Comparison of Female and Male *Batocera lineolata* (Coleoptera: Cerambycidae) Adults with a Combination of Morphological and Mitochondrial DNA Analysis[1]

Zengxia Mei, Jianqing Li[2], and Jie Yang

Shandong Province Key Laboratory on Eco-environments of Yellow River Delta, Binzhou University, Binzhou, 256603, Shandong, China

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Abstract Morphological and genetic characters of male and female adults of *Batocera lineolata* Chevrolat were studied to determine intraspecific sex differences. Morphologically, the 9th and 10th segments of the antennae of male adults have odontoid processes that the female lacks. The longitudinal stripes of each abdominal segment of female adults appear to be connected between each adjacent segment, but those of the male do not appear continuous. Female adults also have a narrow V-shaped longitudinal groove in the 5th abdominal segment, whereas males do not. Amplification of the mitochondrial cytochrome *c* oxidase subunit I gene, cytochrome *c* oxidase subunit II gene, cytochrome *b* gene, and ribosomal 16S rRNA gene of four mitochondrial DNA showed sequences that differed between male and female adults. The similarity of the four genes between male and female adults is 98.7%, 99.1%, 98.4%, and 98.8%, respectively. The A+T contents of the four genes in female adults were all higher than in male adults. The difference in content of A+T versus C+G base pairs in female adults was higher than in males. This method of combined morphological and genetic analysis appears to be an accurate and straightforward tool for distinguishing male and female adults of *B. lineolata*.

Key Words *Batocera lineolata*, characteristic comparison, genetic difference, male and female adults, mtDNA

Batocera lineolata Chevrolat (Coleoptera: Cerambycidae) is one of the long-horned beetles that is found mainly in China, where it attacks ash (*Fraxinus* spp.), poplar (*Populus* spp.), and walnut (*Juglans* spp.) trees. In the Yellow River Delta region, ash trees are widely used for urban "greening" due to their alkali resistance and stress resistance; they also compose a great percentage of tree species in natural forests. The boring activity of *B. lineolata* affects the growth of ash trees, causing serious economic losses in China (Li et al. 2009, Mei and Li 2014). Yet, at present, the research on the biological habits and population differentiation characteristics of *B. lineolata* attacking ash trees is lacking.

Mitochondrial DNA (mtDNA) is widely used to analyze insect phylogeny and geographical distribution due to its adherence to maternal inheritance, no mutation, and high conservation (Cui et al. 2020, Hu and Yang 2019, Huang et al. 2012).

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[2]Corresponding author (email: lijianqing1977@126.com).

mtDNA has a small molecular mass, is easy to extract and amplify, and is especially suitable for research on low classification order. The most commonly used mtDNA include the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, cytochrome *c* oxidase subunit II (COII) gene, cytochrome *b* (Cyt b) gene, and ribosomal 16S rRNA gene (Guo et al. 2009, Kou and Lian 2006, Xu and Hua 2001). Wang et al. (2012) successfully cloned the entire mitochondrial genome sequence of the adult *B. lineolata* from Kunming City, Yunnan Province, China, and systematically analyzed the different gene sequences and their coding proteins on the mitochondria. Yu et al. (2016, 2017) analyzed the mtDNA COI gene sequences of 17 samples of species of *Batocera*, including two samples of *B. lineolata*. The analysis showed that COI could be used to identify the species of *Batocera*. Moreover, the phylogenetic tree constructed showed that the genetic relationship between different species was closely related to geographical distribution (Yu et al. 2016, 2017). Genetic analysis of differences between male and females of *B. aneonigra* Thomson and *B. gerstaecheri* Thomson revealed that the intraspecific genetic distance was 0.007 and 0.026, respectively, which was greater than the minimum interspecific genetic distance of 0.002 (Yu et al. 2016).

At present, the length of *B. lineolata* antennae has been used as a standard to distinguish the male and female adults. The antennae of male adults are longer, usually exceeding one-third of the body length, while those of female adults are short and slightly longer than body length (Xiao 1992). Yet it is often difficult to accurately determine the sex of individual *B. lineolata* adults because there are differences in the characteristics of each individual in the length of antennae. Moreover, identification is more difficult with specimens that lack antennae or have only a part of the antennae intact. Jiang et al. (2014) proposed using a combination of traditional morphology with the molecular analysis of the COI gene for more accurate differentiation of the sexes of *Ectropis grisescens* Warren and *E. obliqua* (Prout). As for *B. lineolata*, no comparative studies have examined genetic differences of the geographical populations or mtDNA differences between male and female adults attacking ash trees in the Yellow River Delta of China.

The aim of this study was to systematically compare the morphological characteristics, the structure of reproductive organs, and the sequence differences of COI, COII, Cyt b, and 16S rRNA genes between male and female adults of *B. lineolata*. Our results will hopefully serve as a reference for intraspecific differentiation, population phylogeny, and a theoretical basis for integrated management of *B. lineolata*.

Materials and Methods

Insects. The male and female adults of *B. lineolata* used in these analyses were collected from ash trees in Binzhou City, Shandong Province, China, in June 2019. Collected specimens were preserved in 100% ethyl alcohol until examined or analyzed.

Morphological comparisons. The external morphological characteristics of male and female adults were examined by photographing structures using a microscope with a large depth of field. Genitalia were dissected from the abdomens

Table 1. Polymerase chain reaction primers for the four mitochondrial genes.

Gene*	Primer Sequence	Reference
COI	LCO1490: 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	Zhang 2012
	HCO2198: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′	
COII	F: 5′-AAT ATG GCA GAT TAG TGC A-3′	Bu et al. 2006
	R: 5′-GCT CCA CAA ATT TCT GAG CA-3′	
Cyt b	CB1: 5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′	Zhang 2012
	CB2: 5′-ATT ACA CCT CCT AAT TTA TTA GGA AT-3′	
16S rRNA	16SAR: 5′-CGC CTG TTT AAC AAA AAC AT-3′	Zhang 2012
	ND1A: 5′-GGT CCC TTA CGA ATT TGA ATA TAT CCT-3′	

* COI, cytochrome *c* oxidase subunit I; COII, cytochrome *c* oxidase subunit II; Cyt b, cytochrome *b*.

of male and female adults and also photographed. The resulting photographic images were compared.

DNA analysis. The total genomic DNA was extracted by using an animal DNA rapid extraction kit (Sangon Biotech Co., Shanghai, China). The DNA was extracted from 30 mg of thorax muscle and stored at −20°C for later use, according to the kit instructions. The extracted DNA was electrophoresed in 0.8% agarose gel to detect the clarity of DNA bands.

The amplified primers were insect-specific universal primers (e.g., COI, COII, Cyt b, and 16S rRNA) synthesized by Sangon Biotech (Table 1). A 25-μl reaction system, as per the polymerase chain reaction (PCR) kit instructions (Sangon Biotech), was used for the amplification reaction procedures of COI, COII, Cyt b, and 16S rRNA primers (Table 2). The amplification products were stored at 4°C.

