadia mistshenkoi*. A similar partial overlap may exist with *E. obsolescens* (650–1600 m a.s.l.). Possibly most other *Ectadia* species are restricted to (isolated) mountains, although for some species the altitude of their occurrence is not known. So the differences in stridulatory organs are not easily explained by interspecific interactions except assuming much wider ranges than today during the ice ages.

Concerning chromosome evolution, the data presented here are the first available for the genus *Ectadia*. The basic model karyotype of Phaneropterinae, present also in most tettigoniids, consists of 31 (male) with the X0 (male) sex determination system (for a review see Warchałowska-Śliwa 1998). In *Ectadia*, similarly to phaneropterids in another genus of the tribe *Elimaeini*, *Elimaea*, and in the related *Ducetia*, the ancestral chromosome number is reduced to  $2n=29$  (X0), including one tandem fusion between two pairs of autosomes, all chromosomes being acrocentric (e.g. Heller et al. 2017, Warchałowska-Śliwa et al. 2011). In *Ectadia diuturna*, the pattern of heterochromatin distribution has revealed size heteromorphism of C-bands and differential intensity of rDNA hybridization-signals between homologous chromosomes of long-sized pair. These differences suggest the occurrence of polymorphism in the number of copies of rDNA sequences as a result of amplification or loss through different mechanisms such as unequal meiotic crossing-over, homologous recombination, tandem duplication of ribosomal genes, or translocation rearrangements (e.g. Cabral-de-Mello et al. 2011). Similar heteromorphism has been observed in other bushcrickets (e.g. Warchałowska-Śliwa et al. 2013, Grzywacz et al. 2014).

## Acknowledgements

Our thanks go to Susanne Randolph, Vienna, for the unsuccessful search for the holotype of *E. pilosa*, and to Andrei Gorochov, St. Petersburg, for information about the files of some *Ectadia* species. The study was partly funded by the National Natural Science Foundation of China (No. 31572308).

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# New distribution records of Orthoptera of Greece

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## Abstract

New distribution data on Orthoptera of Greece are presented. *Acrometopa servillea* (Brullé, 1832), *Platycleis grisea* (Fabricius, 1781) and *Poecilimon propinquus* Brunner von Wattenwyl, 1878 are reported as new to Mt. Kitheron, the last one also as new to Nomos Biotias. *Anterastes serbicus* Brunner von Wattenwyl, 1882 and *Ephippiger ephippiger ephippiger* (Fiebiger, 1784) are reported as new to Mt. Vrontodous. *Platycleis escalerae escalerae* Bolivar, 1899, *Incertana incerta* (Brunner von Wattenwyl, 1882), *Eupholi-doptera kykladica* Heller, Willemse and Willemse, 2009 and *Eumodicogryllus bordigalensis bordigalensis* (Latreille, 1804) are new to Attiki. The last two are also new to Sterea Ellas. *Platycleis affinis affinis* Fieber, 1853, is new to Kiklades. *Gryllus (Gryllus) campestris* Linnaeus, 1758, *Xya variegata* (Latreille, 1809) and *Xya pfaendleri* Harz, 1970 are new records for Nomos Serron. *Trigonidium (Trigonidium) cicindeloides* Rambur, 1838 is reported as new to Macedonia. We confirm the presence of *Mogoplistes brunneus* Serville, 1838 at the Aegean Sea. Lastly, *Paranocarodes chopardi* Pechev, 1965 is reported as new to Macedonia and sound production in that species is discussed for the first time.

## Key words

new localities, stridulation, Macedonia, occurrence

## Introduction

The southern Balkan peninsula is considered as one of the most important core areas of European endemic Orthoptera. The area served as a refugium during the glacial periods (Kenyeres et al. 2009). The orthopteroid fauna of Greece is considered relatively well studied, especially after the fundamental publications of the Willemse family (1984, 1986, etc.). Even so, since then description of new species and subspecies and new records for the country have considerably inflated the number of taxa known from Greece, which now reach about 395 (Willemse and Willemse 2008). In comparison, the number of Orthoptera species of Bulgaria is 213 (Chobanov 2009) and 175 taxa in FYR Macedonia (Lemonnier-Darcemont et al. 2014). The Orthoptera species richness of Greece is a result of the highly diverse topography, dominated by the Hel-

lenids mountain range (south branch of the Dinarids), the complex palaeogeographic and geological history, the fact that the area served as a refugium during glacial periods and the position of the country between different zoogeographic regions.

## Methods

The taxonomic nomenclature follows the Orthoptera Species Files (OSF) (Cigliano et al. 2016).

The material on which this paper is based consists of specimens deposited in the author's collection (cSA). Several specimens will be deposited to the collection of Zoological Museum of University of Athens (ZMUA). The following specimens were transferred to Abant İzzet Baysal Üniversitesi Entomoloji Müzesi, Bolu (AİBÜEM): *Paranocarodes chopardi*, Macedonia, Mt. Vrontodous (Lailias), 16 IV 2016, 1 ♂; 19 V 2016, 1 ♀, for confirmation of identification. Specimens of *Poecilimon propinquus*, Sterea Ellas, Biotia (Boeotia), Mt. Kitheron, c.1400m, 20 VI 2015, were transferred to CH (Collectio Klaus-Gerhard Heller, 2 ♂), CL (Collectio Arne W. Lehmann, 2 ♂) and CW (Collectio Willemse, 2 ♂).

The material has been collected in different parts of Greece by the author himself in the years 2012 to 2016, by hand or with a net or by shifting rocks and leaf litter. Specimens were identified using Willemse (1986) and papers published after that.

## Results

### Tettigonioidea

#### Tettigoniidae

##### *Acrometopa servillea* (Brullé, 1832)

Sterea Ellas: Attiki / Biotia (Boeotia), Mt. Kitheron, 1400m, 20 VI 2015, 2 ♀.

A species common on the eastern half of the Greek mainland (Willemse 1984). New for Mt. Kitheron.

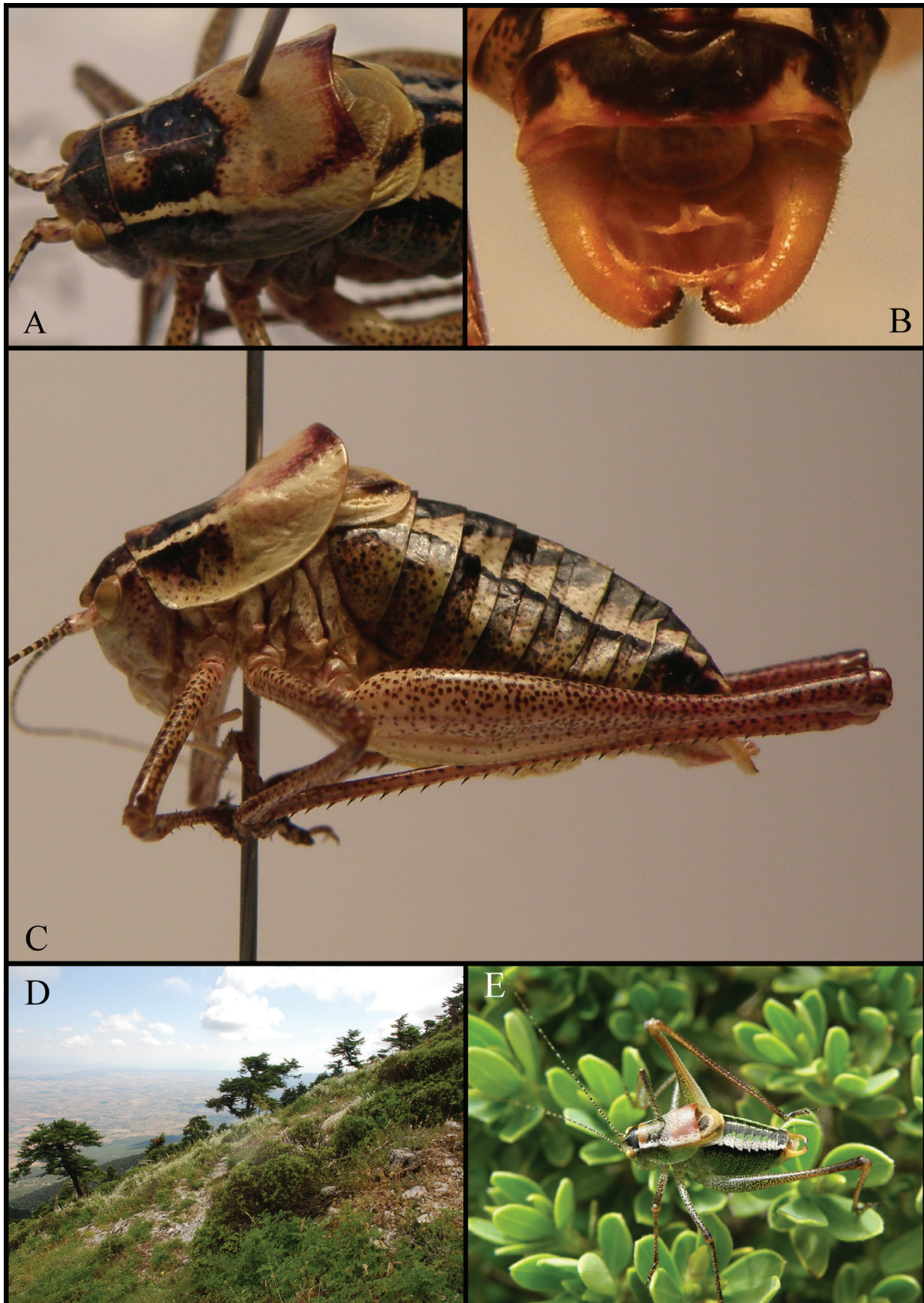


Figure 1. *Poecilimon propinquus*, Sterea Ellas, Mt. Kitheron, 19 V 2013. A. pronotum, B. tip of abdomen, C. habitus, D. collection locality with scattered bushes of *Daphne*, E. habitus.

*Poecilimon propinquus* Brunner von Wattenwyl, 1878

Stereia Ellas: Biotia (Boeotia), Mt. Kitheron, c.1400m, 19 V 2013, 1♂; 20 VI 2015, 8♂, 1♀. Fig. 1A–E.

New for Biotia and Mt. Kitheron. The male cerci (Fig. 1B) and the female basal fold of our material agrees with the figures published in Willemse (1982, 1986). This is the first member of this genus to be collected from Mt. Kitheron. This mountain ridge forms the borders between Attiki and Biotia. The collection locality is on the northern slope of the mountain summit, which belongs to Biotia (Fig. 1D). *Poecilimon propinquus* is endemic to CE mainland Greece (Attiki and NE Peloponnisos), Evvia and some offshore island (Egina, Spetses, Makronisos) (Fig. 2). The species is known from several localities of Attiki (Parnitha, Imittos, Sounion, Boula), most of them on the north and eastern side (Willemse 1984, 1985). The only, yet unpublished, locality from W Attiki, is at Aigosthena, 5 km east of Porto Germenos, not far from Mt. Kitheron (Lehmann and Lehmann unpubl. 2002, A. Lehmann pers. comm. 2017). This is the fourth member of the genus known so far from Biotia, the other three being *P. gerlindae* Lehmann, Willemse and Heller, 2006, *P. obesus* Brunner von Wattenwyl, 1878 and *P. zimmeri* Ramme, 1933, occurring mostly on the west side of Biotia, Mt. Parnassos.

*Poecilimon gerlindae* was described from Domokos, Nomos Pthiotidos and it belongs to the *P. propinquus* group. Its southernmost known locality is Aliartos and Ipsilantis at the heart of Biotian valley. Our locality, only a few km to the south, is obviously situated on, or very close to, the borderline of these two closely related species. Although morphologically very similar, *P. propinquus* can easily be separated by the shape of the male cerci and female basal fold (Lehmann et al. 2006).

The population of Mt. Kitheron seems to be localized. This is the situation also on Mt. Imittos, the type locality of the species, where *P. propinquus* is also found in single sites (Lehmann et al. 2014). Several attempts, in several years, by the author to locate more populations were in vain. All the specimens were collected from an area of a few square meters, a rocky slope dominated by

*Daphne oleoides* shrubs, facing the famous Biotian valley (Fig. 1D). All insects were found on the *Daphne* shrubs, except for a female found moving on the ground.

*Anterastes serbicus* Brunner von Wattenwyl, 1882

Macedonia: Mt. Vrontous (Lailias): 1500m, 20 VIII 2016, 1♂; 1850m, 20 VIII 2016, 2♂, 1♀. Fig. 3.

*Anterastes serbicus* is a mountainous species, with isolated, patchy distribution on summits of mountains of Balkan and W. Turkey (Çiplak et al. 2015, Mol et al. 2016). In Greece, it is known from the high elevation of seven mountains, Olimpos, Ossa, Pieria, Bermio, Barnous, Pangeo and Falakro (Willemse 1984) (Fig. 4).

Mt. Vrontous is a well visited mountain mainly because of the road that leads to the ski resort. It is situated just north of the town of Serres. On Mt. Vrontous, *A. serbicus* was collected



Figure 3. *Anterastes serbicus*, lateral view (scale bar: 5)

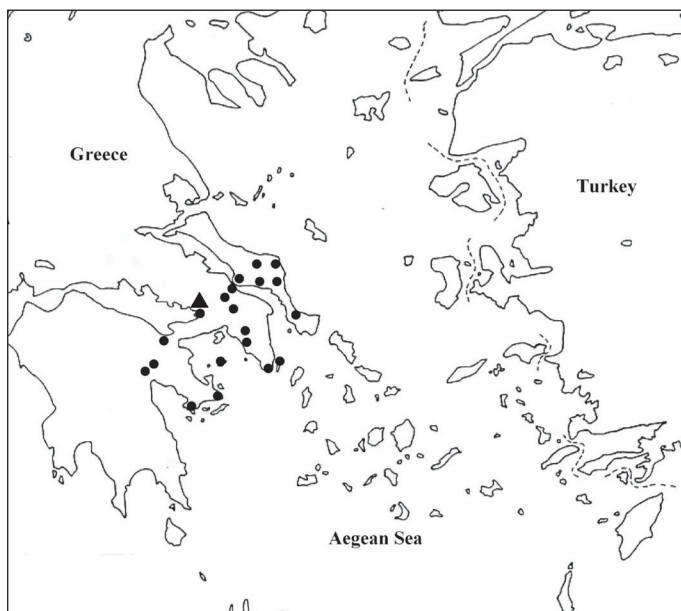


Figure 2. Total known distribution of *Poecilimon propinquus* (• literature localities, ▲ new locality).

