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ORIGINAL ARTICLE

Is there hybridization between 2 species of the same genus in sympatry?—The genetic relationships between *Anoplophora glabripennis*, *Anoplophora chinensis*, and putative hybrids

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Abstract *Anoplophora glabripennis* (Asian longhorn beetle, ALB) and *Anoplophora chinensis* (Citrus longhorn beetle, CLB) are native forest pests in China; they have become important international quarantine pests. They are found using the same *Salix aureopendula* host tree of Cixi, Zhejiang province, China. On this host tree, we collected additional beetles that appeared to be morphologically intermediate between ALB and CLB. By using a stereoscope, we observed that there were several bumps on the base of the elytra, which was inconsistent with ALB, which typically has a smooth elytral base, but was more like CLB, which has numerous short tubercles on the elytral base. Given their sympatry and intermediate morphology, we hypothesized that these may represent ALB × CLB hybrids. We studied the genomic profiles for 46 samples (ALB, CLB, and putative hybrids) using genotyping-by-sequencing (GBS) providing a reduced representation of the entire genome. Employing principal component analyses on the 163 GBS-derived single nucleotide polymorphism data, we found putative hybrids tightly clustered with ALB, but genetically distinct from the CLB individuals. Therefore, our initial hybrid hypothesis was not supported by genomic data. Further, while mating experiments between adult ALB and CLB were successful in 4 separate years (2017, 2018, 2020, and 2021), and oviposition behavior was observed, no progeny was produced. Having employed population genomic analysis and biological hybridization experiments, we conclude that the putative hybrids represent newly discovered morphological variants within ALB. Our approach further confirmed the advantage of genome-wide information for *Anoplophora* species assignment in certain ambiguous classification cases.

Key words *Anoplophora chinensis*; *Anoplophora glabripennis*; genotyping-by-sequencing; invasive pest; mating; putative hybrid

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Introduction

Global trade has been responsible for the introduction and spread of invasive species. Non-native wood-boring insects are some of the most economically impactful invasive species (Aukema *et al.*, 2011). Longhorn beetles (Coleoptera: Cerambycidae) are a threat to deciduous tree species outside their native ranges. The European Plant Protection Organization (EPPO) identified 2 longhorn beetles, the Asian longhorn beetle (ALB), *Anoplophora glabripennis* (Motschlsky) (Coleoptera: Cerambycidae) and the Citrus longhorn beetle (CLB), *Anoplophora chinensis* (Forster) (Coleoptera: Cerambycidae), as quarantined pests, resulting in strict regulations that can result in quarantines for shipment of wood-packaging materials and live plants from Asia, negatively impacting trade from those geographic areas (Gaag *et al.*, 2010; EPPO Global Database).

An endemic cerambycid in China and Korea, ALB is a generalist in Asia, attacking deciduous tree species, primarily *Acer*, *Populus*, *Salix*, and *Ulmus* (Wickham *et al.*, 2012; Wu *et al.*, 2014). Since its initial discovery outside its native range in New York State, USA, in 1996 (Haack *et al.*, 1996), additional ALB infestations have been reported in other countries, including Austria (2001), Canada (2003), France (2003), Germany (2004), Italy (2007), Belgium (2008), the Netherlands (2010), Switzerland (2011), the UK (2012), Finland (2015), and Montenegro (2015) (Javal *et al.*, 2019). In North America and Europe, ALB have caused damage to multiple tree species in the genera *Acer*, *Ulmus*, *Salix*, *Aesculus*, *Populus*, and *Fraxinus* that ultimately can cause tree death (Haack *et al.*, 2010). In 2020, an outbreak of ALB occurred in Japan that caused substantial damage to *Cercidiphyllum japonicum*, *Ulmus parvifolia*, *Aesculus turbinate*, and *Salix* spp. (Sunamura *et al.*, 2022; Yasui *et al.*, 2023).

Citrus longhorn beetle is a destructive native pest in China, Japan, and the Korean peninsula (Haack *et al.*, 2010). It has a broad host range with more than 100 plant species (in 19 families) and poses a threat to various tree species such as *Acer*, *Betula*, *Corylus*, *Carpinus*, and *Alnus* as well as economically important trees such as *Citrus reticulata*, *Malus pumila*, *Pyrus* sp., *Salix* sp., *Melia azedarach*, and *Casuarina equisetifolia* (Liu, 2013; Ge *et al.*, 2014). CLB was first detected outside its native range in the Netherlands in 1980 (Haack *et al.*, 2010), with the first established population detected in 2000 in northern Italy (Colombo & Limonta, 2001).