The PCR products were detected by 1.5% agarose gel electrophoresis. Samples (3 μl) of each amplified product were labeled with a DNA marker and were then subjected to electrophoresis at 120V for 50 min, ethidium bromide (EB) staining for 8 to 10 min, followed by rinsing for 2 to 3 min. The gels were photographed to observe and record whether the DNA bands on the gel were clear. Amplified products of COI, COII, Cyt b, and 16S rRNA extracted from male and female *B. lineolata* were purified and sequenced by Sangon Biotech. The four gene sequences were assembled by the SeqMan program in DNAStar software. The MegAlign program was used to compare the assembled sequences and base differences. The EditSeq program analyzed base content.

Data analysis. The data obtained from SeqMan and MegAlign of DNAStar software were analyzed using Excel and DPS software.

Table 2. Reaction procedures and reaction conditions of polymerase chain reaction for four genes.

Reaction Procedure	COI Gene*		COII Gene		Cyt b Gene		16S rRNA Gene	
	Temperature (°C)	Time	Temperature (°C)	Time	Temperature (°C)	Time	Temperature (°C)	Time
Predegeneration	94	4 min	94	4 min	94	4 min	94	4 min
Degeneration	94	30 sec	94	30 sec	94	30 sec	94	30 sec
Annealing	47	30 sec	52	30 sec	49	30 sec	47	30 sec
Extension	72	30 sec	72	30 sec	72	30 sec	72	30 sec
	72	4 min	72	4 min	72	4 min	72	4 min

* COI, cytochrome *c* oxidase subunit I; COII, cytochrome *c* oxidase subunit II; Cyt b, cytochrome *b*.

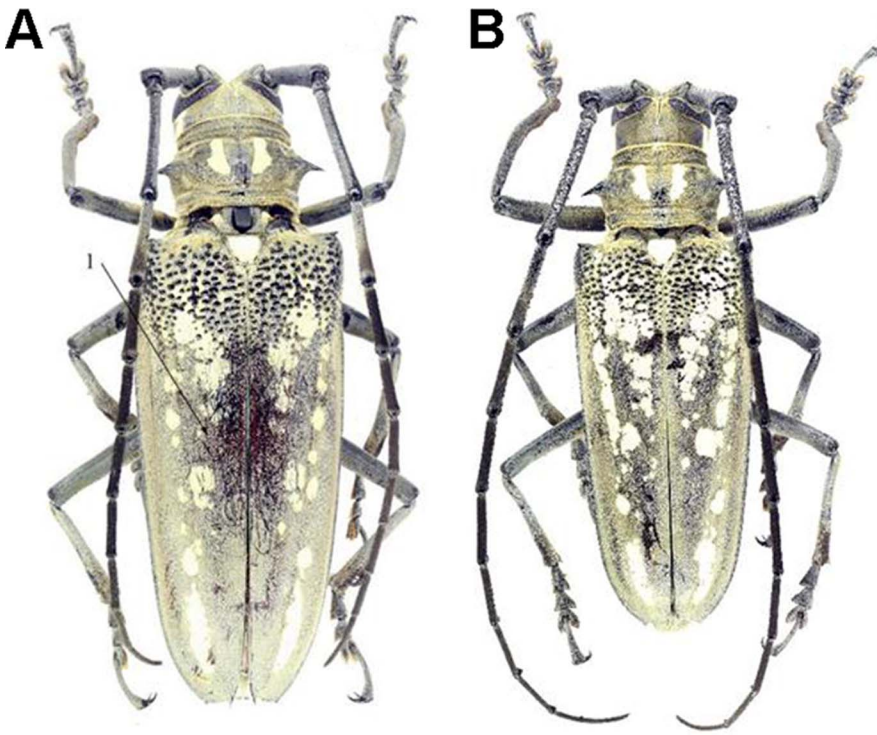

Fig. 1. Male and female adults of *B. lineolata* attacking ash trees. (A) Female adult; (B) male adult. 1, mating spots.

Results

External morphological characteristics. Female adults were slightly larger and more robust in body size than male adults, even when collected from the same host ash tree (e.g., same nutritional conditions) (Fig. 1). Females could be distinguished from males based on the mating spots on the surface of the elytra (Fig. 1) and having antennae shorter than the body length; male antennae are 1.5× longer than body length.

Each segment of the female antennae is covered by villi, and the cuticular surface is relatively even and smooth, whereas the surface of the 1st to 3rd antennal segments of males is uneven, brightly colored, and covered with only a few villi. Moreover, the upper half the 3rd segment near the base of the 4th segment appears the same as the surface of the 3rd segment, with cuticular bumps that are not evenly distributed (e.g., the proximal half near the lower segment has only a few bumps), and the color of villi gradually becomes darker distally. The distribution of the bumps on the surface of antennae (males versus females) is most apparent on the 3rdrd segment (Fig. 2A, B). The distal ends of the 9th and 10th segments of the male antennae have odontoid processes that are most obvious on the 9th segment (Fig. 2C, D), whereas female adults have no odontoid process on the antennae. We

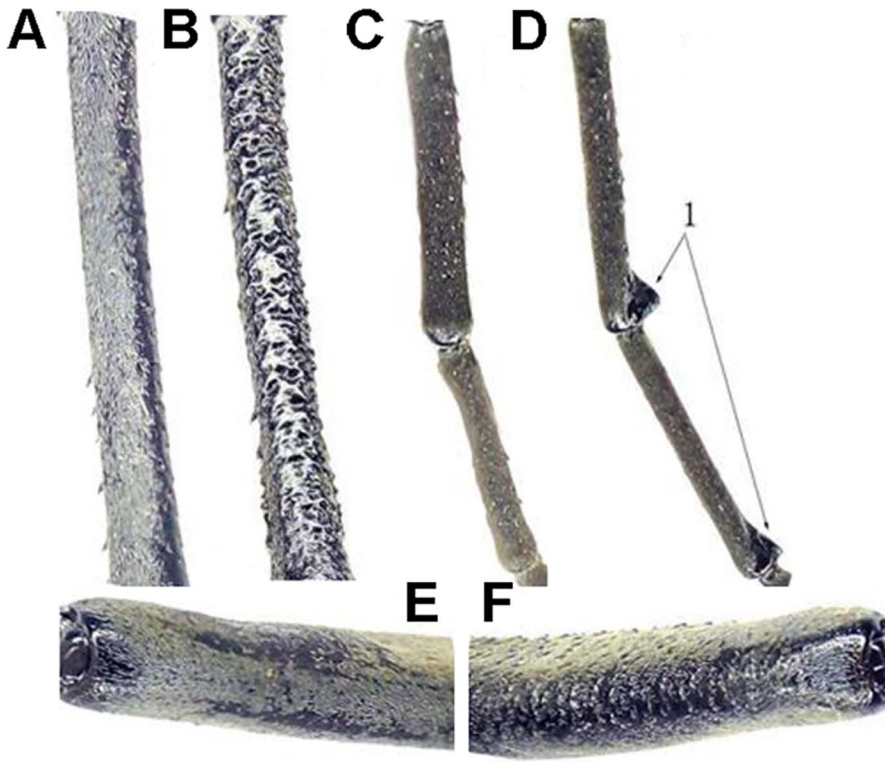

Fig. 2. Surface morphological differences of antennae and forelegs of *B. lineolata* attacking ash trees. (A, B) The third segment of female (A) and male (B) antennae. (C, D) The ninth and tenth segment of female adult (C) and male adult (D) antennae. (E, F) Forelegs of female adult (E) and male adult (F). 1, odontoid processes.

also found that the surface of the forelegs of the female are smooth, whereas the foreleg surface of male adults is uneven and bumpy (Fig. 2E, F).