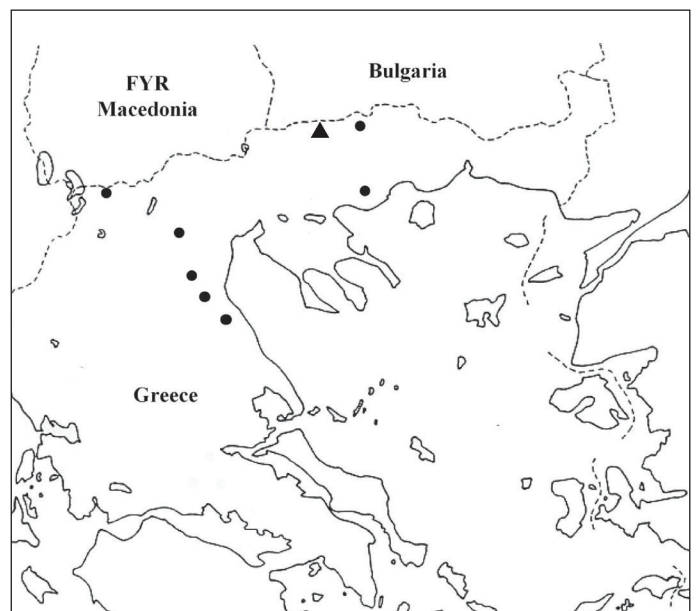


Figure 4. Distribution of *Anterastes serbicus* in Greece (• literature localities, ▲ new locality).

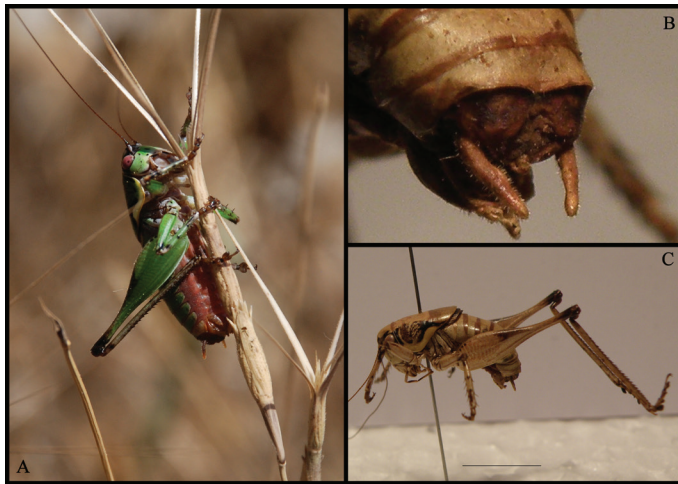


Figure 5. *Eupholidoptera kykladica*, Attiki, Vravra, 11 VI 2013. A. habitus, B. last abdominal segment, dorso-lateral view, C. lateral view (scale bar: 1 cm).

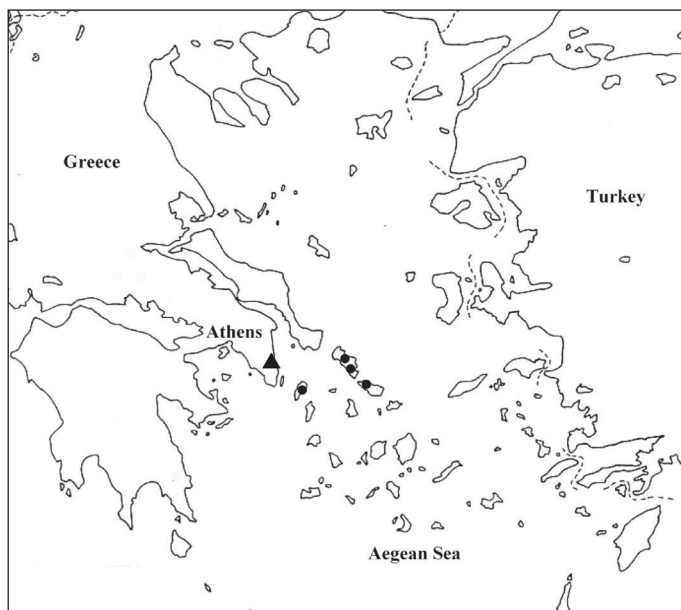


Figure 6. Total known distribution of *Eupholidoptera kykladica* (● literature records, ▲ new record).

from the plateau of the ski center at 1500m, inside dense grass vegetation. The species was most abundant above timberline, at the summit area (1850m), on dry rocky ground with very sparse vegetation. It should be noted that compared to specimens from Mt. Pangeo, those of Mt. Vrondous are 25% smaller, with a body length of 11–13 mm.

*Eupholidoptera kykladica* Heller, Willemse and Willemse, 2009

Stereia Ellas: Attiki, Vravra, wasteland, 11 VI 2013, 1♂. Fig. 5A–C.

New for Attiki. This is a recently described, small sized *Eupholidoptera*, endemic to the west Kikladic islands of Andros, Tinos and Kea (Tzia) (Çiplak et al. 2009) (Fig. 6). Our location is the first mainland population of this species. The specimen collected

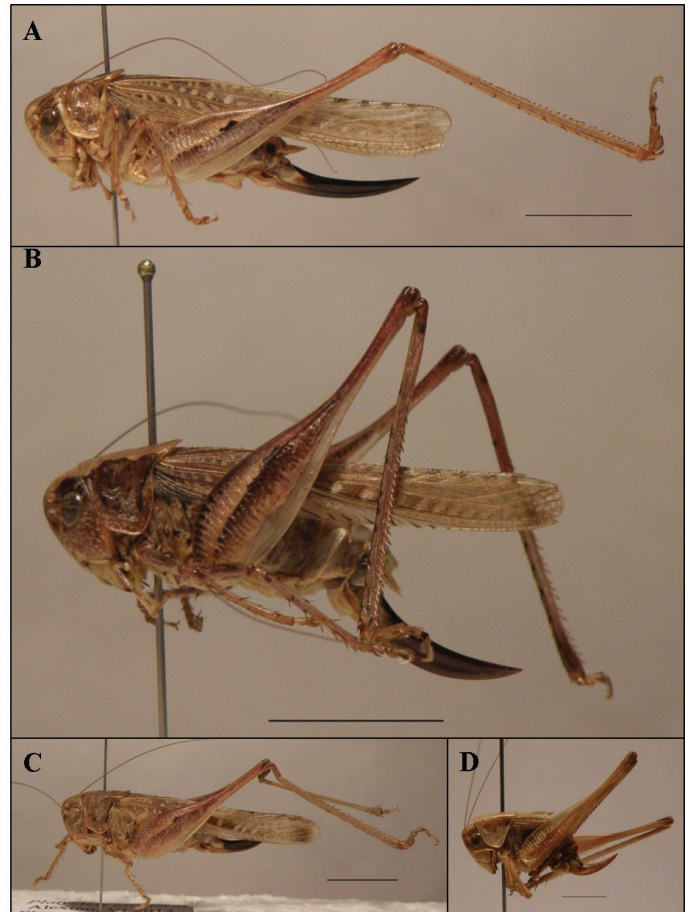


Figure 7. A. *Platycleis affinis affinis*, female, Kiklades, Andros (scale bar: 10 mm), B. *Platycleis grisea*, female, Mt. Kitheron (scale bar: 10 mm), C. *Platycleis escaleraei escaleraei*, female, Attiki, Vravra (scale bar: 10 mm), D. *Incertana incerta*, female, Attiki, Vravra (scale bar: 5 mm).

agrees perfectly with the description regarding small size, coloration (Fig. 5A), cerci (Fig. 5B) and subgenital plate. It was collected from *Sarcopoterium spinosum*, a small, thorny bush, typical of dry coastal habitats of Mediterranean Greece. Measurements (length, in mm): body 18, pronotum 8, hind femur 15.5.

*Eupholidoptera kykladica* belongs to *E. prasina*-group, a group of Aegean and Anatolian species, mostly with localized distribution. The locality reported here represents the first documented presence of a member of this group on mainland Greece, an area otherwise dominated by members of *E. chabrieri*-group (Çiplak et al. 2009).

The presence of *E. kykladica* on Attiki, or the east mainland in general, is no surprise. The zoogeographical connection of west Kikladic islands with mainland Greece is well documented. Two other orthopteran species that have a similar distribution pattern are *Rhacocleis werneri* F. Willemse, 1982, described from Evvia and since then found on east Attiki and Andros island and *Leptophyes lisae* Heller and Willemse, 1989, occurring at E Peloponnisos and Andros (Willemse and Willemse 2008).

The genus *Eupholidoptera* is rare in the area of Attiki. Antonatos et al. (2014) were the first to report members of the genus from Attiki. In particular, they report *Eupholidoptera* sp.<sub>1</sub> from the area of the Athens International Airport, which is located at the



lowland, near Vravrona, and *Eupholidoptera* sp<sub>2</sub>, from Mt. Parnitha. In both locations, *Eupholidoptera* were rare, less than 1% of the sampled specimens. In particular, *Eupholidoptera* sp<sub>1</sub>, which could prove to be *E. kykladica*, represented only 0.002% of the collected specimens, over a period of two years, while *Eupholidoptera* sp<sub>2</sub>, from the mountain location, represented 0.94%, respectively. On the other hand, Lehmann et al. (2014), in their acoustic monitoring of orthopterans on Mt. Imittos, did not report any *Eupholidoptera* species. We collected members of this genus from Mt. Imittos (VI 2014, 1♀) and Mt. Kitheron (VIII 2014, 1♀; 20 VI 2015, 1♀). All collected specimens are females of small body size (less than 15 mm). Since *Eupholidoptera* species are recognized mainly by male genital morphology, we hesitate to assign them to a particular species until further field research results in collection of male specimens.

*Platycleis affinis affinis* Fieber, 1853

Kyklades: Andros, 18 VI 2015, 2♀. Figs 7A, 8A.

New for Kyklades. This circum-Mediterranean subspecies is common on mainland Greece. It has also been recorded from Kriti

and few island. On Andros it was found by the road to Chora, on short grass. This is the second member of this genus recorded from Kyklades, after *P. intermedia intermedia* (Serville, 1839), which is common in this area (Willemse 1984).

*Platycleis grisea* (Fabricius, 1781)

Stereia Ellas: Mt. Kitheron, VIII 2014, 1♀. Figs 7B, 8B.

New for Mt. Kitheron. A mountain taxon, reported from several mountains of mainland.

*Platycleis escalerae escalerae* Bolivar, 1899

Stereia Ellas: Attiki, Vravrona, wasteland. VI 2013, 1♀. Figs 7C, 8C.

New for Attiki. This East Mediterranean subspecies is known from mainland Greece and the islands of Kriti and Rhodos. On Vravrona it was collected from a wasteland, dominated by thorny bushes of *Sarcopoterium* and *Coridothymus*.

*Incertana incerta* (Brunner von Wattenwyl, 1882)

Stereia Ellas: Attiki, Vravrona, VII 2014, 1♀; Schinias, II 2013, 1♂. Figs 7D, 8D.

New for Attiki. This East Mediterranean species is known from mainland Greece and several Aegean islands.

*Ephippiger ephippiger ephippiger* (Fiebiger, 1784)

Macedonia: Mt. Pangeo, 2000m, 7 VIII 2016, 1♂, 1♀. Fig. 9.

New for Mt. Pangeo. Known Greek localities for this central- and south-European species are Mt. Falakro, Mt. Vrontous, Mt. Cholomon, Paleokastron Chalkidikis, Mt. Barnous, Veldos, Mt. Olimbos (Willemse 1984) and Mt. Athos (Tilmans et al. 1989).

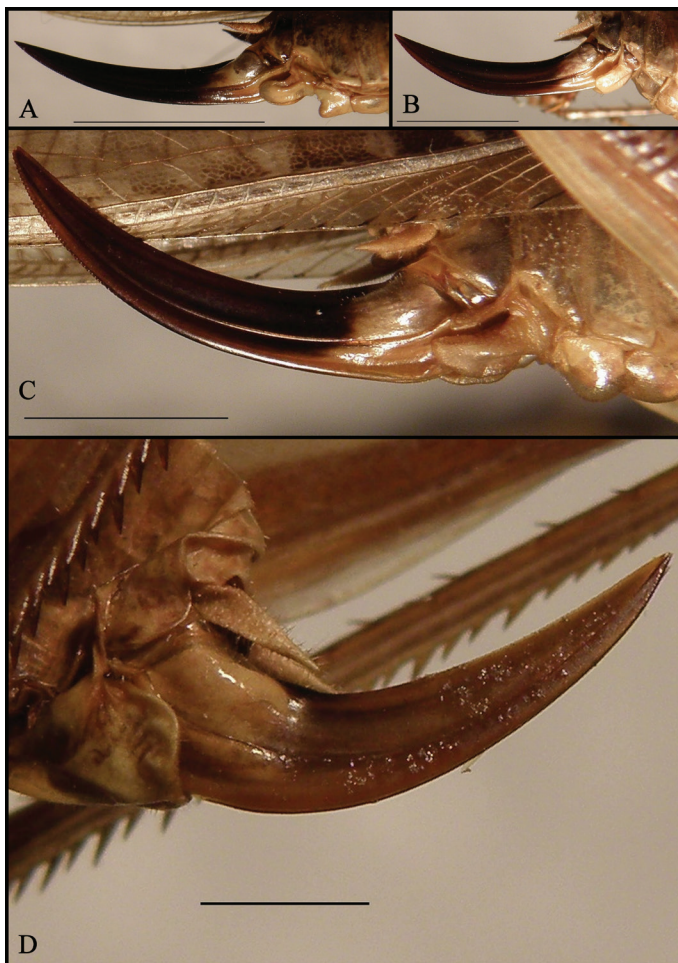


Figure 8. Female, VII and VI sternites, subgenital plate and ovipositor, lateral view A. *Platycleis affinis affinis* (scale bar: 10 mm), B. *Platycleis grisea* (scale bar: 5 mm), C. *Platycleis escalerae escalerae* (scale bar: 5 mm), D. *Incertana incerta*, (scale bar: 2 mm).



