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Acoustic and Molecular Differentiation between Macropters and Brachypters of *Eobiana engelhardti engelhardti* (Orthoptera: Tettigonioidea)

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Yin-Liang Wang, Jian Zhang, Xiao-Qiang Li, and Bing-Zhong Ren (2011) Acoustic and molecular differentiation between macropters and brachypters of *Eobiana engelhardti engelhardti* (Orthoptera: Tettigonioidea). *Zoological Studies* **50**(5): 636-644. This study focused on the wing dimorphism of *Eobiana engelhardti engelhardti* (Uvarov 1926). To examine acoustic differences between macropters and brachypters, we recorded and analyzed the calling songs of the 2 forms. Moreover, the vocal organs of *E. e. engelhardti* were also observed under optical and scanning electric microscopy. As a result, there were 3 "dynamic" song traits which had significant differences between the 2 forms, but no obvious differences were observed in vocal organs. For macropters, we assumed that differentiation of these calling songs showed compensation for a reproductive disadvantage. Finally, some molecular biology experiments were conducted to examine differences between the 2 forms at the molecular biology level; as a result, brachypterous insects were grouped in a clade, while macropterous insects were grouped into another, which indicates that these molecular differences had already occurred before these macropters migrated. http://zoolstud.sinica.edu.tw/Journals/50.5/636.pdf

Key words: Insect songs, Katydid, Toothed flies, Wing dimorphism.

Wing dimorphism independently evolved in most major orders of insects, and many genera are composed of species that are dimorphic for wing length or are monomorphically wingless (Roff 1986 1994). In the Orthoptera, wing length is believed to reflect a tradeoff between mobility and fecundity (Zera and Denno 1997, Saglam et al. 2008). Nevertheless, long-winged individuals are usually fertile (Sänger and Helfert 1975, Ritchie et al. 1987) and therefore able to establish new populations. Although the phenomenon of macroptery has been known since the early 1900s (Karny 1913, Puschnig 1914), its causes are still being discussed (Harrison 1980, Zera and Denno 1997). Some research indicated that macroptery is density-induced (Ando and Hartley 1982, Sasaki 2002, Higaki and Ando 2003, Poniatowski 2009); however, besides environmental factors, there is also a theory that wing dimorphism results from a variation in genotype or from a combination of both genetic and environmental effects (Harrison 1980, Zera and Denno 1997, Sakashita et al. 1998).

Most insects in the Cicadidae and Orthoptera can make sounds, but the methods used to emit sounds are very diverse (Kevan 1955, Uvarov 1966, Seabra 2008). Katydids and crickets, members of the Orthoptera, produce sounds by rubbing together the scaler and a specialized wing vein of their tegmina (Walker and Carlysle 1975, Schmidt and Stelzer 2005). Yet grasshoppers display somewhat different behavior, using the femoro-elytral method that is most widespread in

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*To whom correspondence and reprint requests should be addressed. E-mail:bzren@163.com the suborder Caelifera, producing stridulations by rubbing the posterior femora against the tegmin (Ragge 1986). Other reports claimed that some apterous grasshoppers have developed their own methods to produce sounds (López et al. 2007). The main function of insect songs is in the context of mate choice; song traits also offer females the opportunity to choose between specific males. In this case, preference might lead to directional selection for song traits with females preferring the most extreme values. These song properties should show high between- and within-male variations and might indicate the caller's quality. High within-male variations may predominantly be influenced by physiological (e.g., condition-dependent traits) and behavioral mechanisms (Wollerman 1998). Gerhardt (1991) called these highly variable song traits "dynamic" properties.

To find acoustic differences between calling songs of macroptery and brachyptery, we recorded and analyzed the song traits of *Eobiana engelhardti engelhardti* (Uvarov 1926); moreover, some molecular and physiological experiments were done to explore differences between macropters and brachypters of *E. e. engelhardti*.