Although ALB is widely distributed in China, including 24 provinces (Wang, 2004), it is less frequently found in Anhui, Jiangxi, Hubei, Hunan, Shanghai, Guangdong, Guangxi, Yunnan, Guizhou, and other southern provinces of China (Liu, 2016). CLB is a serious pest in citrus orchards and shelterbelts of *C. equisetifolia* in southern China. In the areas and provinces north of Henan, although there are records of distribution of CLB, there are almost no reports of damage caused by CLB (Wei *et al.*, 2011). ALB and CLB have similar ecological and life histories, including diet and feeding behaviors, similar male pheromone components, and they also share multiple hosts in China (Hansen *et al.*, 2015; Javal *et al.*, 2018; Wang, 2019; Wang *et al.*, 2023). ALB and CLB larvae are difficult to distinguish when they first hatch from eggs, but mature larvae of the 2 species can be distinguished by the distinct shape and size of their pigmented pronotal shields (Haack *et al.*, 2010). Visual loop-mediated isothermal amplification assays have been used distinguish the larvae, frass, and adults of ALB and CLB where they are sympatric, ALB samples are lighter than CLB samples in reaction tubes once the hydroxy naphthol blue dye is added (Rizzo *et al.*, 2020). The major distinguishing morphological characters for adults are that CLB has 20−40 small tubercles on the basal region of each elytron, whereas ALB does not (Haack *et al.*, 2010).

With the continued spread of ALB and CLB, the 2 species have become sympatric in many parts of China, such as Hubei, Zhejiang, and Guizhou provinces (Ren, 2019; Wu *et al.*, 2022; Tang *et al.*, 2023). Recently, we observed ALB and CLB infesting the same *Salix aureopendula* trees, causing extensive damage in coastal shelter belt forests in Cixi, Zhejiang province. This raises the possibility that these 2 species could hybridize. We found 98 longhorned beetles on these trees which we assigned to *Anoplophora* based on their morphological features, but their specific species was not clear (Fig. 1A). Although these specimens were morphologically similar to pure ALB (Fig. 1B) and pure CLB (Fig. 1C) in some respects, there were still several important differences with regards to the pure species. We observed several bumps on the base of the elytra (Fig. 2A), which was inconsistent with ALB that has a smooth elytral base (Fig. 2B), yet more similar to CLB that has numerous short tubercles on the elytral base (Fig. 2C). Chen (1989) also found that some ALB had tuberculous on the shoulder of the elytra. In his discussion, he also believed that ALB with tubercules and ALB without tubercules might be 2 different species. Due to few specimens being observed, Chen (1989) concluded that there was insufficient evidence for describing a new species, and further study was needed. Moreover, over from 1998 to 2013, 22 *Anoplophora* sp. (remained unclassified to species) from China were very likely to have been ALB which were intercepted at the US border (Eyre & Haack, 2017). Given

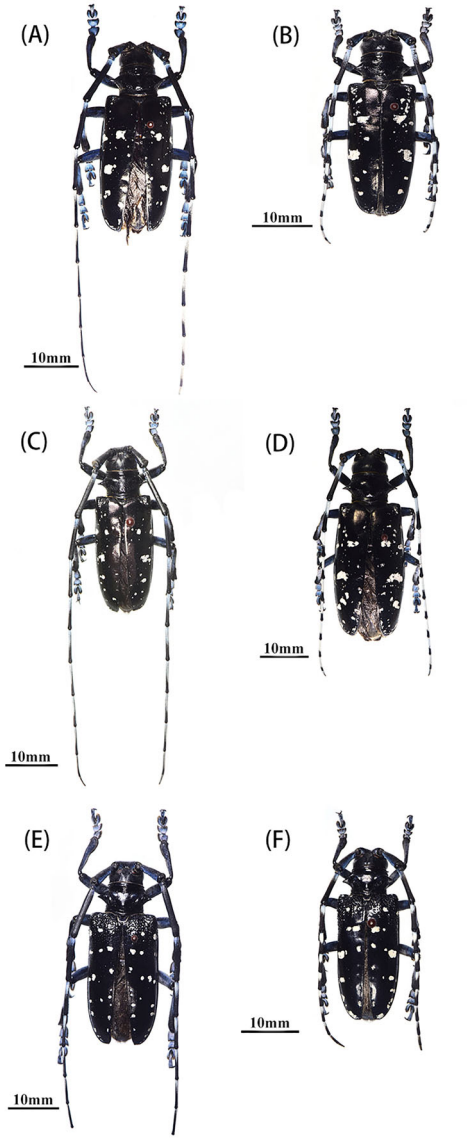

Fig. 1 The uncertain longhorn beetle, ALB and CLB morphology. (A) Adult male of uncertain longhorn beetle; (B) adult female of uncertain longhorn beetle; (C) adult male ALB; (D) adult female ALB; (E) adult male CLB; (F) adult female CLB. Scale bar = 10 mm. ALB, Asian longhorn beetle; CLB, citrus longhorn beetle.

that our uncertain beetle specimens, found on *S. aureopendula*, shared the same habitat with ALB and CLB and had similar morphology to both *Anoplophora* species, we hypothesized that these beetles could be ALB × CLB hybrids.