A long, thin, white longitudinal stripe on both sides of each abdominal segment of the female adult appears connected and continuous, whereas those on the male do not. Upon closer examination, the lines are created by white colorations on each side of the segments. In females, those spots are rectangular in shape and extend from the proximal to distal edges of each segment (Fig. 3A). In males, the spots are ovoid in shape, often not extending to the forward and hind edges of the segments, thus giving the appearance of a noncontinuous line (Fig. 3B). There is also a black longitudinal V-shaped groove in the center of the base of the 5th abdominal segment of females (Fig. 3A), with no brown villi distributed on its surface. Male adults have no such groove (Fig. 3B).

The end of the 5th abdominal segment of females has a tubular extension that protrudes and bends downward. The sternum has a slight depression and sparse hair in the middle of its distal edge. The male lacks such structures and

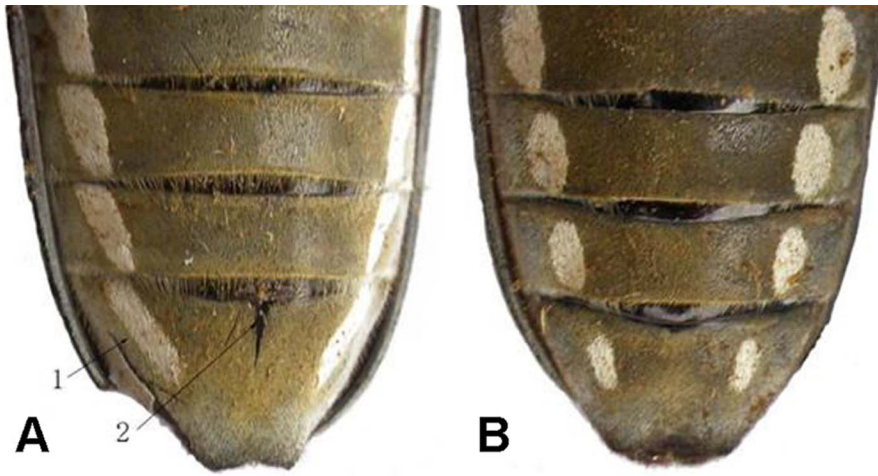

Fig. 3. Comparison of abdominal morphological characteristics of female and male adults of *B. lineolata* attacking ash trees. (A) Female adult; (B) male adult. 1, longitudinal stripe; 2, longitudinal groove.

characteristics and has no tubular extension, but has an arc-shaped depression in the middle of the distal edge of the sternum that is densely covered with hairs.

Reproductive organs. Female ovipositors (Fig. 4) originate from the 8th abdominal segment, the intersegmental membrane between the 8th and 9th abdominal segments, and the 9th abdominal segment at the terminal end of the abdomen. A pair of abdominal structures of the 9th abdominal segment are specifically adapted as a pair of oviposition valvula. There are some longitudinal baculi in the oviposition valvula, which are divided into paraproct baculi, coxite baculi, and dorsal baculi (Fig. 4A). The baculi provide longitudinal support for the oviposition valvula, and the two baculi are alternately arranged. During oviposition, the angle of the oviposition valvula can be adjusted to ensure that the eggs are laid at the correct orientation and position. The end of the oviposition valvula is styli, a process on the end of the oviposition valvula, which mainly detects whether the site is suitable for oviposition.

Female internal reproductive organs include spermatheca, bursa copulatrix, vagina, oviduct, and ovary. The vaginal end is connected with the ovipositor, the base is connected with the middle oviduct and bursa copulatrix, respectively, and the opening is at the base of the oviposition valve. There are two vaginal plates (Fig. 5) on both sides of vagina base near the oviduct, which are highly ossified and dark brown, and oval-shaped. The vaginal plates can embrace the endophallus of the external genitalia of the male during mating, and adjust the opening and closing of the oviduct and vagina during oviposition.

The bursa copulatrix is balloon-shaped; however, its shape may change due to its membranous structure with the base connected to the vagina. It connects the bursa copulatrix through the spermathecal duct on one side of the bursa copulatrix. It is C-shaped and mostly milky white in color, with the outer wall being highly ossified. The spermatheca can be divided into three parts: spermatheca terminal,

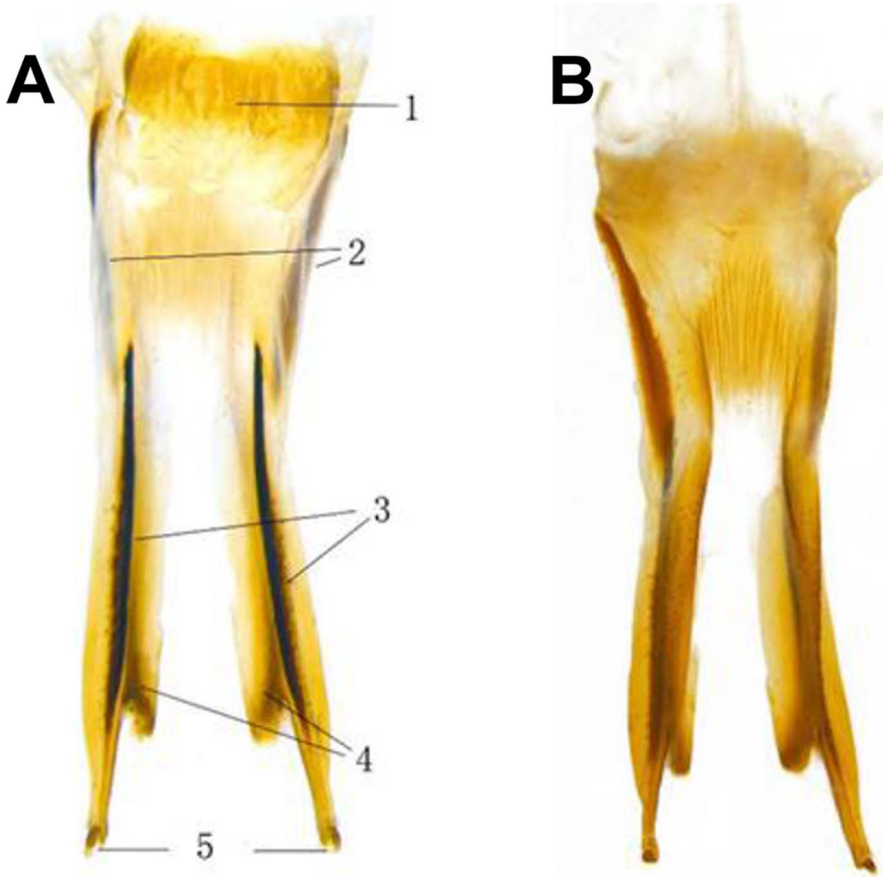

Fig. 4. Ovipositor of female adults of *B. lineolata* attacking ash trees. (A) Dorsal view of ovipositor; (B) ventral view of ovipositor. 1, proctiger; 2, paraproct baculi; 3, dorsal baculum; 4, coxite baculi; 5, styli.

spermatheca median, and spermatheca proximal. The spermatheca proximal is yellowish-brown, as shown in Fig. 6.