MATERIALS AND METHODS

Studied materials

We used adult males of *E. e. engelhardti* as our experimental subjects. This species was formerly known as *Metrioptera engelhardti*, in 1993. The subgenus *Eobiana* was raised to a full generic status and revised by Storozhenko and Yamasaki (1993). This genus includes 2 subspecies: *E. e. engelhardti* and *E. e. subtropica*. Recently, 3 new species of this genus were found in Japan (Ishikawa and Wada 2001). This species is distributed throughout the Korean peninsula, Northeast China, and the Russian Far East (Kim 2010), and the specimens we used were collected in Heilihe (41°34'N, 118°23'E, 1430 m in elevation), a national nature reserve in Chifeng City, Inner Mongolia. The total area of Helihe is 27,638 ha, mainly covered by natural *Pinus tabulaeformis* forests, and with a high level of biodiversity. Samples were collected in 3 different small regions near the aforementioned coordinates; field observations showed that long-winged individuals were only discovered in areas with high population densities. After the calling songs were recorded, the samples were numbered by their location for further experiments.

Song recording

Within 15 d of collection, we recorded the male calling song of *E. e. engelhardti* in the wild as sound files with a digital voice recorder (PCM-D50 digital recorder, Sony, Tokyo, Japan). The sampling rate was 96 kHz; according to Nyquist's law, the frequency response could reach 48 kHz, the signal-to-noise ratio was about 10, and the distance between the specimen and the recorder was about 50 cm. It was reported that the acoustic behaviors and traits of the songs change with temperature (von Helversen 1972), so we recorded the environmental temperature for each sound file. Each recording took 3-5 min; in total, 30 individuals were recorded, including 11 macropterous and 19 brachypterous forms.

Song trait analysis

The song traits were analyzed with Cool Edit (pro vers. 2.1, Adobe Systems, San Jose, CA, USA) and Matlab (vers. 7.0, Mathworks, Natick, MA, USA). In order to remove low-frequency noise, we highpass-filtered the recording before the analysis at a cutoff frequency of 200 Hz (the frequency ranges of calling songs of *E. e. engelhardti* are mostly above 5000 Hz, and the period of the waves is 0.05 s; therefore, 200 Hz could sufficiently remove the noise without disturbing the frequency component of the original signals). Then the song traits of *E. e. engelhardti* were automatically analyzed by the program we wrote based on Matlab.

Observation of toothed files and tegmina

The forewings of male samples were immediately isolated from specimens using ophthalmic scissors. The total area of the tegmin was measured under a stereomicroscope (SMZ-168 stereomicroscope, Motic, Hong Kong, China). The tegmina were first placed in 70% ethanol for 24 h and then dehydrated in a graded alcohol series of 75%, 80%, 85%, 90%, and 100% for 10 min at each concentration, coated with gold, and photographed under a Hitachi scanning electron microscope (S-570, Hitachi Tokyo, Japan) at 20 kV with a 21-mm working distance. Then the clear image was photographed.

DNA extraction, polymerase chain reaction (PCR), and sequencing

For the analysis of orthopteran species groups, we selected genes for which data already existed and which showed sufficient variations between closely related species (mitochondrial genes: 16S ribosomal (r)RNA gene and especially the barcoding gene, cytochrome oxidase subunit I (COI); preliminary work confirmed the suitability of the selected genes) (Hemp et al. 2010). Total genomic DNA was extracted from the hind femur muscles of 10 insects. DNA was extracted by a standard phenol-chloroform-isoamylalcohol (PCI) extraction with a few modifications (Sambrook et al. 1989). The fragment was amplified using C1-J-1718 (GGAGGATTTGGAAATTGATTAGTTCC) and L2-N-3014 (TCCATTGCACTAATCTGCCATATTA) (Knowles 2000). Each PCR sample contained 5 μl of 10× PCR buffer at pH 8.3 (10 mmol/L of Tris-HCl at pH 8.3 and 50 mmol/L KCl), 4 μl of 2.5 mmol/L $MgCl_2$, 1.5 U of Taq DNA polymerase, 1 μl of 10 mmol/L of each deoxynucleotide triphosphate (dNTP) (C, G, A, and T) all from Takara Biotech (Dalian, China), 2 μl of 10 μmol/L of each primer (Sangon Biotech, Shanghai, China), 2 μl of the DNA template, and 33.7 μl H_2O. The regions to be analyzed were amplified using standard PCR approaches with the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, primer-specific annealing at 47°C for 40 s, and extension at 72°C for 1 min; there was a final extension for 5 min at 72°C. This resulted in the amplification of a fragment of approximately 1300 bp long. Amplicons were sequenced using a BigDye Terminator kit (Applied Biosystems, Foster, CA, USA) and an ABI 3730 automated sequencer (Applied Biosystems). Both sense and anti-sense strands were sequenced for all individuals. Gene sequences of *Calliphona konigi*, *Elimaea cheni*, and *Banza mauiensis* (all of the Tettigoniidae) were used as the outgroup and were obtained from GenBank (EF515100.1, GU323362.1, and DQ649486.1, respectively). DNA sequences were aligned using the multiple-sequence program, Clustal 1.8, with default parameters (Thompson et al. 1997). Phylogenetic analyses were performed using PAUP 4.0 b10 (Swofford 2002). Phylogenetic trees were reconstructed by maximum parsimony (MP).