Figure 9. *Ephippiger ephippiger ephippiger*, male, Macedonia, Mt. Pangeo, 7 VIII 2016.

## Grylloidea

## Gryllidae

*Gryllus (Gryllus) campestris* Linnaeus, 1758

Macedonia: Serres, Mt. Vrontous, 600m, IV 2016, 1♀.

New for Nomos Serron. A west Palearctic species, known from mainland Greece and the east Aegean islands of Limnos and Kos. Other Macedonian localities are Mt. Falakro, Pisoderi Florinas, Velvendos Kozanis and Mt. Pieria (Willemse 1984).

*Eumodicogryllus bordigalensis bordigalensis* (Latreille, 1804)

Stereia Ellas: Attiki, Vravra, Erasinis river, 1 nymph, X 2012; Biotia (Boeotia), entrance of cave Kopaida, XI 2014, 3 nymphs. Fig. 10A, B.

New for Attiki and Stereia Ellas, this widespread Mediterranean species has been recorded from a few localities all over Greece and the islands. Our specimens were collected from under stones, in wet places, close to the margin of cultivations. At the first locality, it was found in syntopy with *Gryllus bimaculatus* De Geer, 1773. Identification of our material was achieved through the details of the head (Fig. 10A) as summarized in Cordero et al. (2007).



Figure 10. *Eumodicogryllus bordigalensis bordigalensis*, Attiki, Vravra, Erasinis river, X 2012. A. frontal view of the head, B. lateral view (scale bar: 5 mm).

## Trigonidiidae

*Trigonidium (Trigonidium) cicindeloides* Rambur, 1838

Macedonia: Chalkidiki, near village Afitos, 40m, 11 VII 2016, 2 ♀. Fig. 11.

New for Macedonia. This very characteristic orthopteran species is widespread in many parts of the world. In Greece it has been recorded from several localities of southern and central mainland and some islands, as far north as Kerkira island and Magnisia. The specimens were observed and collected during the day time on the wall of a small hotel surrounded by dense pine forest.

## Mogoplistidae

*Mogoplistes brunneus* Serville, 1838

Dodecanisa: Rhodos, 8 X 2016, 2 ♂, 1 ♀. Fig. 12.

New for Rhodos and the East Aegean islands. This is a mainly west Mediterranean species. Until now its eastern distribution limits were formed by the islands of Kerkira and Thira (Santorini). According to Willemse (1984), especially Thira record needs confirmation. The specimens collected from Rhodos expand the dis-



Figure 11. *Trigonidium cicindeloides*, female, Macedonia, Chalkidiki, Afitos, 11 VII 2016.



Figure 12. *Mogoplistes brunneus*, Dodecanisa, Rhodos, 'Valley of the Butterflies', 8 X 2016.

tribution of the species considerably to the east. The locality is the well known 'Valley of Butterflies', a rivulet of a few hundred meters length on the west side of the island, named by the locals 'Pelecanos'. The area is visited by millions of tourists every summer in order to admire the huge concentration of the moth *Euplagia quadripunctaria*. It is a humid and shady place dominated by *Platanus orientalis* and *Liquidambar orientalis* trees. *Mogoplistes brunneus* was observed and collected at night from inside the leaf litter, under rocks and on the surface of the rocks that were covered with mosses, where it was common. Syntopic, but less common, we also found *Ovaliptila* sp. nov. (Gorochov and Alexiou, in press.). The related *Pseudomogoplistes squamiger* (Fischer, 1853) was reported from nearby Kos and Tilos islands (Baccetti 1992). Our specimens were identified using Willemse (1986).

### Tridactyloidea

#### Tridactylidae

##### *Xya variegata* (Latreille, 1809)

Macedonia: Serres, Strimon river, 25 V 2016, 2 ♂. Fig. 13A.

New for Nomos Serron. Already known from other rivers of Macedonia, like Nestos. Found in syntopy with *X. pfaendleri*.



Figure 13. *Xya* species, Macedonia, Strimon river, 25 V 2016. A. *X. variegata*, B. *X. pfaendleri*.



Figure 14. *Paranocarodes chopardi*, habitus, female, Macedonia, Mt. Vrontous, 19 V 2016.

##### *Xya pfaendleri* Harz, 1970

Macedonia: Serres, Strimon river, 25 V 2016, 1 ♀, 1 nymph. Fig. 13B.

New for Nomos Serron. Already known from other rivers of Macedonia, like Axios and Nestos. Found in syntopy with *X. variegata*.

### Acridoidea

#### Pamphagidae

##### *Paranocarodes chopardi* Pechev, 1965

Macedonia: Mt. Vrontous (Lailias): 1000m, 16 IV 2016, 2 ♂; 19 V 2016, 1 ♀. Figs 14–15.

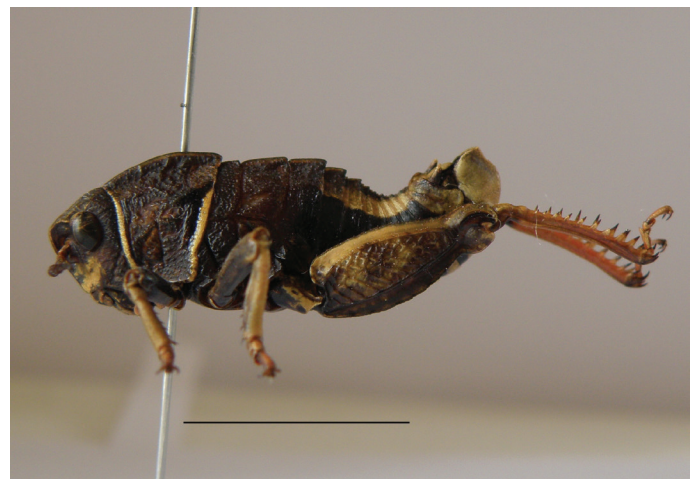


Figure 15. *Paranocarodes chopardi*, habitus, male, Macedonia, Mt. Vrontous, 16 IV 2016, lateral view (scale bar, 10 mm).



Figure 16. Total known distribution of *Paranocarodes chopardi* (• literature records, ▲ new record).

New to Macedonia. This species was described from the extreme southeast Bulgaria, the eastern foothills of Rhodopi mountain, close to the Greek-Bulgarian borders. In 2001 it was reported as new to the Greek fauna, from several localities of Evros, Thraki (Kati and Willemse 2001) (Fig. 16). Its habitat was described as *Quercus* sp. forest, sometimes mixed with *Pinus brutia* and/or *P. nigra*, commonly with dead leaf litter in which the animal was frequently found, and at an altitude of 200-900m a.s.l.

Pamphagids of the Balkan Peninsula, *Paranocarodes* I. Bolívar, and the related *Nocaracris* Uvarov, 1928 (*Paranocaracris* Mistshenko, 1951 was synonymized with *Nocaracris* by Ünal 2016), are ancient relicts, remnants of a thermophilous Tertiary fauna and have restricted ranges (Popov 2007). The locality of Mt. Vrondous is isolated from the populations in Thraki. Our specimens were collected from a meadow with a few scattered bushes, mainly *Juniperus* sp., bordering dense *Pinus nigra* forest, at an altitude of c. 1000m. The males were found very close to each other and were noticed from a distance by their strong stridulation. The female was noticed crossing the asphalt road from the same meadow towards the forest. No other specimen could be found despite several efforts. Apparently nymphs overwinter and adults appear in early spring, which may explain how such an obvious and impressive insect has gone unnoticed for so long on a well accessible mountain. According to L. Willemse (pers. comm. 2016), nymphs of what could be members of *Paranocarodes* have been observed from a couple of adjacent localities of N. Greece.

The male phallic complex is one of the main diagnostic characters used to distinguish the genera of pamphagids (Ünal 2016). A study that took place at Bolu by Ünal on the phallic complex of specimens sent to him confirmed our identification: this specific characteristic is almost the same as the one of the type specimen, kept provisionally at Bolu. Other morphological characteristics, such as the shape of the pronotum and coloration, are also identical with the type specimen. The tympanum size though of our specimen is much smaller than the typical *P. chopardi*, but a variation of this character has been documented in other pamphagids as well (Ünal pers. comm. 2017).

#### Notes on stridulation of *Paranocarodes chopardi*

The stridulation (sound production) mechanism within Pamphagidae is very diverse because of the secondary adaptation of some structures for its function (García et al. 2014). Almost all cases of sound production in this family have been documented from females as part of the mating behavior. Sound production in males has very rarely been observed (López et al. 2008). Our observations in the field, a product of good luck, for the first time document that the males of *P. chopardi* stridulate. As far as we know, no other record of stridulation within that particular genus exists.

The purpose of stridulation and the behavior of the males while producing the sounds is intriguing. No female was in sight. The two individuals were very active, moving along parallel paths, within less than a meter away from each other. An unresolved issue is whether the two males were stridulating as part of aggression behavior towards each other, or because of the disturbance caused by our presence, and not as part of an attempt to attract females for mating. Ünal (2007) reached similar results for the pamphagid genus *Glyphotmethis*, documenting that members of this genus produce sounds through Krauss's organ not to attract the different sex but for defense reasons.

The sound produced consisted of 'clicks' easily audible from several meters away. We observed that they used a rubbing method, involving the movement of the hind "feet" (it is uncertain whether tarsi or femora are involved), up and down on the vertical axis. *Paranocarodes* species are extremely squamipterous, with only traces of tegmina found, so the emission of sounds by the males was not achieved by means of rubbing the tegmina against parts of the hind leg, but through a different way, involving most probably parts of the hind legs and the sides of abdomen. Ünal (2007), in his research on *Glyphotmethis*, observed that the sound was produced by rubbing the hind femur on Krauss's organ.

Massa (2012) studied a large number of Pamphagidae genera for their ability to produce sounds. Krauss's organ is a small plate situated on both sides of the second abdominal tergite in most pamphagids, and is supposed to have a stridulatory function by rubbing hind femurs against it. This method of sound production is considered most primitive. The species of genus *Paranocarodes* Bolívar, 1916 have only a just visible Krauss's organ and no specialized hind femur (Massa 2012), thus have probably abandoned this mechanism, and have secondarily adapted a more evolved method of sound production (García et al. 2014). The tympanum, a structure placed also on both sides of the abdominal tergites, although normally used for hearing only, in this case amplifies the emitted sounds. According to Massa (2012), members of *Paranocarodes* have a wide tympanum. *Paranocarodes chopardi* however does not seem to fit that rule as it has a very small tympanum (Ünal pers. comm. 2017).

#### Acknowledgements

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# Adapting the Dragonfly Biotic Index to a katydid (Tettigoniidae) rapid assessment technique: case study of a biodiversity hotspot, the Cape Floristic Region, South Africa

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## Abstract

Global biodiversity faces many challenges, with the conservation of invertebrates among these. South Africa is megadiverse and has three global biodiversity hotspots. The country also employs two invertebrate-based rapid assessment techniques to evaluate habitat quality of freshwater ecosystems. While grasshoppers (Acrididae) are known indicators of terrestrial habitats, katydids (Tettigoniidae) could be as well. Here, we adapt a South African freshwater invertebrate-based rapid assessment method, the Dragonfly Biotic Index (DBI), for the terrestrial katydid assemblage, and propose a new assessment approach using katydids: the Katydid Biotic Index (KBI). KBI assigns each katydid species a score based on a combination of: 1) IUCN Red List status, 2) geographic distribution, and 3) life history traits (which consist of mobility and trophic level). This means that the rarer, more localized, specialized and threatened katydid species receive the highest score, and the common, geographically widespread and Least Concern species the lowest. As a case study, we calculated KBI across one of South Africa's global biodiversity hotspots, the Cape Floristic Region (CFR). We then correlated KBI/Site scores of individual ecosystems with their ecosystem threat scores. The CFR's katydid assemblage did not differ significantly from that of the overall South African katydid assemblage in terms of its species traits, threat statuses, or distribution among tettigoniid subfamilies. Likewise, KBI/Site scores did not differ significantly among ecosystem threat statuses. This may be explained by the coarse spatial scale of this study or by the lack of specialization of the CFR katydid assemblage. Nevertheless, the KBI holds promise as it is a relatively simple and non-invasive technique for taking invertebrate species composition into account in an assessment of habitat quality. In regions where katydid assemblages are well-known, acoustic surveys and KBI may provide an efficient means for assessing habitats.