The external genitalia of males are located in the cloaca formed by the distal abdominal segment. As shown in Fig. 7, the tergum of the 8th abdominal segment is developed and strong, and the middle part of the abdominal segment is specialized into a “Y” spiculum gastrale. The bifurcated arms of the spiculum gastrale mainly support the opening of the cloaca. There is also a spiculum relictum in the cloaca, which is less developed and small. The central part of the distal edge of the tergum is curved and densely covered with setae.

Male external genitalia include tegmen, penis, and endophallus. The tegmen has two parts: one is a pair of parameres at the top, while the other is phallobase at the base (Fig. 8A, B). The end of the parameres is densely covered with sensory setae. The phallobase is a leathery and translucent basal piece, attached to a pair of

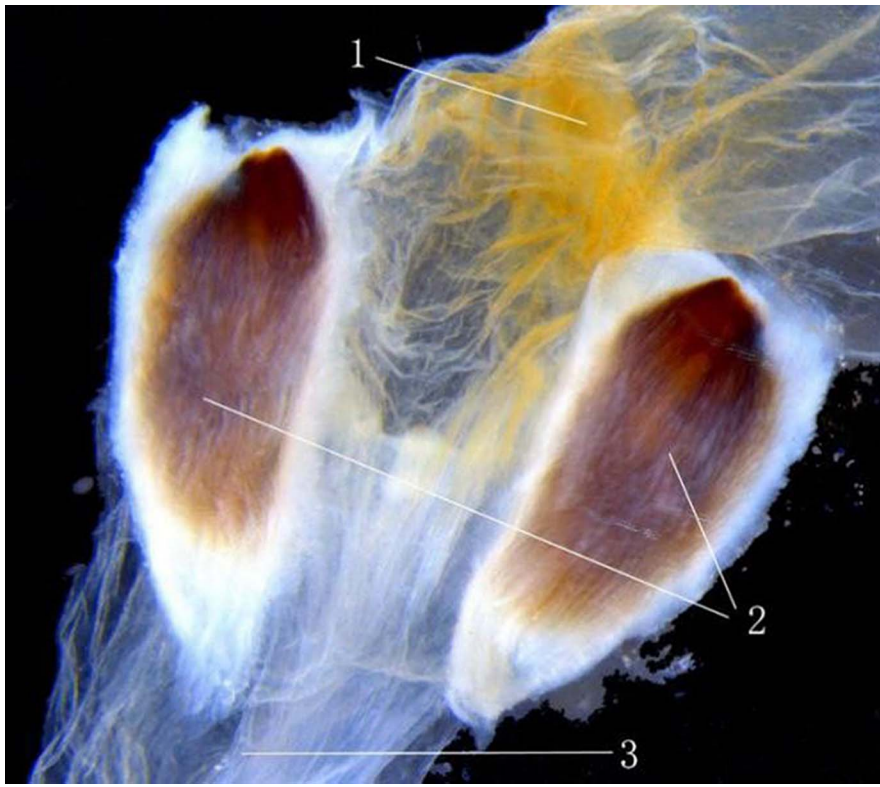

Fig. 5. Vaginal slices of female adults of *B. lineolata* attacking ash trees. 1, middle oviduct; 2, vagina plates; 3, vagina.

lateral arms. The two lateral arms encircle the penis and merge at the base of the penis. In addition, the penis is ossified and curved and is divided into two parts: the penis lobe and the penis struts (Fig. 8C). The penis lobe is highly ossified, hard, and dark brown. The endophallus is ductile and membranous, and connected with the penis. It is usually shrunk in the penis tube and turns outward during mating to enter the bursa copulatrix of the female during mating.

Mitochondrial genes. The OD_{260}/OD_{280} value of the DNA extracted from the adult males was 1.67 and 1.78 in adult females. Concentrations were 83.40 and 89.10 mg/l, respectively, indicating that the extracted DNA had high purity and concentration. The electrophoresis patterns of the mitochondrial genes (COI, COII, Cyt b, 16S rRNA) of male and female adults amplified by PCR showed no differences in gene bands based on sex (Fig. 9). According to the DNA marker, the lengths of the COI, COII, Cyt b, and 16S rDNA genes are about 730 bp, 680 bp, 500 bp, and 850 bp, respectively.

Based on results from the MegAlign analysis, the similarity of gene sequences between males and females for COI, COII, Cyt b, and 16S rRNA differed for the four genes (Table 3). With the Cyt b sequences, we found a similarity of 98.4% between