RESULTS

Results of the calling song analysis

Song traits were examined from 11 macropterous and 19 brachypterous individuals. We measured the following 5 song traits in the time-domain analysis: the durations of the pulse and pulse group, the intervals between pulses and between pulse groups, and the number of pulses per pulse group, as shown in figures 1 and 2. Both wing forms had 26-28 pulses in a pulse group, and 2.2-2.6 s intervals between pulse groups. As a result, songs of the macropters represented a greater “effort” than those of brachypters, because macropterous songs had longer pulses and shorter intervals, and a longer lasting time in the pulse groups. *U*-test ($p = 0.05$) results showed that there were significant differences in the 3 song traits between macroptery and brachyptery: the duration of the pulse, the interval between the pulses, and the duration of the pulse group (Table 1).

We found no significant differences in the frequency domain between the 2 wing forms. As

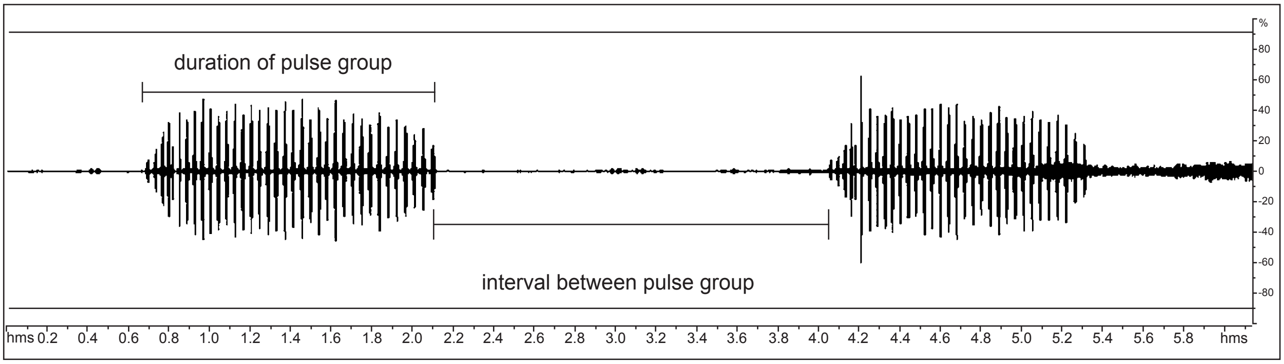

Fig. 1. Definition 1 of song traits of *Eobiana engelhardti engelhardti*; definitions of the duration of the pulse group and the interval between pulse groups. These were actual calling songs of *E. e. engelhardti*.

shown in figure 3, the 2 curves nearly coincided in shape, with the frequency range at 20 dB from 7000 Hz to 35 kHz, and a violent fluctuation in the distribution of frequency; however, macropterous songs had a greater distribution in ultrasonic frequency bands, indicating a better ability for orientation. Montealegre-Z et al. (2006) suggested that these ultrasonic sounds were produced by 1 wing part slipping over another at elevated velocities; the results showed the correctness of our conclusion that the macropters used greater "effort" and produced more-energetic songs than brachypters.