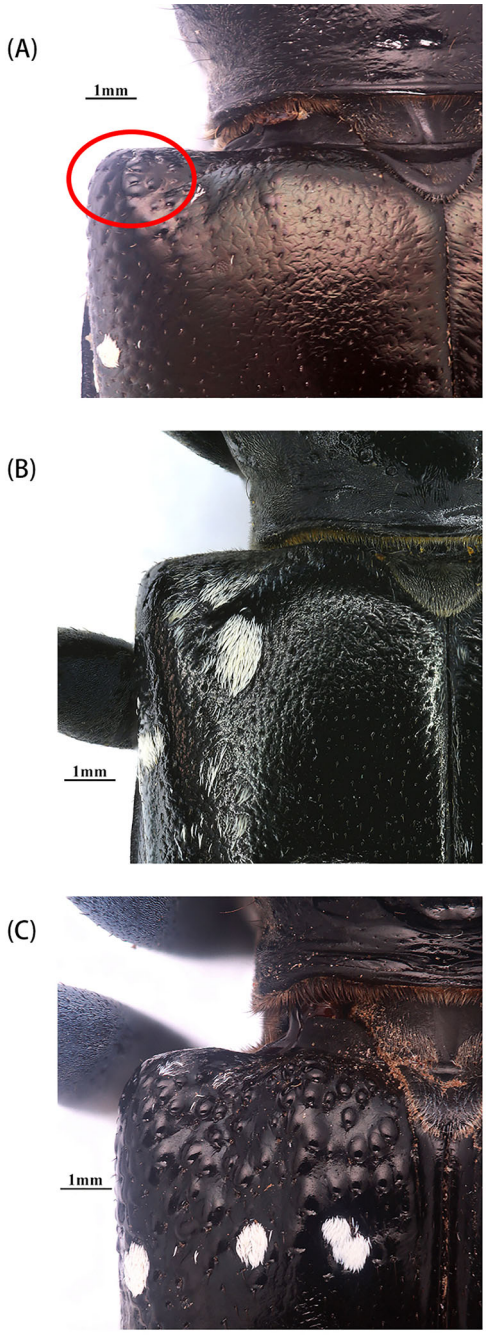

Fig. 2 Differences of the basal portion of the elytra. (A) Uncertain longhorn beetle: several bumps of the basal portion of the elytra; (B) ALB: smooth surface of the basal portion of the elytra; (C) CLB: grainy surface, due to tubercles of the basal portion of the elytra. Scale bar = 1 mm. ALB, Asian longhorn beetle; CLB, citrus longhorn beetle.

As ALB and CLB are both targets for eradication, species identification is critical to triggering management protocols. Moreover, if hybridization does occur in invasive alien species, it could lead to introgression of genes from one species to another and transfer of traits (such as broadened host range, temperature tolerances, etc.) between the 2 species, or result in hybrid vigor or traits that would be even more problematic for pest management. For example, hybrids of cotton bullworm (*Helicoverpa armigera*) and corn earworm (*Helicoverpa zea*) show decreased susceptibility to pesticides and the hybrids have increased host ranges compared with the parent species (Anderson *et al.*, 2018). Hybridization can also affect temperature tolerance (Elkinton *et al.*, 2010). In North America, winter moth (*Operopthera brumata*) is an invasive pest that is known to hybridize with native spanworm (*Operophtera bruceata*). Although spanworm can persist in areas with colder winters than the winter moth, the occurrence of hybrids may affect the future spread of winter moth and success of biological control (Elkinton *et al.*, 2010; Havill *et al.*, 2016; Andersen *et al.*, 2019). Compared with CLB larvae, ALB larvae have higher survival rates at 5 °C (Keena & Richards, 2022). ALB larvae have higher cold resistance and elevated breathing rates, so are better at maintaining body temperature in cold conditions (Feng *et al.*, 2016; Javal *et al.*, 2018). On the other hand, CLB have strong heat resistance and can survive and develop at higher temperatures than ALB (Keena & Richards, 2022). If hybridization does occur, mingling of these traits may allow hybrids to invade areas not currently at risk for either ALB or CLB. Wang and Keena (2021) conducted ALB and CLB species reciprocal crossing experiments in the laboratory, they found CLB from China did not cross successfully with ALB, but *Anoplophora malasiaca* (Japanese citrus longhorn beetle; JCLB) males crossed successfully with ALB females to produce eggs. JCLB is from Japan and has recently been synonymized with CLB. Since gomadalactones are essential contact pheromone components that induce JCLB males mating behavior and ALB females have gomadalactone derivatives JCLB males may recognize ALB females as potential mates (Yasui *et al.*, 2023). However, Sunamura *et al.* (2022) found that mating between Japanese field-collected ALB and JCLB was unsuccessful. None the less, if JCLB is able to hybridize with ALB the risk of such hybridization between ALB and CLB may be possible and could impact management programs. Also, in newly invaded areas species that are isolated in native habitats can be brought together and hybridization can occur that can alter the invasion.