## Key words

conservation, IUCN Red-List, life history, species traits, ecosystem threat

## Introduction

Global biodiversity is facing many challenges, resulting in the extinction of species at rates estimated to be 100 to 1000 times faster than the background extinction rate (Rockström et al. 2009). Biodiversity is often measured, or assessed, to guide conservation

planning. These assessments involve the measurement of various vertebrate or plant taxa. Although invertebrates are often not included in these assessments owing to their high numbers of species, it is sometimes assumed that due to the great numbers of insect-plant interactions that insect diversity may mirror that of the plants (Myers et al. 2000). Also, biodiversity assessments usually overlook species-specific information, so ignoring the intrinsic value of each species, and compromising the economic viability and conservation value of biodiversity assessments (Samways 2002).

South Africa currently employs two robust and rapid biodiversity assessment methods targeting freshwater and riparian habitats: the South African Scoring System (SASS) (Dickens and Graham 2002) and the Dragonfly Biotic Index (DBI) (Samways and Simaika 2016). Both of these methods are simple yet effective ways in which stream condition can be assessed based on the resident aquatic larvae of invertebrates (SASS) or on the adult dragonfly assemblages (DBI). The DBI uses three sub-indices to indicate the quality of a freshwater system: geographical distribution, habitat sensitivity and Red List status of each species at a focal locality. Based on these three sub-indices, each species is individually assessed and assigned a score of 0 to 9. The higher a species score, the higher the sensitivity of the species, the lower its tolerance to habitat disturbance, and the more threatened it is. This results in dragonfly assemblages being directly comparable in terms of their conservation value and allows for the ranking of different habitats according to their level of disturbance (Samways and Simaika 2016).

Grasshoppers (Orthoptera: Acrididae) in South Africa are a good bioindicator group within the grassland ecosystems (Bazelet and Samways 2011a, b) as well as the Grasshopper Conservation Index (GCI) having been developed within the Cape Floristic Region (CFR) (Matenaar et al. 2015). However, katydids (Orthoptera: Tettigoniidae) have not yet been explored in the region, and could potentially also be good biological indicators, especially in more woody environments. There are an estimated 169 katydid species in South Africa and, of these, two thirds are thought to be endemic to the country (Picker et al. 2004). So far, 129 species have been described and, as of December 2014, the threat statuses of these species have been assessed and uploaded onto the IUCN Red List (Bazelet et al. 2016). Coupled with the threat statuses, a

wealth of coarse-scale additional information is available, such as estimates of species distributions and life history information. In-depth studies on the biology of individual species are almost entirely lacking, but confident predictions can be made on the basis of trends among species and within higher taxa.

Most notably, mature male katydids produce characteristic species-specific songs enabling non-invasive species detection in an environment by listening alone (Bailey and Rentz 1990). Combined, these characteristics make katydids an attractive taxon upon which an acoustic rapid assessment method could be based for assessing the quality of terrestrial habitats in South Africa (Grant and Samways 2016).

Rapid assessment techniques are vital tools for detecting biodiversity, particularly in areas which have high species diversity and/or experience extreme threat, such as the biodiversity hotspots (Myers et al. 2000, Alonso et al. 2011). Global biodiversity is not homogenous in its distribution (Gaston 2000), with biodiversity hotspots covering only 2% of Earth's surface. Yet 50% of all plant species and 42% of terrestrial vertebrate species exist in this 2% of land (Mittermeier et al. 2004). These "traditional" biodiversity hotspots do not take into account invertebrate diversity, as it was assumed that insect diversity mirrors that of the plants based on the high numbers of observed insect-plant interactions (Orme et al. 2005). The CFR, one of three biodiversity hotspots in mega-diverse South Africa (Mittermeier et al. 2004), is an example of insect diversity mirroring plant diversity (Procheş and Cowling 2006), although these patterns do vary among insect taxa, with some having significantly higher diversity than others (Wright and Samways 1998, Procheş and Cowling 2006).

Here, a new biodiversity assessment method that employs katydids for monitoring terrestrial habitat quality based on an adaptation of the DBI is outlined. The calculation of the Katydid Biotic Index (KBI) is described, and a subset of museum records is used to conduct a case study to illustrate the efficacy of the KBI for assessing biodiversity and habitat quality across a biodiversity hotspot, the CFR, in South Africa. Ultimately, the KBI is evaluated with regards to its possible use in highlighting ecosystems in need of conservation action.

## Materials and methods

**Data collection.**— In 2014, the Red List threat statuses of 133 katydid species were assessed using records obtained from the MANTIS database (Naskrecki 2008). Geographical ranges of species and species endemism were calculated using the collection localities of the records. Published taxonomic descriptions as well as expert knowledge were used to assess various life history traits of the individual species (Rentz 1988, Naskrecki et al. 2008, Naskrecki and Bazelet 2009, 2012) [see Bazelet et al. (2016) for methods description].

**Development of the Katydid Biotic Index.**— The KBI allows for individual species to be ranked and compared. Based on similar criteria to that of the DBI, katydids were assessed based on three sub-indices: 1) Red List Status, 2) geographical distribution, and 3) life history traits (in which the mobility and the trophic level at which the species feed are scored on the basis of objective criteria, these two values are then summed, and scored accordingly). Each sub-index is scored from 0 to 3, with the life history category being a combination of individual scores for mobility and trophic level. These sub-indices are added together to give the KBI score for a species. These species KBI scores range from 0 for a widespread, habitat tolerant, Least Concern (LC) species to 9 for a narrow-range, highly habitat sensitive and Red Listed species (Table 1; Bazelet et al. 2016).

The sum of the scores in any specified region or at any particular site is the total KBI score. When the site score is divided by the number of species recorded, it gives the KBI/Site score. The KBI/Site score is thus an average value calculated from all the individual KBI species scores, and allows for the ranking of sites based on their katydid assemblages.

**Katydids in the Cape Floristic Region.**— Globally renowned for its botanical diversity, the CFR includes 122 different vegetation types or ecosystems (Government Gazette 2011) and covers <4% of southern Africa or an area of ±90 000 km<sup>2</sup>. Within this relatively small area, an estimated 8640 species of plants occur, of which

**Table 1.** Katydid Biotic Index calculation method.

Species Score	Threat (T)	Distribution (D)	Life History Traits (LH) <sup>†</sup>		
			Mobility (M)	Trophic Level (Tr)	M+Tr Sum
0	LC	Very common: > 75% coverage of SA and sA	Fully-flighted	Omnivorous	0
1	VU	Localized across a wide area in SA, and localized or common in sA: > 66% in SA and > 66% sA -OR- Very common in 1-3 provinces of SA and localized or common in sA: 0 - 33% SA and >66% sA	Only one sex flighted -OR- One or both sexes partially flighted	Predatory	1–2
2	EN	National SA endemic confined to 3 or more provinces: > 33% SA -OR- Widespread in sA but marginal and very rare in SA < 33% SA and > 66% sA	Flightless	Herbivorous, polyphagous	3
3	CR	Endemic or near-endemic and confined to only 1 or 2 SA provinces < 33% in SA alone		Herbivorous, monophagous	4–5

SA=South Africa, Lesotho, and Swaziland and sA = southern Africa (South Africa, Lesotho, Swaziland, Namibia, Botswana and Zimbabwe).

<sup>†</sup> To calculate LH score, M (range 0 - 2) + Tr (range 0 -3) are summed. The sum is assigned a logical species score.



65% are considered endemic to the CFR. The total number of species within the CFR is disproportionate to its small size as the observed number of species is comparable to that of tropical regions (Goldblatt and Manning 2002).

A subset of geo-referenced katydid collection localities ( $n = 207$  and accurate to eight decimal places) for the CFR was extracted from the MANTIS database (Naskrecki 2008; see supplementary material of Bazelet et al. (2016) for raw data records). Using QGIS (Quantum GIS Development Team 2015) katydid records were associated with the CFR ecosystem in which they were found, the threat statuses of the individual ecosystems was available in the list of threatened terrestrial ecosystems (available through the Biodiversity GIS programme of the South African National Biodiversity Institute, map scale was 1:250 000). Duplicate records of the same species were removed from the ecosystems so that there was only one record per species per ecosystem. Average KBI values for each individual ecosystem were calculated. The threat scores and average KBI scores were then mapped using QGIS.

*Statistical analysis.*— A Chi-square contingency table was used to determine whether the distribution of species among threat statuses and level of endemism were significantly correlated for South African and CFR katydid species. A Kruskal-Wallis test in R (R Development Core Team 2013) was used to assess differences in mean KBI scores of the katydid assemblages of the individual ecosystems and the threat categories to which the ecosystems belong (LC, VU, EN and CR). This was done across the entire CFR, separately for the ecosystems on the eastern seaboard and a one-way ANOVA was conducted for those on the western seaboard. Kruskal-Wallis was selected as it is suitable for non-parametric data, as KBI scores were not normally distributed (Shapiro-Wilk's  $W = 0.95$ ,  $p < 0.001$ ). Post-hoc Nemenyi-Tests were then conducted using the package PMCMR in R (Pohlert 2015) to assess pairwise differences among katydid threat statuses, ecosystem threat status and average KBI. After mapping the threat scores and average KBI scores of the ecosystems these two maps were then visually assessed in order to identify any emergent patterns.

## Results

Of the 133 katydid species which were assessed for IUCN Red List threat status, 16 (12%) were Data Deficient (DD) and were therefore excluded here from further analyses. Across all South African katydid species, over 50% are considered to be LC, while 35% of species were assessed as threatened [Vulnerable (VU), Endangered (EN), or Critically Endangered (CR)]; (Fig. 1A). Within the CFR, of the non-DD species, almost three-quarters (73%) of species are LC, and 27% of species are threatened (Fig. 1B).

The CFR katydids did not differ significantly from all South African katydids in terms of the number of species assigned to each threat status, endemism level, mobility class or trophic level ( $\chi^2_{(df=3, n=134)} = 0.88$ ,  $p > 0.05$ ;  $\chi^2_{(df=3, n=38)} = 0.25$ ,  $p > 0.05$ ;  $\chi^2_{(df=2)} = 0.9$ ,  $p > 0.05$  and  $\chi^2_{(df=3)} = 0.07$ ,  $p > 0.05$  respectively; Fig. 1).

Within the total katydid assemblage of South Africa, all species considered to be threatened (VU, EN or CR) were also endemic to the country, this is also true for the CFR katydid species (Fig. 2A, B). Across the CFR, 62% ( $n = 11$ ) of all species are flightless, and within the entire South African assemblage 60% ( $n = 11$ ) are flightless (Fig. 1H, G). No South African flighted species was assessed as either EN or CR (Fig. 2C), and all flighted species in the CFR were assessed to be LC (Fig. 2D). Among the South African katydid assemblage, species with varying trophic levels were evenly spread

across the threat status categories (Fig. 2E). However, within the CFR katydid assemblage, all omnivorous species were classified as LC, while 25% of species ( $n = 8$ ) were monophagous herbivores and these were relatively more prevalent in the threat classes (VU, EN and CR) than in LC (Fig. 2F).

The distribution of species in each subfamily maintained similar patterns in the CFR as in South Africa as a whole, with Phaneropterinae the most abundant subfamily overall, and Pseudophyllinae the least common (Fig. 3).

As expected, LC katydids have significantly lower median species-specific KBI scores than the threatened katydids (VU, EN and CR), but interestingly, these do not differ from each other ( $\chi^2 = 44.18$ ,  $df = 9$ ,  $p < 0.05$ ). There were no significant differences in the mean KBI scores among the ecosystem threat status categories ( $\chi^2 = 3.28$ ,  $df = 3$ ,  $p > 0.05$ ; Fig. 4). Although not significantly different ( $F = 0.91$ ,  $df = 33$ ,  $p > 0.05$ ), through visual inspection, in the western seaboard section of the CFR, there appears to be a slight but non-significant inverse correlation between the KBI score with ecosystem threat status, such that the lower the KBI score, the more threatened the ecosystem threat status. In the eastern seaboard section of the CFR, this relationship is not evident ( $\chi^2 = 0.79$ ,  $df = 3$ ,  $p > 0.05$ ; Fig. 5).

## Discussion

Although no significant differences were observed among the ecosystem threat statuses in terms of their KBI/Site values (i.e. average KBI value), the aim was rather to show how the KBI could be employed in the future once more thorough sampling has been conducted. When mapped, patterns do start to emerge in KBI/Site values among ecosystems. Ecosystems with low KBI/Site scores (mean KBI 0 – 4) tend to be those which are threatened (CR, EN and VU ecosystems) in the western CFR while the LC ecosystems tend to score higher KBI/Site values (mean KBI 5 – 8). This relationship is to be expected, as the more common and less sensitive species will be able to persist in ecosystems that have been transformed from the original state. Whereas the more sensitive and threatened species (those with higher species-specific KBI values) are expected to prefer the natural habitats and not to persist in the transformed systems. However, in the eastern CFR, where the ecosystems appear to be less threatened overall, there seems to be little correlation between the threat status of the ecosystems and their KBI/Site values. The LC ecosystems score relatively low KBI/Site values, between 0 and 4. These discrepancies could be due to numerous factors.

Among the possible explanations for the lack of correlation between ecosystem threat status and KBI/Site value, the small sample size is the most likely. With only 162 unique katydid records being present in 54 of the 122 CFR ecosystems (or 44% of ecosystems), the area is under-sampled. Furthermore, the scale of this study was very coarse and the KBI/Site values were calculated according to ecosystem threat polygons which is not a relevant biological spatial scale for katydids. Future work would need to determine the spatial scale at which the KBI/Site would be an accurate measure, as has been discussed for the DBI (Samways and Simaika 2016).