Results of toothed files and tegmina

Results showed no obvious difference in the morphology of the forewing between the 2 wing forms, except for the total length of the forewings (Figs. 4, 5). The main functions of the wing membrane are to respond to vibrations and amplify sound signals in the preliminary experiments, and the mirror surface was studied to determine its main areas of activity (Sismondo 1979). In SEM observations, we examined the tooth files and the surface of the wing membrane, and found no obvious differences between the 2

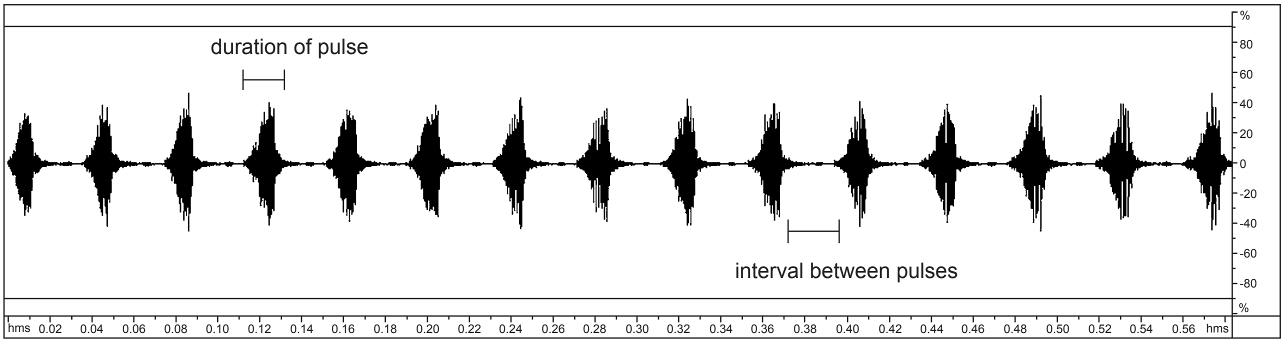

Fig. 2. Definition 2 of song traits of *Eobiana engelhardti engelhardti*; definitions of the duration of the pulse and the interval between pulses. These were actual calling songs of *E. e. engelhardti*.

Table 1. *U*-test of time-domain traits of the calling songs in *Eobiana engelhardti engelhardti*

Wing form		Duration of pulse (ms)	Interval between pulses (ms)	Duration of the pulse group (ms)	Pulses per pulse group	Interval between pulse groups (ms)
Macroptery	No. of samples	278	267	102	57	46
	Average	10.9	21.6	1130	27.2	2290
	Variance	2.4	2.7	3.5	5.1	8.8
	Relative error (%)	22	13	31	19	39
Brachyptery	No. of samples	198	190	57	102	83
	Average	9.7	32.6	890	26.8	2510
	Variance	2.4	3.3	1.6	8.7	14
	Relative error (%)	25	10	18	32	56
U-test ($p = 0.05$)		5.57	37.81	6.12	0.3627	1.49
Critical value		1.65	1.65	1.65	1.65	1.65
Significant difference		Yes	Yes	Yes	No	No

Note: When the sample number was large enough, we used the *U*-test to examine differences between the 2 forms, defined as follows:

U-test, $U = \left| \frac{\bar{x} - \bar{y}}{\sqrt{\frac{s_m^2}{m} + \frac{s_n^2}{n}}} \right| > z_{1-\frac{p}{2}}$, where $s_m = \sqrt{\frac{\sum_{i=1}^{m} (x_i - \bar{x})^2}{m-1}}$, Variance = $s_m / \sqrt{m}$. $\bar{x}$ and $\bar{y}$ are the average data of the 2 wing forms, m and n are the numbers of samples of the 2 forms, and p is the confidence coefficient; when $p = 0.05$, from the table, the critical value is 1.65. When the calculated value is > 1.65, there is a significant difference between the 2 data points.

forms except for the intervals between the teeth. It was interesting that the 2 wing forms had similar lengths and numbers of teeth, while the lengths of the forewings greatly differed (Figs. 6-11). Previous studies suggested that the entire toothed file is used for sound production, and there is nearly a 1: 1 correspondence between the number of cycles in a pulse and the number of teeth on the file (Montealegre-Z and Mason 2005). Macropters had longer intervals between the teeth than the brachypters, and as Montealegre-Z et al. (2006) discussed, enhanced ultrasonic wing velocities of insects are achieved by scraper distortion, a mechanism that stores and releases elastic energy. We supposed that macropters reserve more space for the elastic deformation of the scraper for the purpose of producing more-ultrasonic songs. The surface of the wing membrane is covered with imbricate scales, the ultrastructure of which may effect the Q-values of calling songs of orthopteran insects. We analyzed these phenomena in additional orthopteran insects. These data will be presented in a subsequent paper. All morphological data mentioned above are listed in table 2.