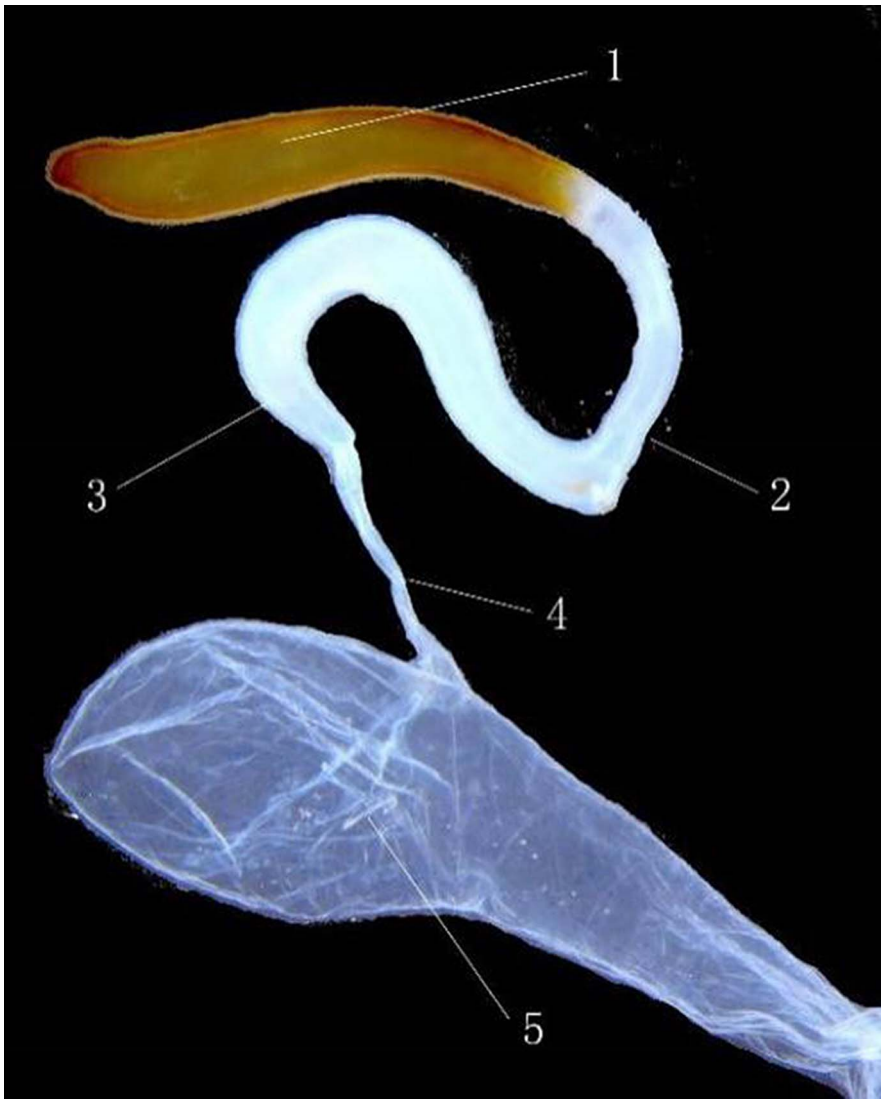

Fig. 6. The spermatheca and bursa copulatrix of female adults of *B. lineolata* attacking ash trees. 1, spermatheca terminal; 2, spermatheca median; 3, spermatheca proximal; 4, spermathecal ducts; 5, bursa copulatrix.

males and females, which was the greatest difference of the four genes. The COII sequences showed a 99.1% similarity between males and females. Similarities of the COI and 16S rRNA genes were intermediate.

The sequence lengths of COII, Cyt b, and 16S rRNA were longer in females than in males (Table 3). The length of the COI gene was slightly shorter (i.e., 4 bp) than that of the males. The maximum number of bases of the COII gene of females was

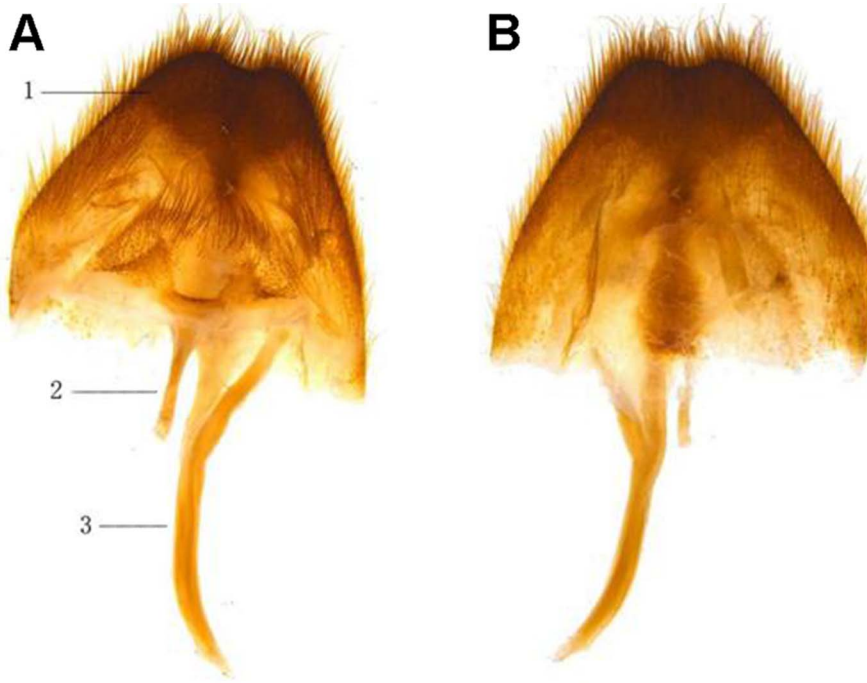

Fig. 7. Tergite VIII of male adults of *B. lineolata* attacking ash trees. (A) Ventral view; (B) dorsal view. 1, the 8th tergum; 2, spiculum relictum; 3, spiculum gastrale.

23 bp more than that of males, and the minimum number of bases of the 16S rRNA gene of females was 3 bp more than that of males.

As depicted in Fig. 10, the similarity of the COI gene between males and females was 98.7%, the length of the sequence used for comparison was 705 bp, and the total length of the base sequence in a female adult was 705 bp, starting from the 1st base to the 704th base. The total length of the base sequence of males was 709 bp (compared bases 6th–709th). Of the 705 bases compared, two were deleted—one for males and one for females. There are, in total, five inconsistent bases in all compared bases, including two bases located in the 10 bases after the start site and 10 bases before the end site, and three bases located in the middle.

The similarity of the COII gene between males and females was 99.1%, and the length of the sequence used for comparison was 654 bp. The total length of the base sequence of females was 676 bp, and the bases for comparison were 16th to 699th. The total length of the base sequence of males was 653 bp, and the comparative bases were 1st to 653th (Fig. 11). Of the 654 bases compared, one base of a male adult was deleted. There were a total of four inconsistent bases in all compared bases, including three bases located in 10 bases after the start site and 10 bases before the end site, and one base located in the middle.

As shown in Fig. 12, the similarity of the Cyt b gene between males and females was 98.4%, and the length of the sequence used for comparison was 490 bp. The total

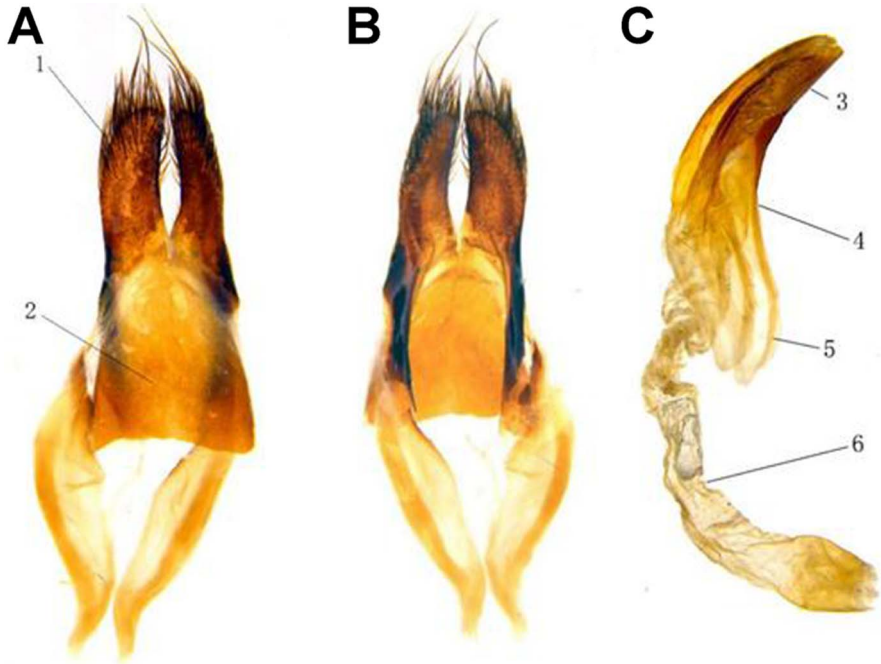

Fig. 8. Internal genitalia of male adults of *B. lineolata* attacking ash trees. (A) Dorsal surface of tegmen; (B) ventral surface of tegmen; (C) penis and endophallus. 1, paramere; 2, phallobase; 3, penis lobe; 4, penis struts; 5, penis; 6, endophallus.

length of the base sequence of females was 497 bp, and the bases for comparison were 7th to 495th. The total length of the base sequence of male adults was 489 bp, and the comparative bases were 1st to 489th. Of the 490 bases compared, two were deleted—one for males and one for females. There were a total of four inconsistent bases among all compared bases, including one base located in 10 bases after the start site and 10 bases before the end site, and three bases located in the middle.