Furthermore, the CFR is an arid biome characterized by a matrix of agriculturally transformed landscapes and the native fynbos vegetation, which is characterized by evergreen plants in the Ericaceae, Restionaceae and Proteaceae families. Large trees are naturally almost absent from the CFR (Rebelo et al. 2006). In turn, katydids are known to be most diverse and abundant in tropical forest habitats and some subfamilies, like the Pseudophyllinae, show a strong degree of adapta-

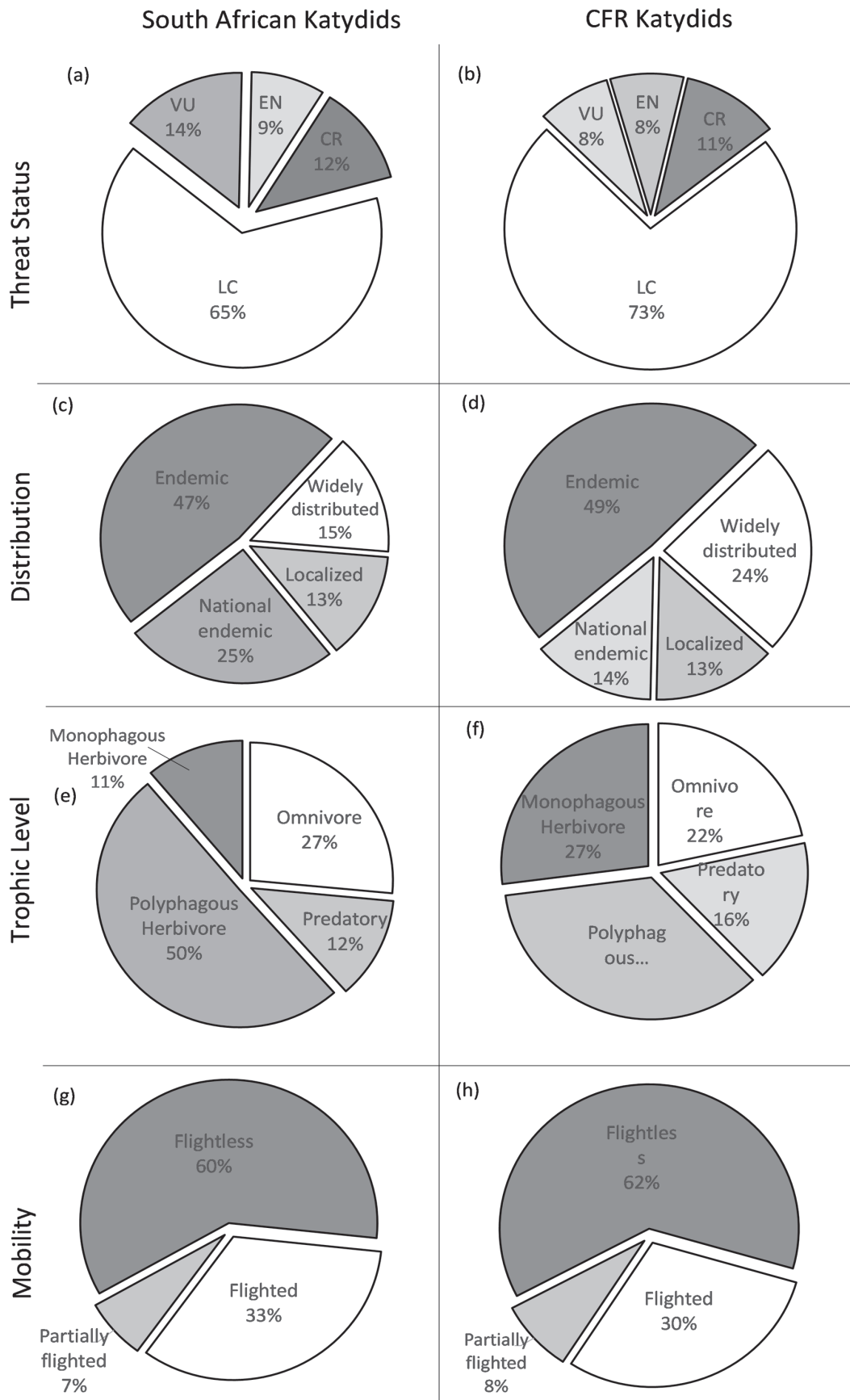


Figure 1. Proportion of South African (A, C, E, G) and Cape Floristic Region (B, D, F, H) katydid assemblages as characterised by the KBI assessment criteria (Threat Status, Distribution, Trophic level and Mobility).

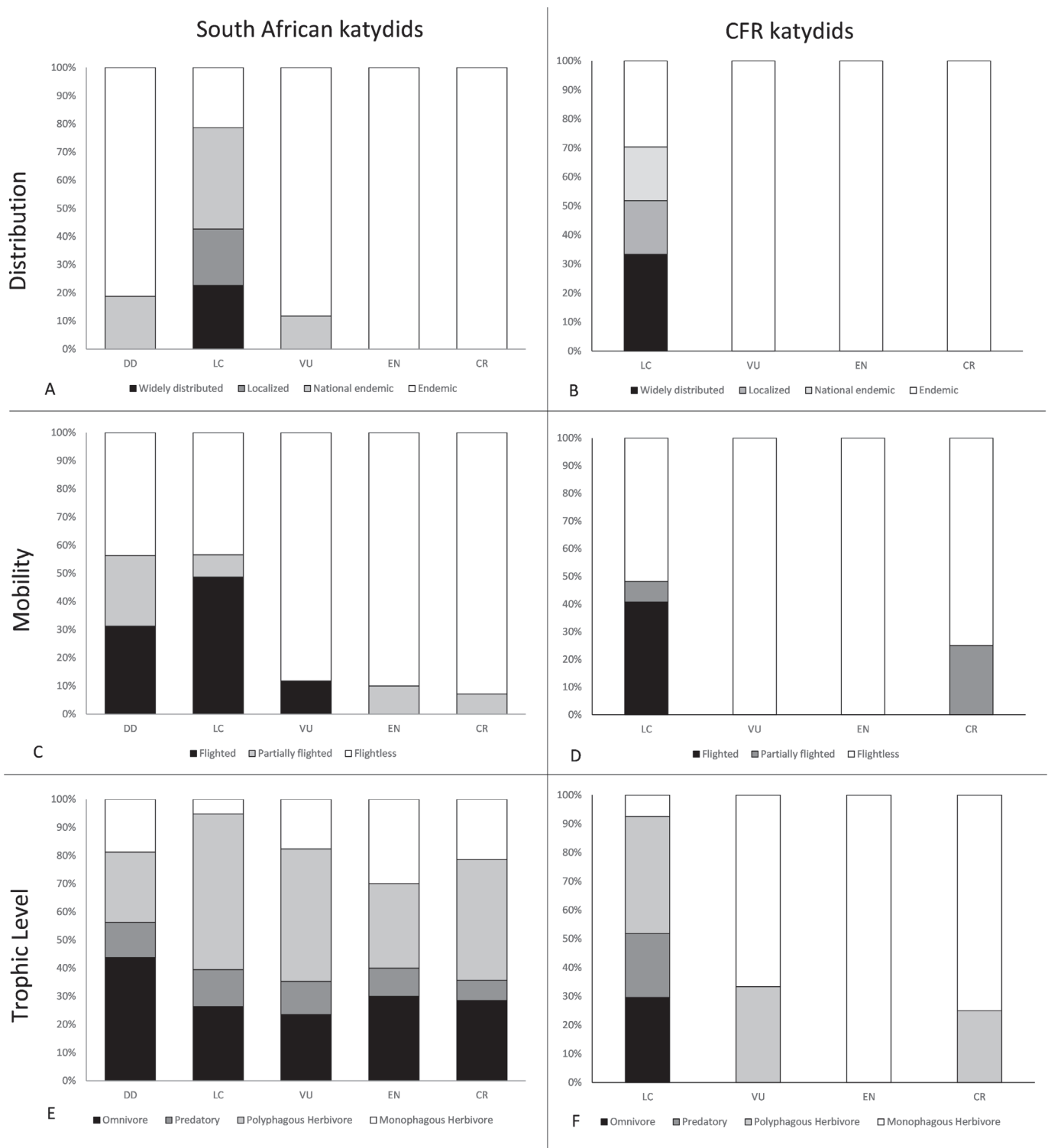


Figure 2. Composition of South African (A, C, E) and Cape Floristic Region (B, D, F) katydid assemblages as characterised by their distribution (A, B), mobility (C, D), and trophic level (E, F) relative to their IUCN threat status.

tion to tree environments, often bearing a strong cryptic resemblance to their tree habitats. Understandably, Pseudophyllinae are extremely rare in the CFR and in South Africa in general, of which only 1% is native forest habitat (Mucina and Rutherford 2006).

South African katydids are relatively well-documented (Naskrecki, unpublished data). Information regarding the ecology

and habitat requirements of the species is relatively well-known, and where information is lacking, it is possible to infer a species' biological characteristics based on well-documented related species. Indeed, most species could be assessed for the IUCN's Red List (Bazelet et al. 2016). Although some habitats and katydid groups are more diverse than others, katydids are found in nearly

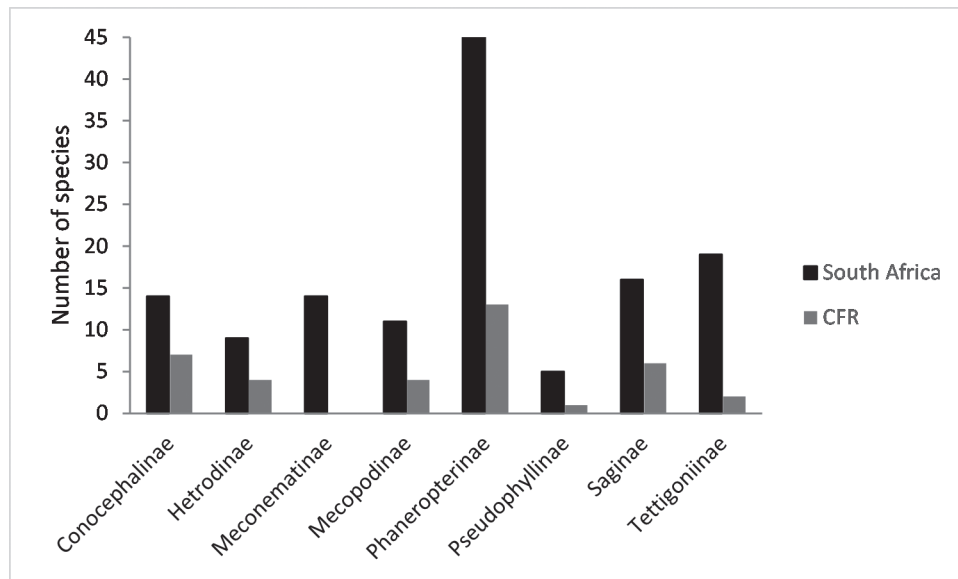


Figure 3. Distribution of South African and Cape Floristic Region katydid species among Tettigoniidae subfamilies.

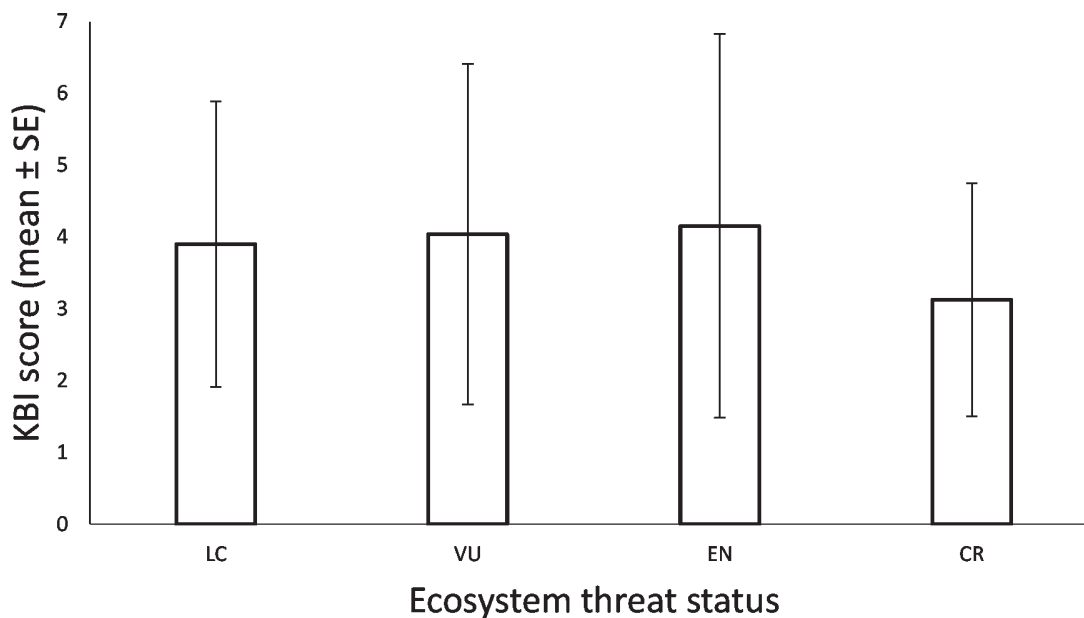


Figure 4. Distribution of Katydid Biotic Index (KBI) among ecosystem threat statuses (mean  $\pm$  s.e.).

all terrestrial ecosystems in South Africa and thus present themselves as a favourable taxon upon which to base a rapid assessment method.

All threatened South African katydids (VU, EN and CR) are either national endemics or are localised endemic species. One of the weaknesses of the DBI and of the current method is that distribution is taken into account in the Red List assessment (when species are scored according to Criterion B which was the case for almost all katydids) and is then used again for scoring of the KBI. This leads to a potential problem of intercorrelation between two of the three categories of the KBI. The Dragonfly Biotic Index (DBI) is a very powerful assessment tool used in South Africa and is based on the threat status, distribution and sensitivity to habitat

change (Samways and Simaika 2016), even here there exist intercorrelations between the distribution and threat status and yet this provides accurate assessments of habitat quality.