Using molecular biology to reveal the genetic relationship between putative hybrids and ALB/CLB populations is a vital content of this study. Genotyping-by-sequencing (GBS) is a promising technology that offers great potential for screening large uncharacterized gene pools of non-model specimens with few genomic resources. GBS was specifically developed for the Illumina sequencing technology and identifies single nucleotide polymorphisms (SNP) on the basis of a reduced representation of the overall genome (Elshire *et al.*, 2011). Compared with other complexity reduction methods, such as reduced representation libraries and restriction-site-associated DNA sequencing, GBS is a relatively easy and quick method for generating a larger amount of SNP data (Davey *et al.*, 2011; Sonah *et al.*, 2013). It has a simple protocol for the generation of genotyping libraries without a specific gel-based size-selection step, avoids the use of divergent Y-adaptors, and is amenable to parallelization using either manual or automated liquid handling approaches (Velmurugan *et al.*, 2016). GBS reduces genome complexity by employing appropriately chosen restriction enzymes, to avoid targeting uninformative repetitive regions of genomes during the sequencing process (Elshire *et al.*, 2011). GBS has already been successfully applied to crops, such as maize, rice, soybean, potato, barley, and wheat, to resolve phylogenetic relationships and assess genetic diversity (Poland *et al.*, 2012; Uitdewilligen *et al.*, 2013; Escudero *et al.*, 2014; Arbelaez *et al.*, 2015; Torkamaneh *et al.*, 2016; Su *et al.*, 2017; Manching *et al.*, 2017; Alipour *et al.*, 2019). However, there are very few GBS studies for insects (Veale *et al.*, 2018).

Our objective was to determine if hybridization is occurring between ALB and CLB. We employed GBS to identify genome-wide SNP to assess the genetic profiles of ALB and CLB populations and to uncover suspected species admixture. We also investigated the ability of ALB and CLB individuals to mate and produce hybrid offspring. Finally, we merged the results obtained from our morphological examinations, attempted hybridizations, and performed molecular screening to classify the putative hybrid beetles.

Materials and methods

Sample collection site

We collected all beetles from Cixi, which is in the east of Zhejiang province, China in 2017, 2018, 2020, and 2021. In 2020 and 2021, we found another overlapping area of ALB and CLB in Bengbu, Anhui province, and we also collected 2 species and putative hybrid individuals for hybridization experiments. Cixi belongs to the north subtropical monsoon climate zone, therefore, its climate

Fig. 3 Information of sample collection site. (A) The habitat of beetles in Cixi, Zhejiang province, China; (B) the habitat of beetles in Bengbu, Anhui province, China.

is mild and humid all year around (Liu, 2013). The site where we collected the beetles is within the Cixi coastal protection forests (30°20′ N, 121°18′ E) with *C. equisetifolia*, *S. aureo-pendula*, *Nerium indicum*, *Sabina chinensis*, *Hibiscus hamabo*, *Fraxinus chinensis*, *M. azedarach*, *Keolreuteria bipinnata*, and *Agave sisalana* as the main tree species (Fig. 3A). The Bengbu collection site is located in a willow forest (32°57′ N, 117°22′ E) along the Huaihe River (Fig. 3B).

Beetle collection for molecular analyses

We collected 96 beetles (after morphological identification using a stereoscopic microscope, 32 specimens each from ALB, CLB, and putative hybrids were obtained) in 2017 and 2018 and placed them into ethanol alive and subsequently stored them at −20 °C. We extracted genomic DNA (gDNA) from the adult leg or thoracic tissue using the DNeasy® Blood & Tissue Kit (250) (Qiagen, Hilden, Germany) according to manufacturer's instructions. We assayed concentration and purity of extracted gDNA by spectrophotometer. The A_{260}/A_{280} value ranges we obtained were in the range from 1.8 to 2.0 which are of sufficient quality for use. We used a total of 200 ng per sample in the genotyping experiments (see below).

GBS procedure

Our general protocol consisted of the following main steps: (a) restriction enzyme digestion, (b) adapter ligation, and (c) polymerase chain reaction (PCR) amplification. Our GBS library was a multiplex library consisting of 46 samples. We prepared GBS libraries at Laval University's Genomic Analysis Platform (Institute of Integrative Biology and Systems, Quebec City, Canada) for the Ion Torrent sequencing technology, using the same methods as previously described for analyzing the barley genome (Abed *et al.*, 2019), with the important exception of using a 3-restriction enzyme system, that is, *Nsi*I, *Pst*I (rare cutters), and *Msp*I (common cutter) with the purpose of increasing the number of assayed SNP. For each

sample, we used a total of 200 ng of gDNA, digested using 5 Units of *Pst*I HiFi, *Nsi*I, and 5 Units of *Msp*I (NEB, Ipswich, MA, USA) in a 30-μL reaction mixture, incubated at 37 °C for 2 h, and then held at 8 °C.