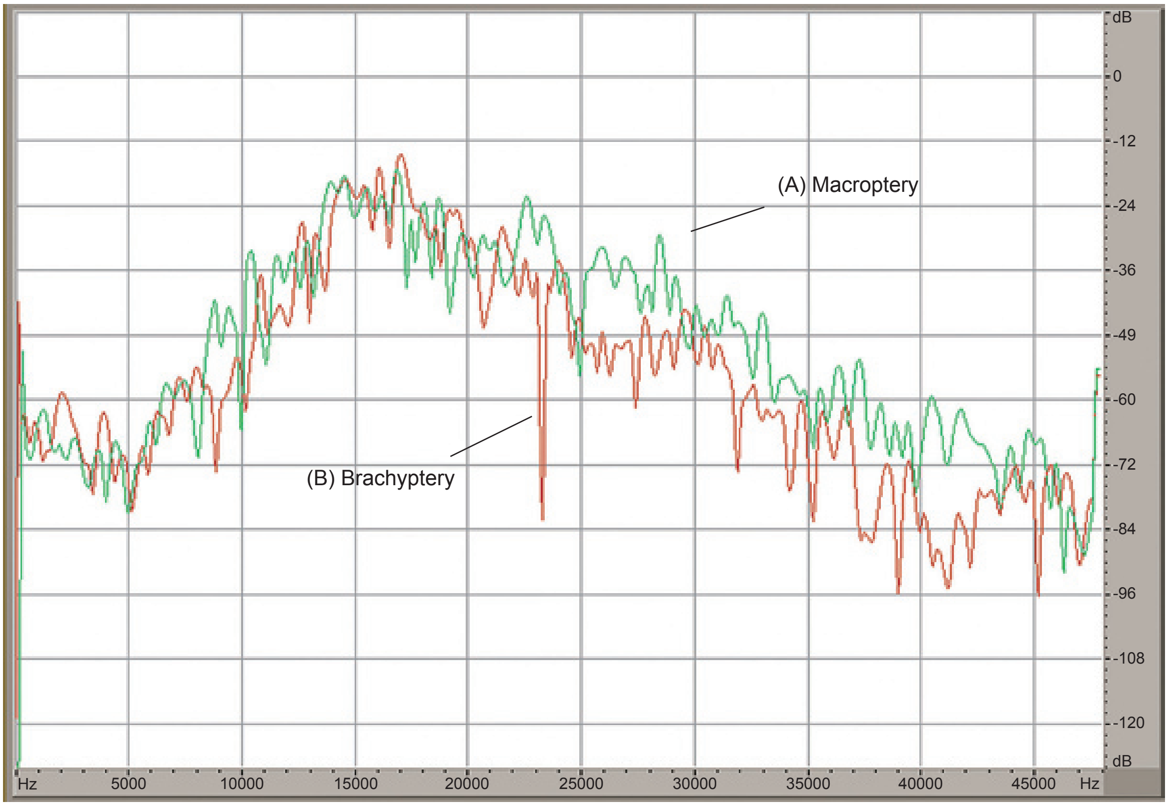

Fig. 3. Frequency-domain measurements of *Eobiana engelhardti engelhardti*. Curve A is the frequency component of macropters, curve B is the frequency component of brachypters, and the 2 curves nearly matched in shape, thus showing a similar distribution of frequency.

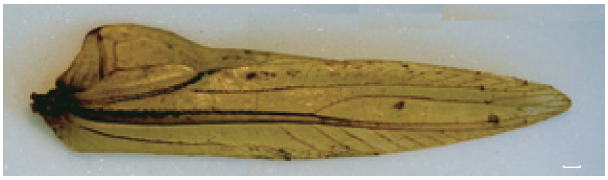

Fig. 4. Forewing of brachypters of *E. e. engelhardti*. Scale bar = 1 mm.

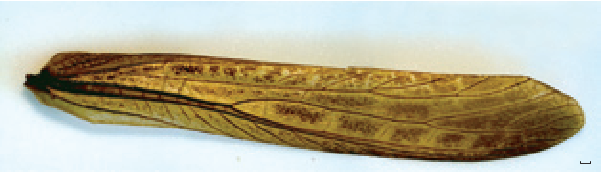

Fig. 5. Forewing of macropters of *E. e. engelhardti*. Scale bar = 1 mm.