Patterns are seen in the effective mobility of a species, with the less mobile species featuring more prominently in the threatened classes. These patterns are also then maintained within the CFR katydids. Katydid traits are shown here to correlate with threat status, thus providing further evidence that the KBI will be an effective way to monitor habitat quality through the resident katydids.

Katydid are known to be highly cryptic as a result of excellent leaf mimicry and, when combined with their predominantly nocturnal habitats, they are notoriously difficult to locate in the

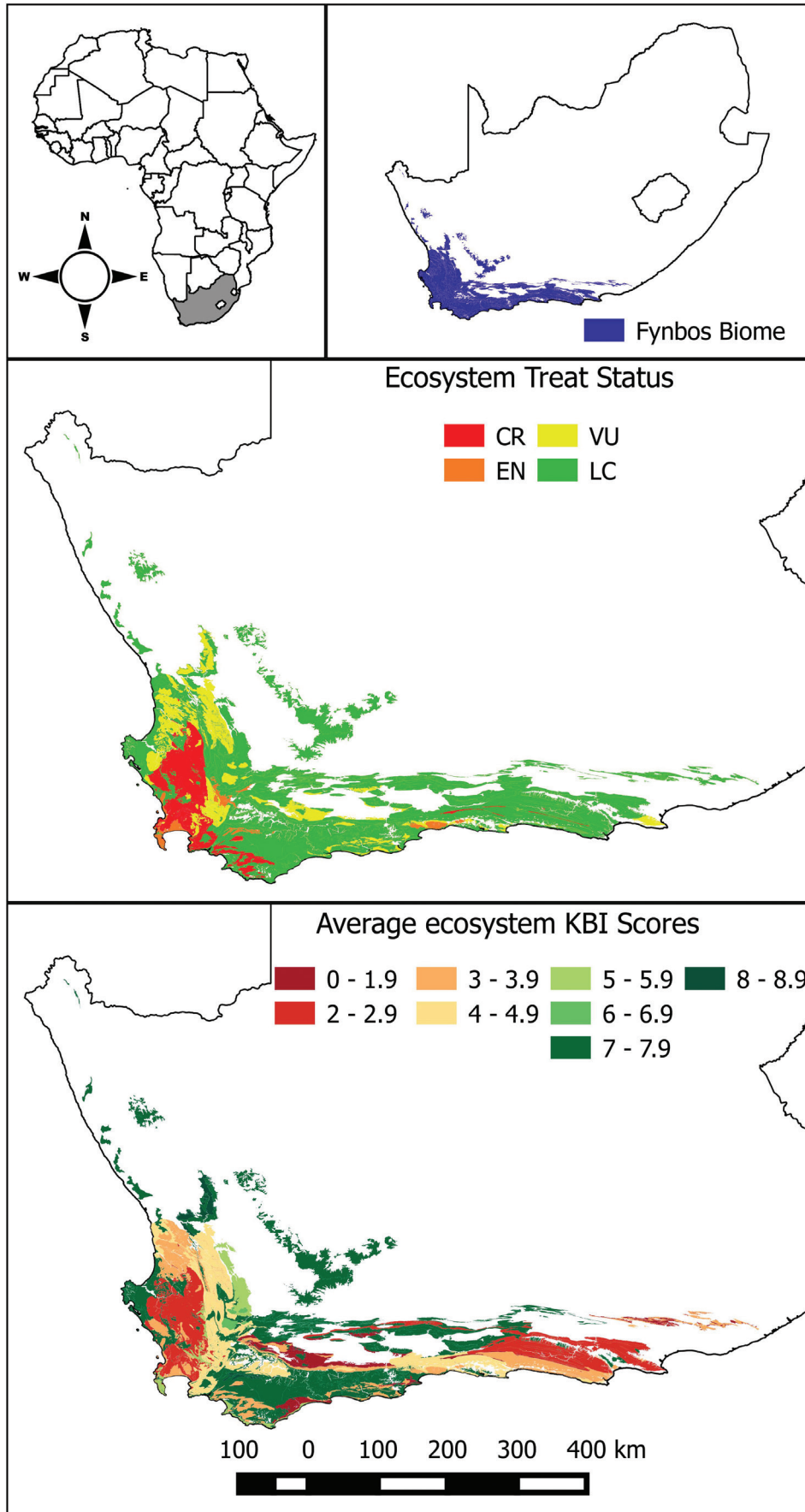


Figure 5. Map of ecosystem threat statuses and the average KBI scores (i.e. KBI/Site) of each ecosystem.

wild. This means that they are not a popular taxon for assessment in comparison with other charismatic invertebrate groups such as dragonflies and butterflies. For this reason, museum records of katydids become a very important source of information. The MANTIS database contains records of all 126 valid species of katydids in South Africa, so allowing for the individual species to be assessed for KBI assessments as accurately as possible.

Although cryptic and difficult to locate, katydids are perhaps best known for the species-specific songs produced by mature adult males (Bailey and Rentz 1990). There has been considerable research into monitoring and tracking of katydid species, as well as other acoustically communicating insects, through acoustic monitoring (Riede 1993, 1998, Diwakar et al. 2007a, Grant and Samways 2016). Acoustic monitoring can be conducted using a variety of techniques, ranging from simple listening exercises (Diwakar et al. 2007b) to complex microphone arrays (Stevenson et al. 2015). In South Africa, acoustic monitoring of katydids is an attractive option as the acoustic environment in which they sing is not such a complex chorus as in tropical forests. The CFR, in particular, has a simple acoustic assemblage, but very complex Mediterranean-type vegetation structure consisting of a majority of thorny and difficult to access bushes and shrubs. This provides ample hiding space for katydids, and increases the need to detect singing individuals.

In view of these conditions, South African katydids can be monitored using inexpensive and simple equipment. A well-trained listener is able to distinguish between the different calls of both katydid and gryllid species (Diwakar et al. 2007b). It is not possible for these listeners to pick up any ultrasonic calls, yet by using a bat detector, inaudible ultrasonic calls can be down-scaled and rendered audible so that real-time identification of katydid species is possible in the field. Although time is required for the listener to learn the various calls, time will be saved in the long-term as, once a reliable voucher collection with associated song library has been constructed, there will be no need to locate the individual insect to correctly identify it. Simple and relatively cheap recorders are also available for long-term deployment, allowing for passive, non-invasive monitoring that, once an operator is well trained, provides an effective way in which to remotely monitor katydid distributions.

Despite a few apparent weaknesses of the KBI, this study aimed to simply determine whether the DBI could be adapted to katydids as it has been proven to be a decidedly powerful tool in similar regions to this study. Further comparisons to existing assessment methods will be required in order to accurately determine the effectiveness of the KBI. As this study relied on museum records only, the "rarity" component of the GCI could not be accurately assessed.

## Conclusions

With improved monitoring of katydids, perhaps on a smaller scale and with controlled measuring of environmental parameters, it could be possible to demonstrate the further value of this scoring system as a monitoring technique. This is a preliminary study aimed only to introduce the idea of a rapid assessment method for terrestrial habitats based on katydid song. It has identified some of the advantages of the approach but has emphasized that much more data gathering is required. However, it does appear as if the KBI may be a promising method, particularly for regions where katydids are abundant and diverse, but relatively well-known.

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# Ground dwelling pygmy grasshoppers (Orthoptera: Tetrigidae) in Southeast Asian tropical freshwater swamp forest prefer wet microhabitats

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## Abstract

Tetrigidae are an ancient group of grasshoppers and, similar to many other insects, have associations and preferences for specific microhabitats and habitats. The ecology of pygmy grasshoppers in Southeast Asia is generally under studied, especially in threatened habitats such as freshwater swamp forests. A study in Nee Soon Swamp forest, Singapore, was conducted to investigate association of limno-terrestrial pygmy grasshoppers with waterbodies and microhabitat. Specifically, we looked at the abundance and species assemblage of all pygmy grasshoppers. We correlated the abundance with major gradients of variation summarizing substrate and vegetation types along belt transects where sampling was performed. We found that pygmy grasshoppers in general are associated with wetter microhabitat conditions rather than the main streams in the swamp forest (i.e., water bodies). This is despite differences in microhabitat conditions of belt transects nearer to and further away from the main streams. We also found that pygmy grasshopper abundance is associated with the wetness of dicot leaf litter. We inferred that the abundance of food resources and suitability for egg development may explain their preference for wet microhabitats. We also found that the same patterns applied for adults and juveniles, suggesting that there is no demographic difference or ontogenetic shift in microhabitat association. Lastly, the adult assemblage can also be correlated to microhabitats. Based on our findings, we propose that pygmy grasshoppers can also be suitable bio-indicators for the freshwater swamp forest, owing to their sensitivity to microhabitat conditions.

## Key words

abundance, ecology, limno-terrestrial, microhabitat association, Singapore

## Introduction

Insects are highly dependent on their environment. Plant communities within an area can vary in diversity, assemblage and productivity, and such heterogeneity can drive dynamics among arthropod communities and influence arthropod-plant interactions (e.g. Knops et al. 1999, Schaffers et al. 2008, Haddad et al. 2009). At a smaller scale, insects are also known to have preference for specific microhabitat conditions (e.g. Wardhaugh et al. 2013,

2014, Pincebourde and Casas 2015, Thom and Daniels 2017). Likewise, orthopterans have associations with vegetation and microhabitat conditions (Joern 1982, Badenhauer et al. 2015).

Orthoptera (grasshoppers, crickets and katydids) are among the largest group of terrestrial insects, comprising more than 27,000 described species (Cigliano et al. 2017). The Tetrigidae, or pygmy grasshoppers, are a monophyletic clade and forms a unique lineage among the Caelifera (grasshoppers) (Song et al. 2015). These grasshoppers are known to prefer moist areas and semi-aquatic habitats like marshes, margins of waterbodies and floodplains. Amédégnato and Devriese (2008) considered most species of pygmy grasshoppers as limno-terrestrial that require an aqueous matrix (moist areas) in strictly terrestrial habitats while some are more dependent on water (such as for egg laying and nymphal development). They appear to be good swimmers and/or divers (Paranjape et al. 1987, Gröning et al. 2007, Amédégnato and Devriese 2008). Different levels of dependency on water correspond to varying degrees of adaptations to swimming, including expanded hind tibia with reduced spines in more water dependent species (Amédégnato and Devriese 2008). Being a group of terrestrial invertebrates which are associated with water, they thus provide an important trophic exchange between aquatic and terrestrial food webs (Bastow et al. 2002, Balian et al. 2008).

However, there is still a dearth of information on the pygmy grasshoppers from Southeast Asia. This is made worse with the taxonomy being particularly problematic, rendering accurate species identification difficult (Tumbrinck 2014). Most of our current understanding of the ecology of pygmy grasshoppers is limited to their diet, ranging from observational (e.g. Paranjape and Bhalerao 1985, Blackith and Blackith 1987, Reynolds et al. 1988) to quantitative data (e.g. Kuřavová et al. 2017b). Quantitative studies on the habitat/microhabitat association of pygmy grasshoppers are rare and restricted to a few species (e.g. Hochkirch et al. 2000, Gröning et al. 2007, Kuřavová and Kočárek 2015, Musiolek and Kočárek 2016), and even fewer in Southeast Asia. Southeast Asia consists of three biodiversity hotspots (Myers et al. 2000), and is threatened by large-scale deforestation and land use conversion (Brooks et al. 2002, Sodhi et al. 2010, Wilcove et al. 2013). At the same time, Southeast Asia is also rich in orthopter-

ans, possibly equally affected by the crisis as are other organisms (Tan et al. 2017a). Along with fragmentary understanding of the orthopteran diversity in Southeast Asia (Tan et al. 2017a), there is a pressing need to study the ecology of Southeast Asian orthopterans.

Here, an attempt was made to investigate the association of the Southeast Asian pygmy grasshoppers with microhabitats and proximity to water bodies. Specifically, such ecological association of the pygmy grasshoppers from freshwater swamp forest were examined for the first time. Studies on pygmy grasshoppers in aquatic habitats focus mostly on species occurring in salt marshes, ponds and rivers (e.g. Hochkirch et al. 2000, Gröning et al. 2007, Amédégno and Devriese 2008). There has not been a study on species from a tropical region and hence tropical freshwater swamp forests up to now. Our study of the water association of the pygmy grasshoppers can provide us with a better understanding of the ecological preferences and association of these tropical orthopterans.

To investigate the association of the pygmy grasshoppers with the microhabitats in the freshwater swamp forests, two hypotheses were tested. Firstly, we investigated if the pygmy grasshoppers in freshwater swamp forest were associated with the water body by comparing the abundance of pygmy grasshoppers near vs. further away from the main streams. If the unique semi-aquatic lifestyle of the pygmy grasshoppers required a close association with existing waterbodies in the swamp forest, this would suggest they may tend to concentrate near streams. Secondly, we also aimed to investigate if the pygmy grasshoppers preferred specific microhabitat conditions. The microhabitats were quantified by a series of vegetation and surface types, as well as surface temperature and relative humidity. Nee Soon Swamp Forest (NSSF), Singapore, was used as the study site. NSSF is the last remnant of freshwater swamp forest in Singapore but remains ecologically significant (Ng and Lim 1992, O'Dempsey and Chew 2013). It also holds unique and endemic biodiversity (e.g. Gorochoy and Tan 2012, Jäch et al. 2013). As the pygmy grasshoppers were usually small and had small dispersal range, we expected that they would form close associations with their preferred microhabitats.

## Materials and methods

**Study area.**— Four sites, situated along the main stream within the NSSF were opportunistically sampled. In each site, eight 20 × 10 m belt transects were opportunistically demarcated based on accessibility. Within each site, four belt transects were situated along the stream banks less than 1 m away from the main stream and four other belt transects were situated at least 5 m from the main stream. In total, 32 belt transects were surveyed. Each belt transect was surveyed once.