We performed the ligation step in a 50-μL reaction volume containing 5 μL of 10 × T4 DNA ligase reaction buffer, 400 Units of T4 DNA ligase, and 5 μL from the corresponding well of the working adapter (20 μL Barcoded Adapters at 0.1 μmol/L, 10 μL Common Adapter at 10 μmol/L, 10 μL 10× Annealing buffer and 60 μL H_2O). We incubated the ligation mixture at 22 °C for 2 h, followed by inactivation of the enzyme at 65 °C for 20 min, and holding at 8 °C when completed. We then purified about 750 μL of the pooled DNA using a QIAquick PCR purification kit (Qiagen, Germany) as per the manufacturer's guidelines and eluted into a total volume of 30 μL using EB buffer. In the amplification step, we used 5 μL of eluted DNA in each PCR, 10 μL of 5 × Q5 buffer, 10 μL of Q5 enhancer solution, 1 μL of 10 mmol/L dNTP, 0.3 μL of 10 μmol/L Iron Forward PCR Primer, 0.3 μL of 10 μmol/L Iron Reverse PCR Primer, 0.5 μL of Q5 High-fidelity polymerase, and 22.9 μL of H_2O. Our PCR amplification was performed using an initial denaturation step at 75 °C for 5 min, followed by 5 cycles at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s; 7 cycles at 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s; and a final elongation step at 72 °C for 5 min, and subsequently held at 4 °C (Abed *et al.*, 2019; Cui *et al.*, 2022).

GBS data analysis

After checking the quality of the raw sequence reads with FastQC (Andrews, 2010), we indexed the reads for the alignment, based on the initial barcode sequences, with the corresponding sample names. We ran the pipeline Fast-GBS (Torkamaneh *et al.*, 2017) for subsequent SNP calling using the published ALB genome (McKenna *et al.*, 2016) in the following way: first, we aligned the clipped reads to the reference genome by using Burrows-Wheeler-Alignment (Li & Durbin, 2010). Next, we prepared the pileup file using all the BAM files with samtools mpileup (Li *et al.*, 2009) to perform genetic variant-calling using PLATYPUS (Rimmer *et al.*, 2014).

We used VCFtools (Danecek *et al.*, 2011) to filter for biallelic SNP and low-frequency alleles, and with the amount of missing data set to 5% per locus. We set minimum allele count to 3, to remove singletons and low frequency alleles (–mac 3). We generated the final VCF file and performed principal components analysis (PCA) using adegenet (Jombart, 2008) in R (R Core Team, 2020).

Morphological analyses

We made morphological analyses with all species. We measured some principal distances (body length and width) with vernier calipers and took photos with a Nikon NS2000 camera. We used Leica S4E stereoscopic microscope to observe the base of elytra, which was the most important distinguishing feature of ALB, CLB, and the putative hybrid. We used Adobe Photoshop CC 2018 for post-production editing.

Hybridization experiments between ALB, CLB, and putative hybrids

To further examine the relationships among these groups of beetle species, we performed hybridization experiments in the Key Laboratory of Beijing for the Control of Forest Pests, College of Forestry at the Beijing Forestry University (China) in 2017, 2018, 2020, and 2021, respectively. We collected 141 ALB male, 163 ALB female, 98 CLB male, 110 CLB female, 60 putative hybrid male, and 71 putative hybrid female adults from the same site in Cixi, Zhejiang province and Bengbu, Anhui province. We divided the beetles into 9 groups and fed each of them fresh willow branches (approximately 0.5 cm diameter × 20 cm length): (1) CLB♂ × ALB♀, (2) ALB♂ × CLB♀, (3) putative hybrid♂ × ALB♀, (4) ALB♂ × putative hybrid♀, (5) putative hybrid♂ × putative hybrid♀, (6) putative hybrid♂ × CLB♀, (7) CLB♂ × putative hybrid♀, (8) ALB♂ × ALB♀, (9) CLB♂ × CLB♀. We used 8 feeding boxes (with dimensions of 28 cm length × 21 cm width × 17 cm height) with 3 pairs each except for the CLB♂ × CLB♀ group (using only 6 feeding boxes with 3 pairs). When a male or female died, we immediately replaced it with an active adult to maintain 3 pairs in each box. For each of these groups, if we observed a male and a female copulating for close to 3 min, it was considered a successful mating (Keena & Sánchez, 2018), subsequently, we moved the female to a net cage (with dimensions of 80 cm length × 60 cm width × 120 cm height) and provided her with willow trunk sections (80 cm length × 8 cm diameter) as an oviposition substrate and fresh twigs for food. We sealed both ends of the oviposition bolts with wax to preserve moisture content. We held the trunk sections at 25 °C and under 14 h : 10 h light : dark photoperiod to allow the eggs to hatch and larvae to develop; then we dissected the bolts. Because the beetles were collected from the same field location, they could have mated before the study, so we used the genotyped by alleles to test on any resulting progeny to

look for the presence of genes from both species in the offspring, which would confirm that hybridization had occurred.

Results

Morphological analysis in the putative hybrid

We confirmed by morphological examination that the putative hybrids belonged to the genus *Anoplophora*. The ALB, CLB, and putative hybrid beetles were black with about 20 irregular, white spots on the elytra. The base of the elytra of CLB had numerous tubercles whereas that of ALB was smooth. The putative hybrids had several less obvious bumps at the base of its elytra, which were different from the other described species in *Anoplophora* (Chen *et al.*, 1959; Lingafelter & Hoebeke, 2002).