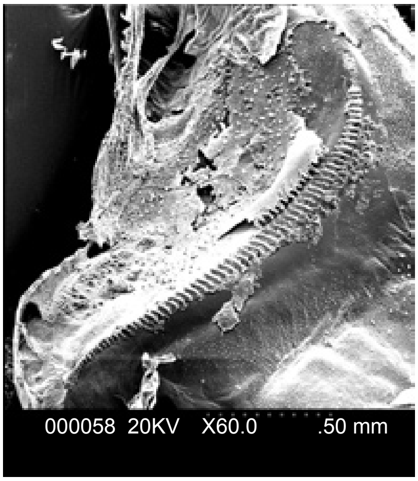

Fig. 6. Full view of toothed files of brachypters of *E. e. engelhardti*. Scale bar = 500 μm.

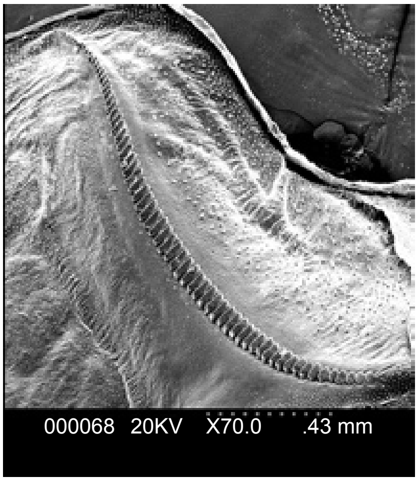

Fig. 7. Full view of toothed files of macropters of *E. e. engelhardti*. Scale bar = 430 μm.

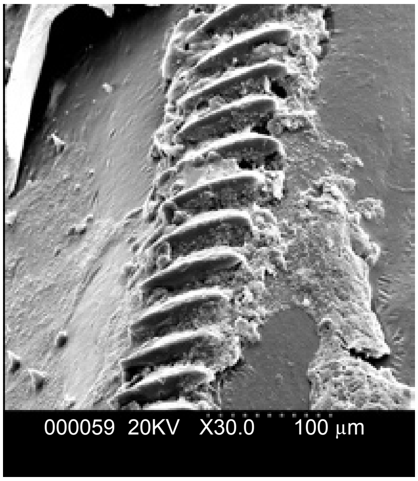

Fig. 8. Near view of toothed files of brachypters of *E. e. engelhardti*. Scale bar = 100 μm.

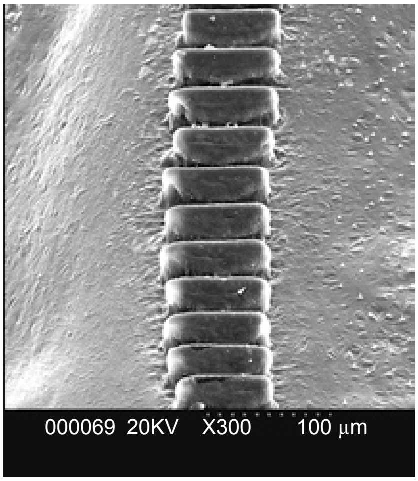

Fig. 9. Near view of toothed files of macropters of *E. e. engelhardti*. Scale bar = 100 μm.

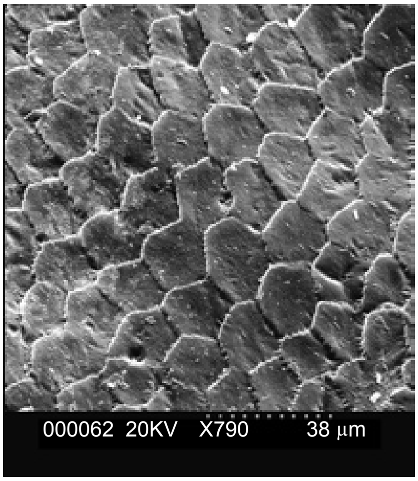

Fig. 10. Surface of the wing membrane of brachypters of *E. e. engelhardti*. Scale bar = 38 μm.