The similarity of the 16S rRNA gene between males and females was 98.8%, and the length of the sequence used for comparison was 832 bp. The total length of the base sequence of the females was 835 bp, and the bases for comparison were 1st to 832nd. The total length of the base sequence in males was 832 bp, and the comparative bases were 2nd to 832nd (Fig. 13). Of the 832 compared bases, one for males was deleted. There were, in total, seven inconsistent bases among all compared bases, including two bases located in 10 bases after the start site and 10 bases before the end site, and five bases located in the middle.

Base composition analysis shows that the four mitochondrial genes of males and females have obvious base preference, and the base pair A+T is higher than that of C+G (Table 4). The content of A+T in the four genes exceeds 65%, with the content of C+G about 30%, which is consistent with the characteristic high A+T content in insect mitochondrial sequences. However, the difference in the content of A+T versus C+G in

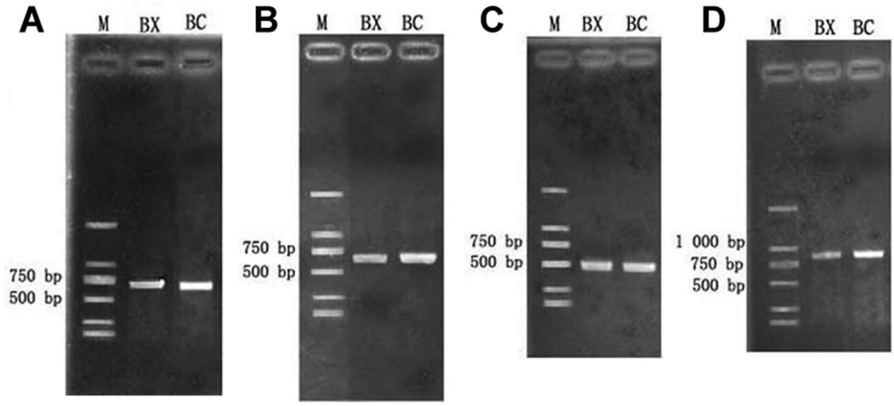

Fig. 9. Electrophoresis of four mitochondrial DNA genes of male and female adults of *B. lineolata* attacking ash trees. (A) cytochrome *c* oxidase subunit I gene; (B) cytochrome *c* oxidase subunit II gene; (C) cytochrome *b* gene; (D) 16S rRNA gene. M, DNA marker; BC, female adult; BX, male adult.

females was higher than that observed in males. For COI, the content of A+T in females was 31.34% higher than that of C+G, while the content of A+T in males was 31.18% higher than that of C+G. For COII, the content of A+T in females was 38.76% higher than that of C+G, while the A+T content in males was 38.44% higher than that of C+G. For Cyt b, the A+T content in females was 34.40% and in males was 34.16% higher than that of C+G. For 16S rRNA, the difference in females and males was 42.76% and 42.54%, respectively. The difference in A+T versus C+G content of the 16Sr RNA gene in males and females was higher than that of COII, followed by Cyt b and COI. The content of A+T was consistently higher in females than in adult with levels of 0.08%, 0.16%, 0.12%, and 0.11% higher for COI, COII, Cyt b, and 16S rRNA

Table 3. Comparison of four gene base sequences of male and female adults of *B. lineolata* attacking ash trees.

Gene*	Female Adult Sequence Length (bp)	Male Adult Sequence Length (bp)	Compared Sequence Length (bp)	Number of Deleted Bases	Number of Inconsistent Bases	Similarity (%)
COI	705	709	705	2	5	98.7
COII	676	653	654	1	4	99.1
Cyt b	497	489	490	2	4	98.4
16S rRNA	835	832	832	1	7	98.8

* COI, cytochrome *c* oxidase subunit I; COII, cytochrome *c* oxidase subunit II; Cyt b, cytochrome *b*.

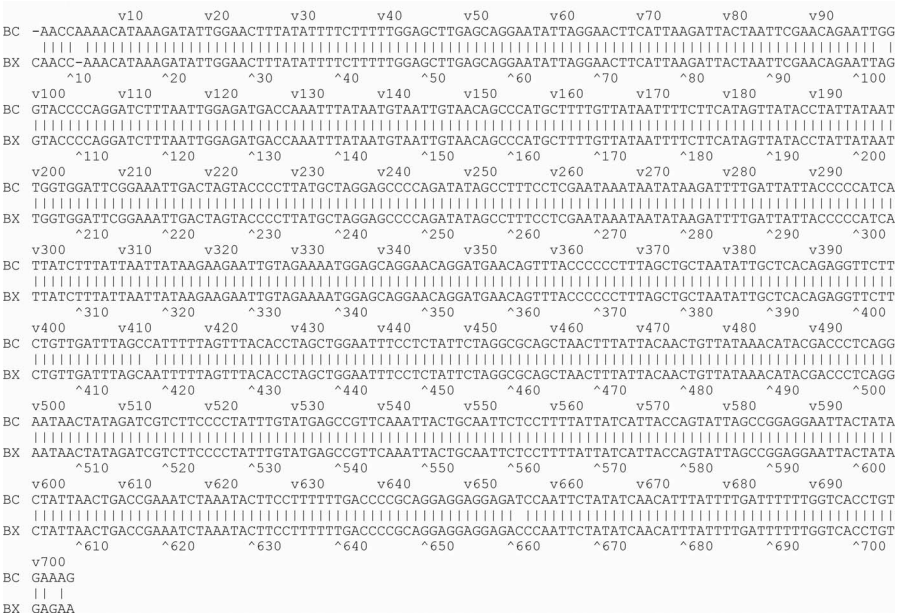

Fig. 10. Sequence comparison of cytochrome *c* oxidase subunit I gene in female and male adults of *B. lineolata* attacking ash trees. BC is a female adult, BX is a male adult, and the letter without vertical link is an inconsistent base.

than in males, respectively. A+T content in the COII gene is higher in females than in males, followed by Cyt b, 16S rRNA, and COI.

Discussion

Antennae and abdominal distal segments are the main characteristics that can be used to distinguish the sex of long-horned beetle adults. In general, the antennae of male adults are significantly longer than those of female adults. Moreover, the distal antennal segment in female adults is longer compared to that of male adults, whose distal segment is wider and shorter. In addition, the female adults have a narrow longitudinal groove in the center of the distal segment, whereas the male adults have no longitudinal groove; however, males have thicker and denser distribution of hair at the end of the distal segment (Qi 1999).