The genetic relationship between putative hybrid, ALB, and CLB

We analyzed GBS sequences for 35 samples (7 samples were eliminated because of poor data quality and 4 because of bacterial contamination, detected by BLAST search), which resulted in 25 869 350 raw de-multiplexed reads with an average of 663 317 reads per sample. We successfully mapped between 0.13% and 96.65% of the reads to the reference genome of *A. glabripennis* (McKenna *et al.*, 2016). After removing SNP that were present in repetitive regions of the genome, we detected 876 636 SNP. Removal of SNP and samples above 5% missing SNP data resulted in a final data set of 163 SNP from 30 samples (14 ALB, 8 CLB, and 8 putative hybrids), which were subsequently used to perform PCA (Fig. 4).

To obtain an overview of genetic relationships among ALB, CLB, and putative hybrid, we performed a PCA using the GBS data. The first 3 axes accounted for 79.25% of the total variance, with axes 1, 2, and 3 representing 71.51%, 4.25%, and 3.48%, respectively. (Fig. 4). The PCA first axis clearly separated 2 groups, the first encompassing all the ALB individuals and putative hybrids and a second, encompassing the CLB individuals. Axes 2 and 3 showed separation of the CLB individuals, whereas the ALB and putative hybrid individuals tightly clustered together on all 3 axes.

Hybridization tests in ALB, CLB, and putative hybrids

We performed crossing experiments to assess the ability of ALB, CLB, and putative hybrids to hybridize. All 9 groups showed mating behavior (Fig. 5A−I). After successful mating between CLB♂ × ALB♀, ALB♂ × CLB♀, putative hybrid♂ × CLB♀, and CLB♂ × putative hybrid♀ groups, the females laid eggs singly on the trunks. However, there were no larvae or feeding traces found when the oviposition bolts were split, so the eggs that were laid did not hatch. In the putative hybrid♂ × ALB♀ and ALB♂ × putative hybrid♀ groups, they had 28 eggs hatched, in the next year, 16 adults emerged. For the within-species mating groups (putative hybrid♂ × putative hybrid♀, ALB♂ × ALB♀, and CLB♂ × CLB♀), they had offspring successfully (Table 1).

In our reciprocal crossing experiment, percentage of successful copulation of both within species matings and matings between ALB and the putative hybrid individuals were above, at 65%. The percentage copulation of putative hybrids with each other and with CLB was 62% or less. Percentage copulation between species was higher when the male was ALB than when the male was CLB. However, no eggs hatched in ALB × CLB and putative hybrid × CLB treatments. Percentage of eggs that hatched in the ALB × putative hybrid treatments was about 56%. Percentage of individual offspring that eclosed as adults in the ALB × putative hybrid treatments was above 50% and above 60% for pure putative hybrid, ALB, and CLB pairings (Table 1).

Discussion

No evidence of hybridization in sympatric populations of ALB and CLB

Based on morphological characteristics (Lingafelter & Hoebeke, 2002), we found a putative hybrids belonging to genus *Anoplophora* but they were not a clear match to known species. The putative hybrids, however, did genetically cluster with individuals from the ALB population and separated from individuals of the CLB population. This result indicates that the uncertain samples were most likely ALB, and not F_1 hybrids between ALB and CLB. Our crossing experiment further supports the rejection of the hybrid hypothesis and confirms that ALB and CLB are reproductively isolated in China. There appears to be some post-copulatory reproductive isolation between ALB and CLB because they copulated and oviposited, but no hatch was observed. Wang and Keena (2021) also confirmed that ALB collected from China could not successfully cross with CLB from China, which is consistent with our results. This provides customs and pest management evidence to classify the morphological variant that