**Sampling of pygmy grasshoppers.**— Sampling was conducted between late August 2013 and early February 2014, during the cooler and wetter part of the year in Singapore. Surveys were conducted between 7:30 pm (after last light) and 10 pm. Nocturnal survey was found to be more appropriate for representative estimation of pygmy grasshopper abundance as they are more easily sighted at night than in the day. Two belt transects from a single site were opportunistically sampled per survey night. Each site was surveyed across the entire span of the sampling period, rather than restricted to a single month, to minimize the effect of temporal autocorrelation. Each belt transect was surveyed systematically for 20 min by two equally trained and efficient surveyors (MKT and HY) working together at the same time, from one end of the belt transect to the other. Adults and juveniles were actively searched using both headlamps and hand torches. The ground, low-lying vegetation and dead logs were searched. Pygmy grasshoppers were thor-

oughly sampled within the time frame and belt transect. Abundance was quantified as the total number of individuals found in each belt transect. Adult specimens were tentatively identified to morpho-species because Tetrigidae in Singapore are generally not well studied with species delimitation remaining unresolved and many species undescribed (Tan et al. 2017b). All adults were likely to be capable of flight owing to the presence of developed hind wings whereas the nymphs were flightless.

**Microhabitat quantification.**— At every 1 m along the belt transect, the surface and vegetation types were recorded. Surface types include: (1) dry dicot leaf litter, (2) wet dicot leaf litter, (3) dry muddy (fine and claying substrate), (4) wet muddy, (5) dry sandy (coarser and porous substrate), (6) wet sandy, (7) gravel and rocks, (8) wet dead woody structure (i.e. log and exposed roots), (9) dry woody structure (i.e. log, trunk and exposed roots), (10) water-logged, (11) wet ceramics (e.g. broken ceramic tiles left over from past settlement) and (12) dry monocot leaf litter. We distinguished dicot leaf litter, muddy surface and sandy surface between wet and dry. For surface types 7 to 12, they were always either wet or dry and hence not distinguished between wet and dry. We summarized different plant forms as vegetation types that included: (1) herbaceous plants and grass, (2) woody plants (i.e. trees, woody shrub), (3) creepers and vines, (4) rattans (including *Pandanus*), (5) ferns and (6) palms. The prominence of each microhabitat (both surface and vegetation type) was quantified by counting the number of times they were recorded within the belt transect. Within each belt transect, the ambient surface temperature and ambient surface relative humidity were also recorded three times, at approximately the two ends and midpoint, and averaged.

**Data analyses.**— As the different types of substrate and vegetation were not mutually exclusive, they were likely to be highly intercorrelated. To summarize substrate and vegetation into major gradients of variation, non-metric multidimensional scaling (NMDS) using Bray-Curtis distance was performed using the 'metaMDS' function implemented with the community package vegan version 2.3-1 (Oksanen et al. 2015). Stress values of 0.20 and below were deemed acceptable for interpretation of patterns (Clarke 1993), and where this was exceeded, the plots were ordinated on three dimensions. NMDS axes representing the microhabitat variation were labelled Microhabitat NMDS1 and Microhabitat NMDS2. The scores for the NMDS axes were scaled and checked for collinearity. Variance inflation factor (vif) values of below 3.0 were deemed acceptable (Zuur et al. 2010).

To test if the pygmy grasshoppers were associated with specific microhabitat axes, the abundance of pygmy grasshoppers was fitted as a generalized linear mixed-effects model using Poisson error structure using the 'glmer' function. We included the scores on the two scaled NMDS axes and proximity to main stream (either close or far) as fixed effects and the site location as random effect.

We postulated that pygmy grasshoppers had habitat preferences, and thus occurred at higher abundances associated with specific microhabitat conditions. Additionally, owing to their preference for aquatic habitat, we also postulated that more pygmy grasshoppers would be found on belt transects closer to the main stream. The following models were proposed and ranked accordingly to Akaike information criterion with adjustments (AICc) using the 'MuMIn' package (Barton and Barton 2015):

1. ~ Microhabitat NMDS1
2. ~ Microhabitat NMDS2

3. ~ Microhabitat NMDS1 + Microhabitat NMDS2
4. ~ Proximity to main stream
5. ~ Microhabitat NMDS1 + Proximity to main stream
6. ~ Microhabitat NMDS2 + Proximity to main stream
7. ~ 1 (null model)

We interpreted the models with delta (difference in the AICc of a particular model and that of the best model) less than 2.0 (Burnham and Anderson 2002) in tandem. Subsequently, we also selected surface and vegetation types based on the NMDS plot that might have been important to explain the abundance. To better understand how each of these environmental variables correlated with the abundance, we proposed various models containing each of these environmental variables as fixed effect and ranked accordingly to Akaike information criterion with adjustments (AICc).

Since adults and juveniles could have different microhabitat requirements and dispersal abilities, we performed separately the same analyses using the subset of (i) adult and (ii) juvenile abundances to investigate if the same patterns persisted. The assemblage (or beta-diversity) of adults were correlated with major gradients of variation in microhabitat conditions (i.e., Microhabitat NMDS1 and NMDS2). This was done by performing canonical analysis of principal coordinates (CAP) with Euclidean distance using the 'capscale' function implemented with the community package vegan version 2.3-1 (Oksanen et al. 2015).

All statistical analyses were done in the R software version 3.3.3 (R Development Core Team 2017).

## Results

A total of 94 adult and juvenile pygmy grasshoppers were collected during the sampling of 32 belt transects (19 from Site 1 (= Woodcutter Trail), 13 from Site 2 (= Nee Soon interior), 28 from Site 3 (= Nee Soon main pond) and 13 from Site 4 (= Upper Seletar). In total, 62 adults and 32 juveniles were collected. We identified the adults into six morpho-species, with one dominant morpho-species. All morpho-species were collected across the entire sampling period and there appeared to be no clear evidence of species turnover with time.

The first two scaled NMDS axes summarized the microhabitat conditions (including vegetation and surface types) and had a stress value of 0.17 (below the threshold of 2.0), indicating that they were sufficient to represent the variation in the microhabitat conditions (Fig. 1). Subsequently, we obtained the scores for the two axes to test our hypotheses. The Microhabitat NMDS1 showed that belt transects with wetter microhabitats (waterlogged, wet muddy and wet dicot leaf litter) had higher scores than those with drier microhabitats (dry sandy, dry muddy and dry dicot leaf litter). The Microhabitat NMDS2 showed that belt transects with coarser surface type (i.e. sand, tiles, rock and gravel) had a lower score than those with finer surface type (i.e. mud). Microhabitat NMDS2 also summarized the vegetation types. A PERMANOVA also showed that the microhabitat conditions were dissimilar between the belt transects nearer to the main streams and those further away (F-value = 4.81,  $R^2 = 0.14$ , p-value = 0.003) (Fig. 2).

When we correlated the total abundance of pygmy grasshoppers with the NMDS axes and proximity to main streams, the two best models with delta less than 2.0 both had NMDS1 as fixed effect (Table 1). The best model also had NMDS2 as fixed effect. The best model explained 35% of variance, about 10% more than that explained by the second best model (Table 1). Total abundance had a strong positive relationship with Microhabitat NMDS1

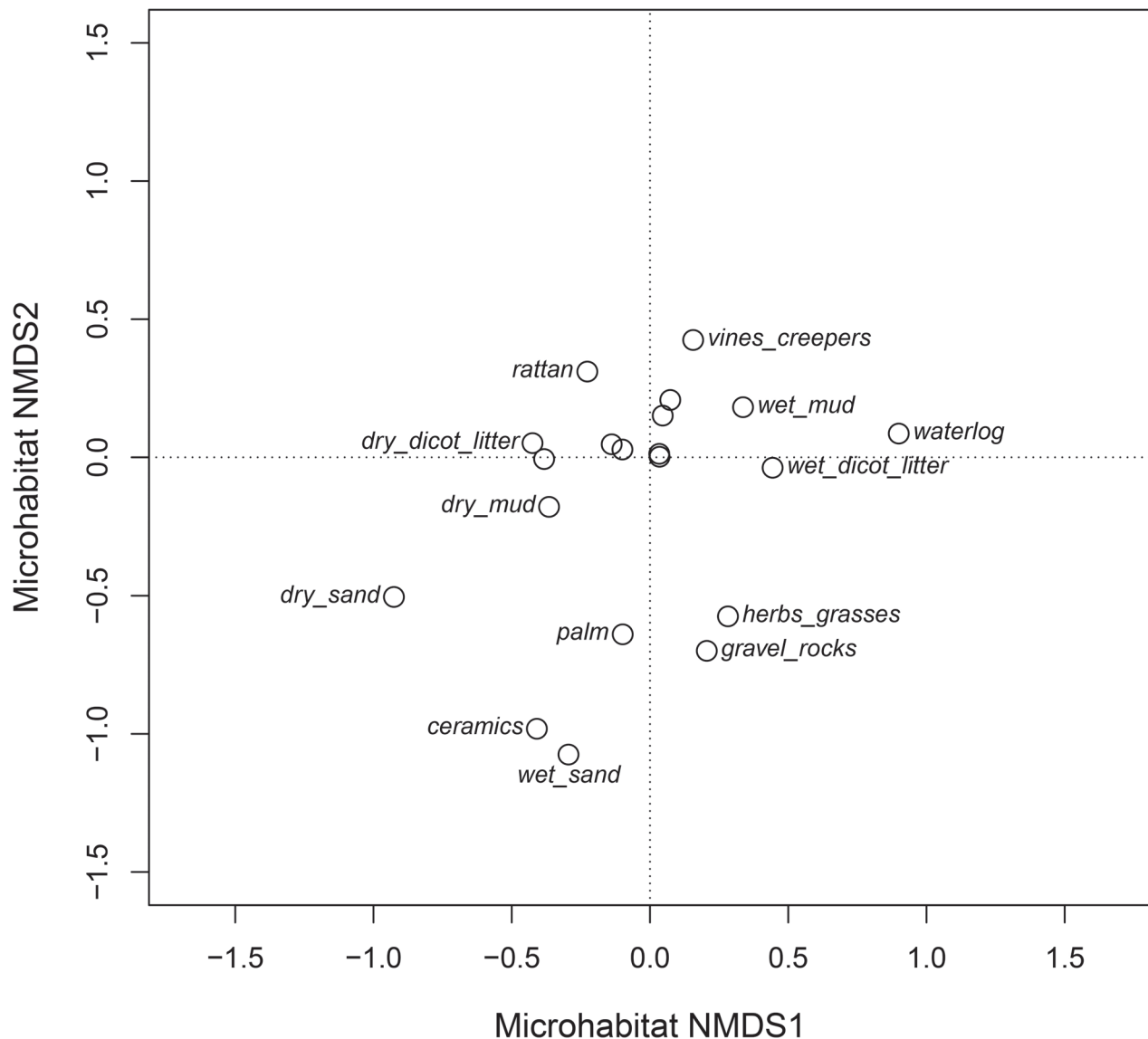
(97.5% CI[0.75, 2.06] in best model and 97.5% CI[0.71, 2.10] in second best model) and a weak negative relationship with Microhabitat NMDS2 (97.5% CI[-1.81, 0.09] in best model). This showed that higher abundance of pygmy grasshoppers could be found in wetter microhabitats (waterlogged, wet muddy and wet dicot leaf litter) than in drier microhabitats (dry sandy, dry muddy and dry dicot leaf litter). Proximity to main stream was not among the best models.

Since total abundance of pygmy grasshoppers had a strong positive relationship with NMDS1, we proposed further models, each model with a single environmental variable as a fixed effect: (1) dry dicot leaf litter, (2) wet dicot leaf litter, (3) wet muddy, (4) dry sandy, (5) waterlog and (6) herbaceous plants and grasses. Upon ranking them using AICc, the best models with delta < 2.0 had dry and wet dicot leaf litter as fixed effects and explained 40% and 37% of variance, respectively (Table 2). Total abundance had a strong negative relationship with dry dicot leaf litter (97.5% CI[-0.12, -0.05] as best model (Fig. 3) and a positive relationship with wet dicot leaf litter 97.5% CI[0.05, 0.11] as second best model). This showed that wetness of dicot leaf litter had a strong effect on the abundance of pygmy grasshoppers.

When we performed the analyses using adults and juveniles separately, the same patterns were observed. Higher abundance of adult and juvenile pygmy grasshoppers were found in wetter microhabitats than in drier microhabitats (Suppl. material 1: Tables 1 and 2). Juvenile abundance had a strong positive relationship with Microhabitat NMDS1 (97.5% CI[0.35, 2.63] in best model and 97.5% CI[0.39, 2.56] in second best model) and a weak negative relationship with Microhabitat NMDS2 (97.5% CI[-2.14, 0.94] in second best model). Likewise, adult abundance had a strong positive relationship with Microhabitat NMDS1 (97.5% CI[0.58, 2.15] in best model and 97.5% CI[0.59, 2.26] in second best model) and a strong negative relationship with Microhabitat NMDS2 (97.5% CI[-2.20, -0.03] in best model). Specifically, we found that wetness of dicot leaf litter had a strong effect on the abundance of both adults and juveniles (Suppl. material 1: Tables 3 and 4). Juvenile abundance had a strong relationship with dry dicot leaf litter (97.5% CI[-0.16, -0.03] and wet dicot leaf litter 97.5% CI[0.04, 0.15]). Adult abundance had a strong relationship with dry dicot leaf litter (97.5% CI[-0.14, -0.05]). Proximity to main stream was not among the best models. Lastly, we found the assemblage of pygmy grasshoppers was significantly correlated to Microhabitat NMDS1 and Microhabitat NMDS2 (proportion variance explained = 0.17, pseudo-F-value = 2.89, number of permutations = 999, p-value = 0.013) (Fig. 4). No apparent difference was detected between the six morpho-species.