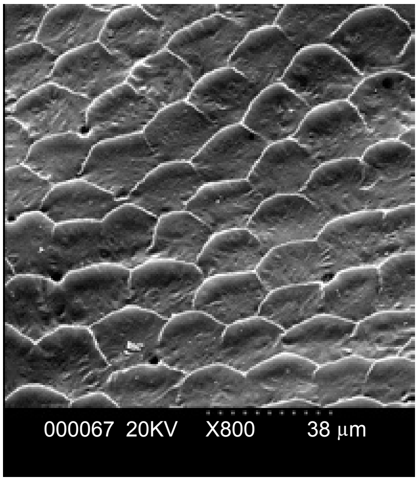

Fig. 11. Surface of the wing membrane of macropters of *E. e. engelhardti*. Scale bar = 38 μm.

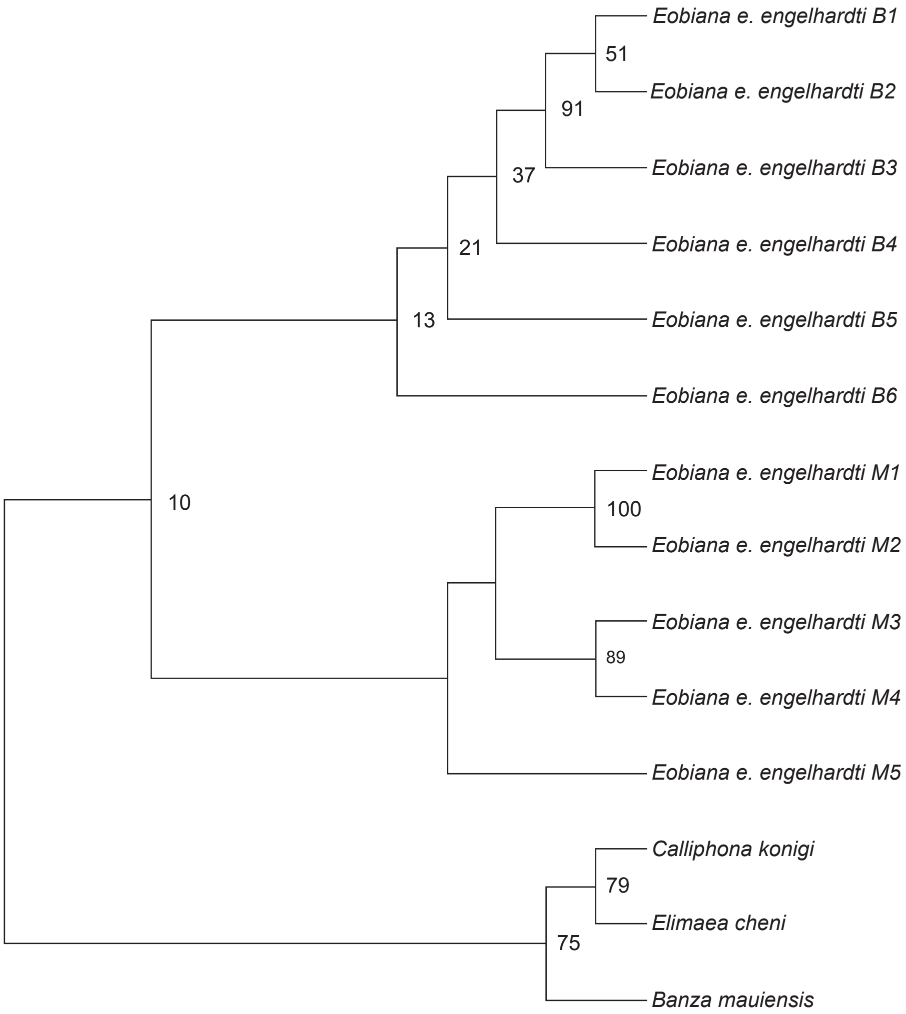

Fig. 12. Phylogenetic analysis of the CO1 mtDNA sequences of the 2 wing forms of *Eobiana engelhardti engelhardti*. Numbers above the branches indicate bootstrap values of > 50%. B, brachypters; M, macropters.