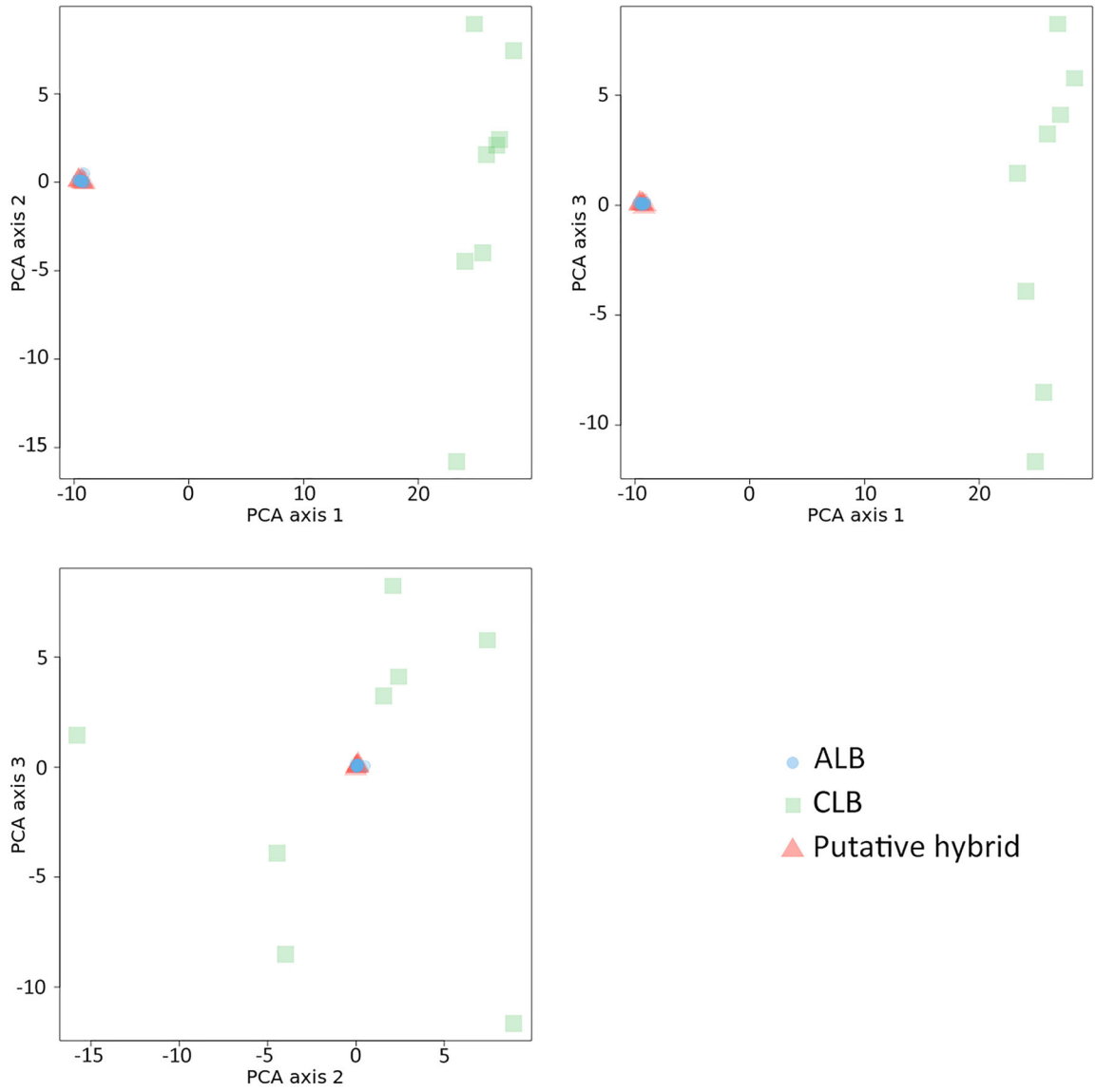

Fig. 4 PCA among specimens for GBS-SNP data. 2-dimensional scatter plots are shown of the PCA of GBS-SNP data of 14 ALB, 8 CLB and 8 putative hybrids. The first components from PCA, PC1, PC2, and PC3, define the *x* and *y* axes of the 2-dimensional plot, so the distance between any 2 points indicates the variances between them. ALB, Asian longhorn beetle; CLB, citrus longhorn beetle; GBS, genotyping-by-sequencing; PCA, principal component analyses.

have bumps on the anterior portion of the elytra as ALB. Additional work should also be done to determine if this morphological variant of ALB is more heat adapted than the smooth elytral variant of ALB and so should be distinguished when intercepted.

In this study copulation occurred between CLB and ALB, females produced eggs after mating, but the eggs were sterile. This is consistent with the findings of Wang and Keena (2021). The previous findings that JCLB will mate and lay eggs in the laboratory (Wang & Keena, 2021) but not when field collected (Sunamura *et al.*, 2022) may have to do with the hosts the individuals came from. Some host compounds are acquired from the *Anoplophora* beetles' elytra as attractants or repellents, and feeding of hosts may alter their sexual attractiveness. If beetles feed on different hosts, they may not know each other (Yasui *et al.*, 2007; Fujiwara-Tsujii *et al.*, 2013; Yasui & Fujiwara-Tsujii, 2016; Sunamura *et al.*, 2022). However, all of the ALB, CLB, and putative hybrid individuals in this study were collected from the