In this study, we found some morphological differences in the antennae and abdomen of male and female adults of *B. lineolata*. In addition to the difference in antennal length, the surface of the 1st to 3rd segments of the male antennae is uneven and bumpy, whereas the 9th and 10th segments have odontoids. These characters were not observed in females. In addition, we found two distinguishing features in the abdominal region, namely the white longitudinal stripes on both sides of each abdominal segment and the longitudinal groove at the base of the 5th

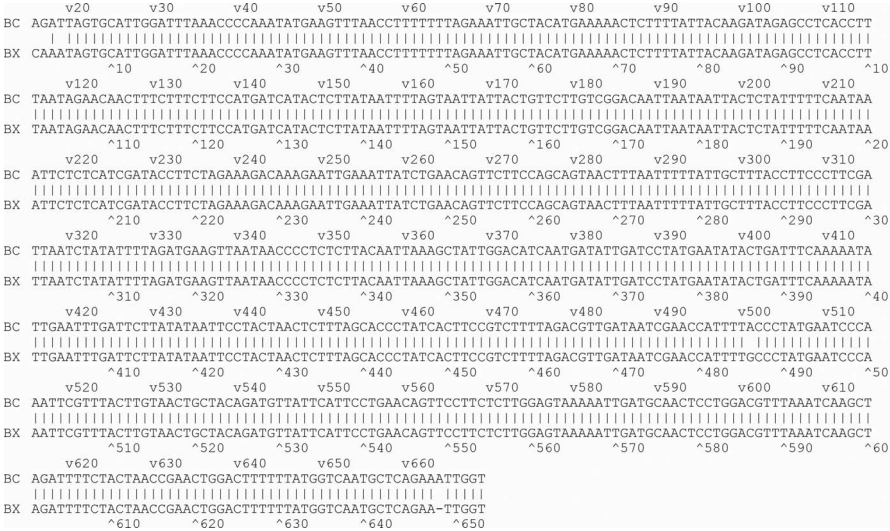

Fig. 11. Sequence comparison of cytochrome *c* oxidase subunit II gene in female and male adults of *B. lineolata* attacking ash trees. BC is a female adult, BX is a male adult, and the letter without vertical link is an inconsistent base.

abdominal segment, which can be useful in distinguishing males from females. These qualitative indices can be observed without dissection or magnification.

Zhang et al. (2017) reported that the males of *Batocera horsfieldi* (Hope) attacking *Carya cathayensis* Sarg in the Lin'an area of Zhejiang Province of China were characterized with antennae that extended beyond the end of the abdomen and exceeded the length of the body. The distal abdominal segment (5th segment) of the female adult possessed an obvious vertical line, whereas the males did not.

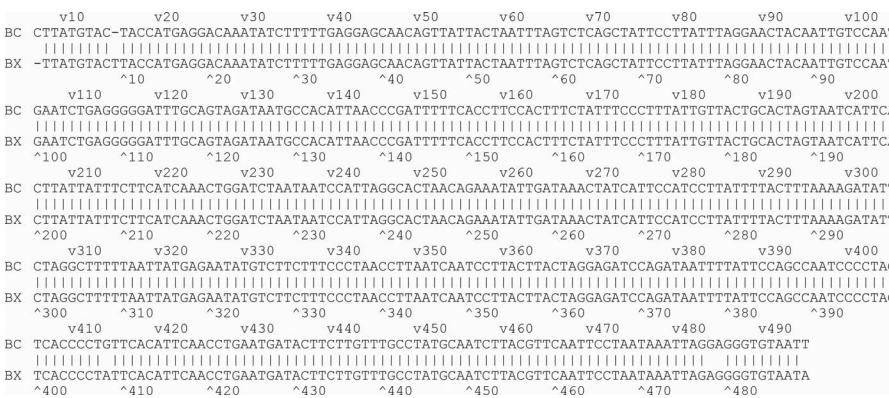

Fig. 12. Sequence comparison of cytochrome *b* gene in female and male adults of *B. lineolata* attacking ash trees. BC is a female adult, BX is a male adult, and the letter without vertical link is an inconsistent base.

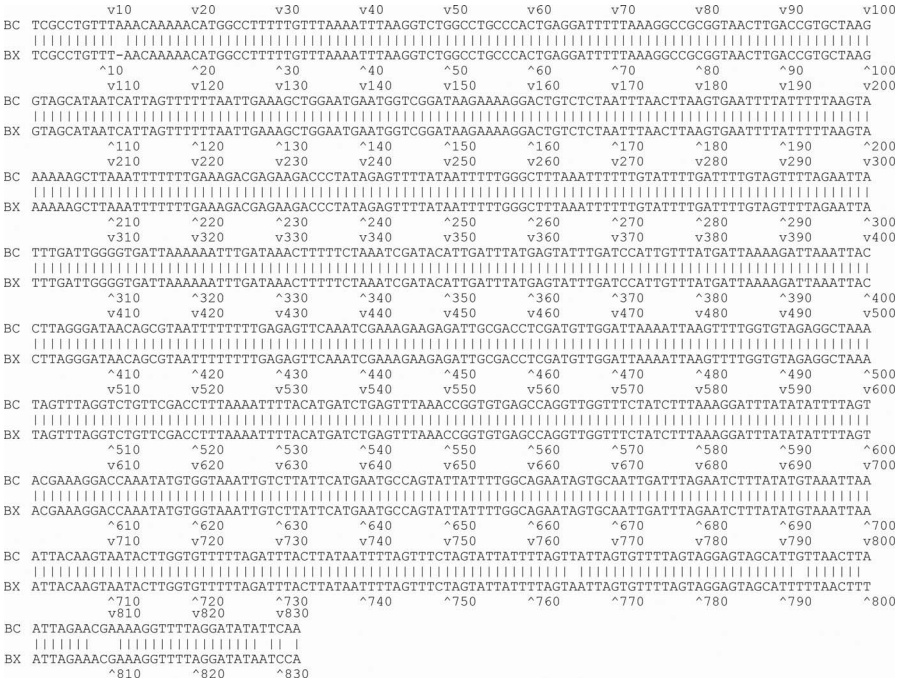

Fig. 13. Sequence comparison of 16S rRNA genes between female and male adults of *B. lineolata* attacking ash trees. BC is a female adult, BX is a male adult, and the letter without vertical link is inconsistent base.

Table 4. Comparison of base contents of four genes of male and female adults of *B. lineolata* attacking ash trees.

	COI* (%)		COII (%)		Cyt b (%)		16S rRNA (%)	
Base	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
A	30.64	30.61	31.66	31.24	29.78	29.86	31.74	31.61
G	15.74	15.80	11.98	11.64	12.07	12.07	18.80	18.75
T	35.04	34.98	37.72	37.98	37.42	37.22	39.64	39.66
C	18.58	18.62	18.64	19.14	20.72	20.86	9.82	9.98
A+T	65.67	65.59	69.38	69.22	67.20	67.08	71.38	71.27
C+G	34.33	34.41	30.62	30.78	32.80	32.92	28.62	28.73

* COI, cytochrome *c* oxidase subunit I; COII, cytochrome *c* oxidase subunit II; Cyt b, cytochrome *b*.