## Discussion

Our study in NSSF demonstrated that pygmy grasshoppers in general were not associated with the main streams in freshwater swamp forests. Instead, the pygmy grasshoppers were found to be associated with wetter microhabitats in general. This is despite the microhabitats between the belt transects nearer to and further away from the main streams being different. In Southeast Asia, many Scelimeninae can be found to occupy river banks and forage for submerged food resources (Kuřavová et al. 2017a). The pygmy grasshoppers from the freshwater swamp forest in Singapore, which include Scelimeninae, however, had different microhabitat associations. This was to be expected since the freshwater swamp forest is a different habitat to that of a typical river. The highly



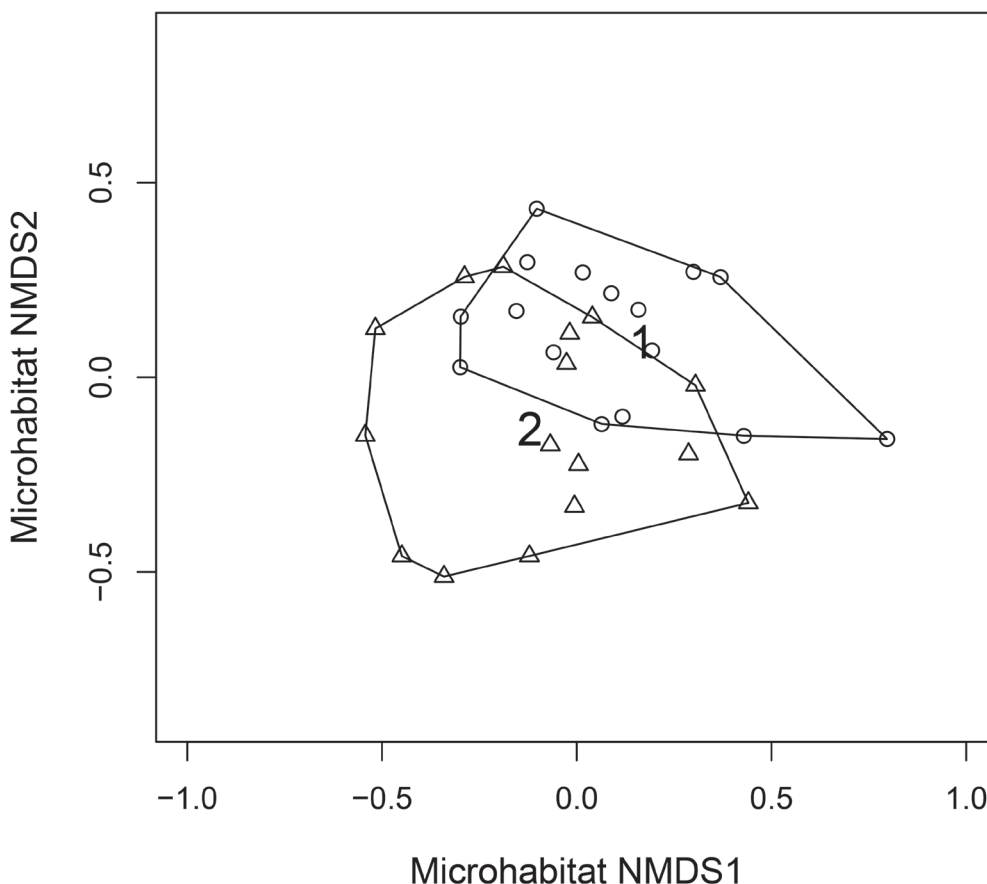
**Figure 1.** A non-metric multidimensional scaling (NMDS) using Bray-Curtis distance to summarize microhabitat condition data, indicating the different microhabitat conditions. Stress value for first two axes = 0.17.

dynamic water level within the freshwater swamp forest leads to the formation of a dense network of main streams and smaller ephemeral streamlets (O'Dempsey and Chew 2013). This also indicates that different pygmy grasshoppers from different habitats and lineages can have very different life histories.

As the diet of pygmy grasshoppers generally consists of algae, moss, fungi and lichen that thrive on wetter conditions (Paranjape et al. 1987, Hochkirch et al. 2000, Kočárek et al. 2008, Bidau 2014, Kuřavová and Kočárek 2015, Kuřavová et al. 2017a, 2017b), our findings also suggested that greater abundance of food sources in wetter microhabitats can account for higher abundance of pygmy grasshoppers in general. We also found that specifically, the wetness of dicot leaf litter is strongly positively correlated with the abundance of pygmy grasshoppers. We inferred that areas with wet dicot leaf litter provided suitable microhabitats for pygmy grasshoppers owing to the availability of food resources. Additionally, wet microhabitats could also provide suitable conditions for egg

development (Paranjape et al. 1987). Since pygmy grasshoppers are known to lay eggs on substrate (Hartley 1962, Paranjape et al. 1987), wet leaf litter, in particular, might reduce rate of drying of the underlying substrate. From our observational studies, we could only infer these behaviors, and how precisely pygmy grasshoppers from tropical Southeast Asia utilize their microhabitats should be further investigated under laboratory conditions. This will then provide better mechanistic understanding of the biology of these pygmy grasshoppers.

On the other hand, pygmy grasshoppers did not appear to be associated with the vegetation types in the swamp forest since their food sources were often not affected by the vegetation types (Paranjape 1985, Paranjape and Bhalerao 1985). This is contrary to previous studies showing diet preference of orthopterans is often closely linked to their association with specific vegetation or host plants (Joern 1982, Schaffers et al. 2008, Badenhauer et al. 2015), although these studies do not focus on pygmy grasshop-



**Figure 2.** A non-metric multidimensional scaling (NMDS) using Bray-Curtis distance to summarize microhabitat condition data, showing two hulls representing belt transects (1, circle) close to and (2, triangular) far from the main stream. Stress value for first two axes = 0.17.

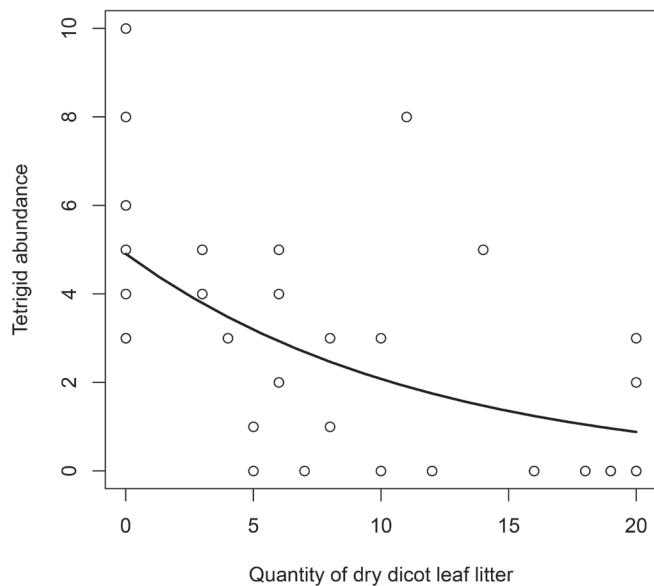
**Table 1.** Effect of Microhabitat NMDs1 and NMDs2 and proximity to main stream on total abundance of pygmy grasshoppers. Marginal R<sup>2</sup> (R<sup>2</sup>m) represents variance explained by fixed effects whereas conditional R<sup>2</sup> (R<sup>2</sup>c) represents variance explained by both fixed and random effects.

	df	logLik	AICc	delta	weight	R <sup>2</sup> m	R <sup>2</sup> c
~ Microhabitat NMDs1 + Microhabitat NMDs2	4	-70.70	150.9	0.0	0.51	0.35	0.35
~ Microhabitat NMDs1	3	-72.29	151.4	0.6	0.38	0.27	0.28
~ Microhabitat NMDs1 + Proximity to main stream	4	-72.24	154.0	3.1	0.11	0.27	0.28
~ Microhabitat NMDs2 + Proximity to main stream	4	-76.57	162.6	11.8	0.00	0.25	0.25
~ 1	2	-80.08	164.6	13.7	0.00	0.00	0.06
~ Microhabitat NMDs2	3	-79.25	165.3	14.5	0.00	0.10	0.10
~ Proximity to main stream	3	-79.55	166.0	15.1	0.00	0.03	0.10

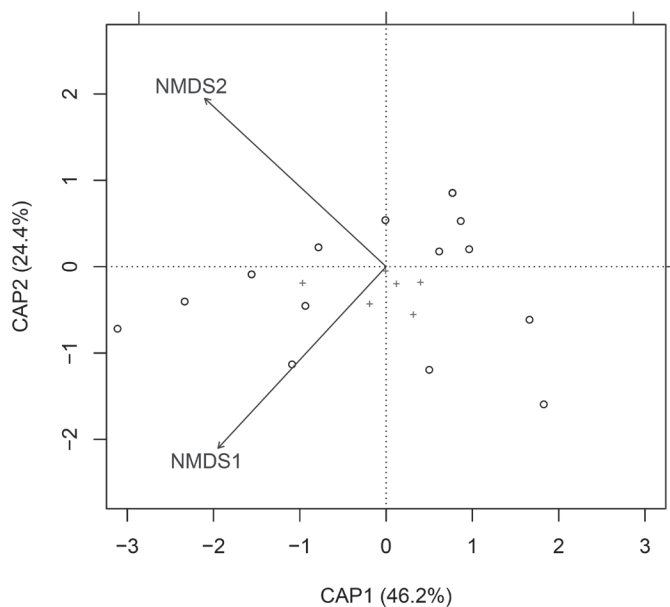
**Table 2.** Effect of specific environmental variables on total abundance of pygmy grasshoppers.

	df	logLik	AICc	delta	weight	R <sup>2</sup> m	R <sup>2</sup> c
~dry dicot leaf litter	3	-66.80	140.5	0.0	0.54	0.40	0.45
~wet dicot leaf litter	3	-66.97	140.8	0.3	0.46	0.37	0.45
~wet mud	3	-77.38	161.6	21.2	0.00	0.07	0.18
~dry sand	3	-77.54	161.9	21.5	0.00	0.06	0.16
~herbaceous plants and grasses	3	-77.99	162.8	22.4	0.00	0.14	0.14
~1	2	-80.08	164.6	24.1	0.00	0.00	0.06
~waterlog	3	-79.96	166.8	26.3	0.00	0.01	0.06

pers. Nonetheless, comparing our findings to other ecological studies of pygmy grasshoppers also revealed that association with the environment can be different among Southeast Asian species and with counterparts from other regions. For example, *Tetrix undulata* was shown to associate with habitats with preferred thermal properties (Ahnesjö and Forsman 2006) whereas *Tetrix tenuicornis* had differing preferences for microhabitats in accordance to changes in the weather conditions (Musiolek and Kočárek 2016). On the other hand, a riverine species, *Tetrix ceperoi*, prefers hotter, damper and bare areas, similar to species from NSSF (Gröning et al. 2007). Unlike previous studies (Ahnesjö and Forsman 2006, Gröning et al. 2007, Musiolek and Kočárek 2016) which focussed



**Figure 3.** Correlation between total abundance of pygmy grasshoppers and dry dicot leaf litter. The model was fitted using generalized linear mixed-effects model using Poisson error structure.



**Figure 4.** A canonical analysis of principal coordinates (CAP) with Euclidean distance to show association of adult assemblage with NMDS1 and NMDS2 representing microhabitat conditions. The circle represents belt transects and cross represents morpho-species.

on a single species of pygmy grasshopper, we investigated the overall abundance of all pygmy grasshoppers, while also examining possible differences between adults and juveniles. The main reason was because of the difficulty to identify closely related species and juveniles among Southeast Asian pygmy grasshoppers owing to unstable taxonomic status of many species (Blackith 1992, Kim and Kim 2004, Tan and Artchawakom 2015). Juveniles of many species were almost impossible to identify using morphology.

Interestingly, we did not find any difference in microhabitat association between the adults and juveniles. While adult and juvenile pygmy grasshoppers can have differing life history (e.g. dispersal ability, dependency on moisture), we did not find evidence for ontogenetic shift for the pygmy grasshoppers in NSSF. It appears that the species of pygmy grasshoppers had very similar association for specific microhabitat conditions since the assemblage of adults also showed similar trends as that of their abundance. We speculate that NSSF may be a unique ecosystem in which small insects such as the pygmy grasshoppers occupy very unique microhabitats.

Owing to the sensitivity of orthopterans to their environment, orthopterans have been proposed as bio-indicators of forests (Fartmann et al. 2012). Nevertheless, the tropical ecology of Southeast Asian orthopterans, along with many other invertebrates, are still under studied. Here, we demonstrated for the first time that pygmy grasshoppers from Southeast Asian freshwater swamp forests are not associated to waterbodies, unlike counterparts from the temperate and subtropical regions. Instead, these pygmy grasshoppers in general prefer wetter microhabitats. Loss or changes in these microhabitats owing to climate change or anthropogenic disturbances can potentially affect the populations (Inamke et al. 2016, Sueyoshi et al. 2016). This is especially so for the freshwater swamp forest which is currently small and isolated and for the pygmy grasshoppers with limited dispersal ability. We propose that pygmy grasshoppers can indeed be potential bio-indicators of freshwater swamp forest. Extending similar studies into other ecosystems can confirm if pygmy grasshoppers can also provide the same service. We hope that our study can help to fill in knowledge gaps in the ecology of pygmy grasshoppers in Southeast Asia, thus providing information for better management of threatened habitats, such as the NSSF.

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### Supplementary material 1

Authors: M.K. Tan, H. Yeo, W.S. Hwang

Data type: Table

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