Table 2. *t*-test of morphological data of forewings and toothed files of *E. e. engelhardti*

	Brachypters (19 individuals)		Macropters (11 individuals)		*t*-test (threshold value: 2.05)	
	Average	Variance	Average	Variance	Test value	Significant difference
Length of forewing (mm)	27.8	4.7	52.5	5.6	3.2886	Yes
Total length of toothed files (mm)	1.76	0.03	1.81	0.04	0.2175	No
Number of teeth	65.3	2.7	69.6	3.1	1.0079	No
Interval between teeth (μm)	26.42	0.62	31.13	0.74	4.7512	Yes
Width of teeth (μm)	19-110	91 (range)	16-97	81 (range)	-	-

Note: *t*-test, $T = \left| \frac{\bar{x} - \bar{y}}{\sqrt{(m-1)s_m^2 + (n-1)s_n^2}} \times \sqrt{\frac{mn(m+n-2)}{m+n}} \right| > t_{1-\frac{p}{2}}(m+n-2)$, where $s_m = \sqrt{\frac{\sum_{i=1}^{m}(x_i - \bar{x})^2}{m-1}}$, Variance = $s_m / \sqrt{m}$. $\bar{x}$ and $\bar{y}$ are average data of the 2 wing forms, *m* and *n* are the numbers of samples of the 2 forms, and *p* is the confidence coefficient; when *p* = 0.05, from the table, the critical value is 2.05. When the calculated value is > 1.65, there is a significant difference between the 2 data points. Because the files greatly differed in width, we used the range here to show the results, Range = $X_{max} - X_{min}$.

Results of the phylogenetic analysis

The 2 wing forms formed 2 distinctly separate clades in the MP analysis, with 6 brachypterous insects grouped into a clade and 5 macropterous insects grouped into another (Fig. 12). The G + C content was 36.58% ± 0.2% in brachypters and 36.25% ± 0.2% in macropters, suggesting that there is indeed some differentiation between the 2 wing forms at the molecular level, which exceeded our previous expectations. These molecular differences were greater than differences within a species but less than interspecific differences. Because all samples were collected in the same area, this differentiation should not have been due to geographical isolation.

DISCUSSION

Previous comparative studies across a wide range of polymorphic winged insects suggested a fitness tradeoff between flight capability and reproduction (Roff 1986 1990, Denno et al. 1989, Roff and Fairbairn 1991); however, the results of our calling song analyses revealed that macropters of *E. e. engelhardti* had more-energetic songs than did brachypters, revealing a compensation in songs traits instead of a complete reproductive disadvantage. In general, females preferred “energy-concentrated” songs. Some behavioral experiments proved that song loudness, the interval/pulse ratio, and the interval between pulses were directly correlated with attractiveness (Klappert and Reinhold 2003). Durations of pulse groups were positively correlated with the overall energy of male songs. The interval/pulse ratio might indicate male quality. Females preferred longer pulses and shorter intervals, which might require more energy to produce than short pulses interrupted by longer intervals. A higher energy cost for preferred higher pulse rates of male song was found, for example, in the cricket *Gryllus lineaticeps* and wax moth *Achroia grisella* (Hoback and Wagner 1997, Reinhold et al. 1998). Intervals between pulses were longer in males that used only 1 leg to produce songs, owing to a lost leg, wing deformation, or inaccurate movement of the legs, and might therefore indicate poor male quality in the sense of ‘good’ genes. Females copulated significantly less often with those stunted males as shown by Kriegbaum (1989), who conducted behavioral choice and field experiments. In the present case, calling songs of macropters had longer pulses and shorter intervals, showing more-energetic and attractive characteristics (Table 1). We found no evident morphological differences in the forewing or toothed files between the 2 wing forms; in other words, the differentiation of song traits was more likely to be caused by behavioral mechanisms, not by physiological factors. Previous studies showed that the morphology of the toothed files influenced the number of cycles per pulse, as in Montealegre-Z and Mason’s (2005) work: the entire toothed file is used for sound production, and there is nearly a 1: 1 correspondence between the number of cycles in a pulse and the number of teeth on a file. The morphology of toothed files might also have some effect on the frequency components of songs. The phylogenetic analysis revealed significant differences between the 2 forms. According to our results, these molecular differences had occurred prior to these macropters flying to other areas and becoming new geographical populations. It was interesting that both wing forms had some distributions in ultrasonic frequency bands, and this phenomenon was also found in other orthopteran insects (Montealegre-Z 2006). The function of the ultrasonic range in insect songs is under study in our lab.

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