The ratio of antennal length to body length in male *Bacchisa atritarsis* (Pic) was significantly greater than that of females. The 5th abdominal segment of the female adult was wider and longer, and there was a longitudinal line in the center of the ventral surface. In contrast, the 5th segment of the adult male was narrower and shorter, and there was no longitudinal line in the center of the ventral surface.

The central longitudinal line of the 5th abdominal segment is the best method for identifying the sex of *B. atritarsis* (Huang et al. 2020). In our study, these newly discovered external morphological characteristics of male and female adults of *B. lineolata* mostly conformed to the characteristics used to differentiate the sexes of other cerambycid beetles. Our study results are basically consistent with the relevant research of other scholars.

Thus far, only a few studies have analyzed the intraspecific difference of mitochondrial genes between male and female individuals. Certain differences between male and female mitochondrial genes have been found in bivalve mollusks, such as *Hyriopsis cumingii* Lea, and some suggest using COII as a marker to distinguish male and female individuals of certain bivalve mollusks (Chapman et al. 2008, Curole and Kocher 2005, Ya et al. 2018). In our study, four mitochondrial genes—COI, COII, Cyt b, and 16S rRNA—of male and female adults of *B. lineolata* were compared, revealing that the four genes have some differences in base sequence and base composition. The sequence gene similarity between male and female adults ranged from 98.40% to 99.10%. We also compared the differences among these four mitochondrial genes in males and females of a *B. lineolata* population attacking poplars, obtaining similar results, with the sequence similarity between males and females ranging between 98.3% and 99.2% (Mei and Li 2021).

In summary, we used traditional morphological characteristics and molecular techniques to systematically and comprehensively analyze and compare the differences between male and female individuals of *B. lineolata* that are known to damage ash trees. Two new abdominal identification features—white longitudinal stripes on both sides of each abdominal segment and the longitudinal groove at the base of the 5th abdominal segment—resulted as very useful for distinguishing *B. lineolata* sexes. This identification method is easy to perform and has high practical value in the investigation and control of *B. lineolata*. Moreover, the systematic comparison of the differences of four mitochondrial genes between males and females has certain theoretical value for studying the internal differentiation of the population.

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References Cited

Bu, Y., Y. Jia and Z. Zheng. 2006. Molecular systematic study on Pentatomidae (Hemiptera, Heteroptera) from China based on mitochondrial cytochrome oxidase II gene. Acta Zootaxonomica Sinica, 31(2): 239–246.

Chapman, E.G., H. Piontkivska, J.M. Walker, D.T. Stewart, J.P. Curole and W.R. Hoeh. 2008. Extreme primary and secondary protein structure variability in the chimeric male-transmitted cytochrome c oxidase subunit II protein in freshwater mussels: Evidence for an elevated amino acid substitution rate in the face of domain-specific purifying selection. BMC Evol. Biol. 8: 165.

Cui, Z., Q. Zhou, Y. Liu, P. Si and Y. Wang. 2020. Molecular identification of citrus fruit flies and genetic diversity analysis of *Bactrocera minax* (Diptera: Tephritidae) populations in China based on mtDNA COI gene sequences. Acta Entomol. Sin. 63(1): 85–96.

Curole, J.P. and T.D. Kocher. 2005. Evolution of a unique mitotype-specific protein-coding extension of the cytochrome c oxidase II gene in freshwater mussels (Bivalvia: Unionoida). J. Mol. Evol. 61(3): 381–389.

Guo, X., N. Sun and Y. Zhang. 2009. Application of mitochondrial COI gene in insect molecular systematics. Int. J. Genet. 32(5): 79–81.

Hu, X. and Z. Yang. 2019. Analysis of the genetic differentiation among different geographic populations of *Loxostege sticticalis* (Lepidoptera: Crambidae) in China based on mitochondrial COI, Cytb and COII genes. Acta Entomol. Sin. 62(6): 720–733.

Huang, L., H. Que, C. Chen, X. Kuang and F. Xue. 2012. Variation of mtDNA COI sequences in different geographic populations of the Asian corn borer, *Ostrinia furnacalis*. Chin. J. Appl. Entomol. 49(6): 1508–1512.

Huang, X., P. Luo, W. Liu, B. Xiao and G. Wang. 2020. Screening and determination of the best method in identifying the sexes of *Bacchisa atritarsis* adults. Acta Agric. Univ. Jiangxiensis 42(2): 266–274.

Jiang, N., S. Liu, D. Xue, M. Tang, Q. Xiao and H. Han. 2014. External morphological and molecular identification of two tea geometrid moth from southern China. Chin. J. Appl. Entomol. 51(4): 987–1002.

Kou, J. and Z. Lian. 2006. Characters of animal MtDNA and application in phylogenetic analysis of crickets. J. Yanan Univ. (Nat. Sci. Ed.) 25(1): 56–59.

Li, J., Z. Yang, Z. Mei and Y. Zhang. 2009. Pest risk analysis and control countermeasures of *B. horsfieldi*. For. Res. 22(1): 148–153.

Mei, Z. and J. Li. 2014. Occurrence, injury and control of *B. lineolata* in Yellow River Delta Region. J. Binzhou Univ. 30(3): 58–62.

Mei, Z. and J. Li. 2021. Differences in four mitochondrial genes in male and female *Batocera lineolata*. Chin. J. Appl. Entomol. 58(2): 326–334.

Qi, C. 1999. A Survey of Insects Cerambycidae in Shandong. Shandong Science and Technology Press, Jinan, China.

Wang, C., Y. Feng and X. Chen. 2012. Complete sequence and gene organization of the mitochondrial genome of *Batocera lineolata* Chevrolat (Coleoptera:Cerambycidae). Chin. Sci. Bull. 57(27): 3578–3585.

Xiao, G. 1992. Forest Insects in China. 2nd ed. China Forestry Publishing House, Beijing.

Xu, Q. and B. Hua. 2001. Application of mtDNA in phylogenetic analysis of insect. J. Northwest Sci-Tech Univ. Agric. For. (Nat. Sci. Ed.) 29(S1): 79–83.

Ya, C., W. Yayu, W. Guiling, H.E. Fangshu and L.I. Jiale. 2018. Full-length cDNA cloning of M and F-type COII genes and expression in different age freshwater mussel *Hyriopsis cumingii*. J. Fish. China 42(1): 29–38.

Yu, H., Y. An, X. Guo, M. Sun, N. Xu, X. Yang, M. Xu and J. Ran. 2017. Preliminary study on gene barcode characteristics and phylogeny of different species of *B. lineolata*. Jiangsu Agric. Sci. 45(13): 90–94.

Yu, H., M. Xu, N. Xu, M. Sun and Y. An. 2016. Establishment of DNA barcode of species of *Batocera*. J. Fujian For. Sci. Technol. 43(2): 90–94, 116.

Zhang, D., S. Zhou, N. Zhu and J. Fan. 2017. Damage investigation and an identification method for different species of *Carya cathayensis* longhorned beetles. J. Zhejiang A&F Univ. 34(1): 42–49.

Zhang, Y. 2011. Comparsion and phylogentic analysis using sequences of mitochondrial genes in some Rutelidae species. Shenyang China:Shenyang University.