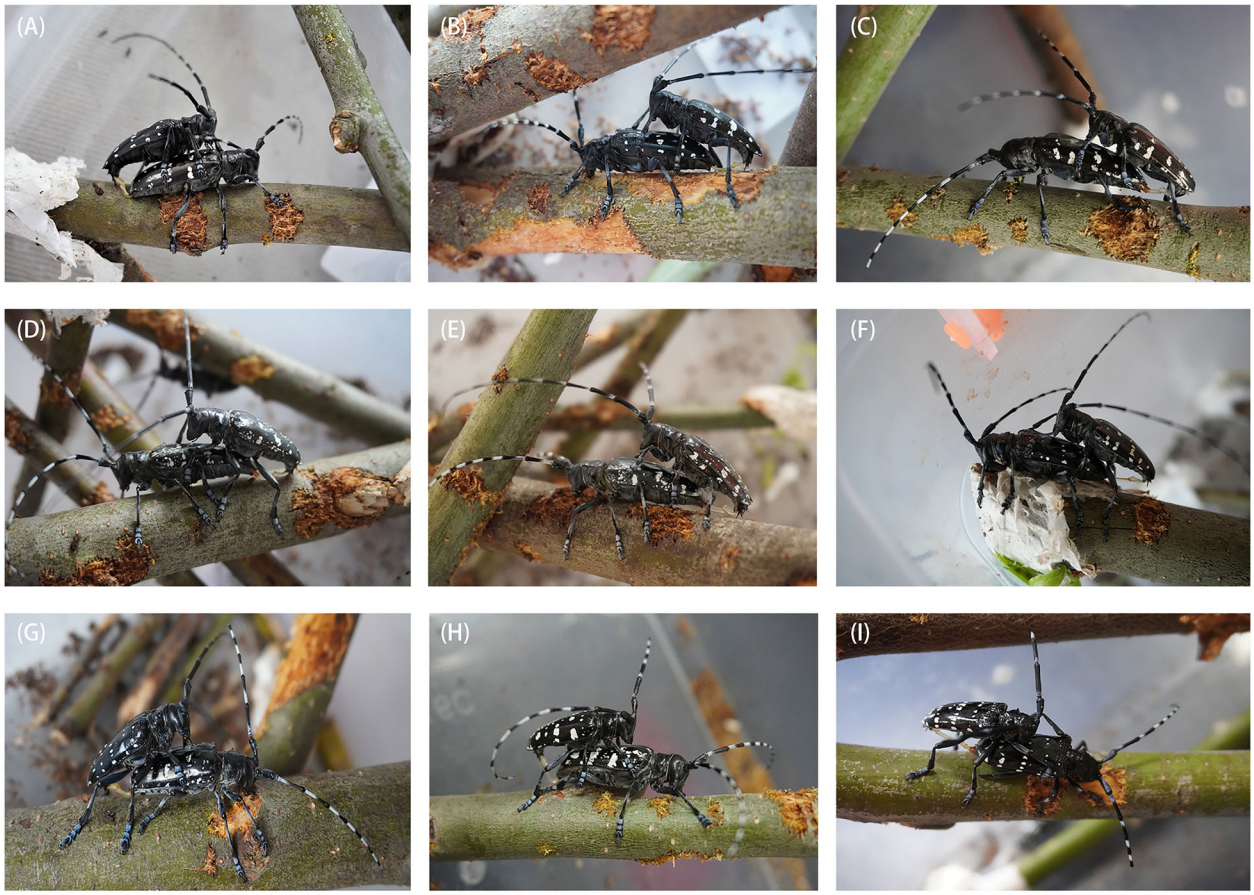

Fig. 5 The crossing experiment of ALB, CLB, and putative hybrid. (A) Male CLB copulating with a female ALB; (B) male ALB copulating with a female CLB; (C) male putative hybrid copulating with a female ALB; (D) male ALB copulating with a female putative hybrid; (E) male putative hybrid copulating with a female putative hybrid; (F) male putative hybrid copulating with a female CLB; (G) male CLB copulating with a female putative hybrid; (H) male ALB copulating with a female ALB; (I) male CLB copulating with a female CLB. ALB, Asian longhorn beetle; CLB, citrus longhorn beetle.

same host in the same collected sites, which might increase the attractiveness and chance of ALB and CLB mating with each other even if no viable offspring result. If the 2 species are sympathetic on the same hosts the heterospecific matings could result in some reproductive interference since males mate guard after completing copulations (Keena & Sánchez, 2018) and so reduce the number of offspring produced in a year because heterospecific matings result in females laying infertile eggs. Also, since the heterospecific matings that result in the most copulations involve female CLB and male ALB the impact on fertility would be primarily on CLB. However, care should be taken in extrapolating laboratory mating results to the field because there could be differences in where or when each species mates on the host that could prevent these interspecific matings in nature.

There are many pre- and post-mating barriers that could be involved in the heterospecific matings and the lack of viable offspring that were observed in our crosses. Mating recognition seemed to play a role in the reduced copulations seen between CLB males and ALB females but not in the ALB male by CLB female matings. The copulations between ALB males and CLB females were normal in length and, based on the Wang and Keena (2021) article, were normal in behavior. So, the mating barrier must involve a lack of sperm transfer or some genetic incompatibility that results in either no fertilization or aborted development. Further work would be needed to determine the cause. Although our work supports the rejection of the hybrid hypothesis and confirms that ALB and CLB are reproductively isolated. Hybridization can occur in phylogenetically close species, so it is vital to consider the possibility of

Table 1 Observed copulations between Asian longhorn beetle (ALB), citrus longhorn beetle (CLB), and putative hybrid adults conducted in 2017, 2018, 2020, and 2021.

Cross*	Numbers of pair-mating attempts	Numbers of pairs copulating (% of matings)	Replicates of pairs	Numbers of eggs laid	Numbers of eggs hatched	Numbers of offspring
AA	103	71 (68.9)	37	80	48	30
CC	30	26 (86.7)	12	32	18	11
CA	43	13 (30.2)	7	28	0	0
AC	56	25 (44.6)	12	33	0	0
PP	21	13 (61.9)	4	6	5	3
PA	42	30 (71.4)	9	17	10	6
AP	45	30 (66.7)	20	32	18	10
PC	17	10 (58.8)	5	13	0	0
CP	15	8 (53.3)	5	6	0	0

Resulting oviposition by females and observed hatch.

*AA, ALB male by ALB female; CC, CLB male by CLB female; CA, CLB male by ALB female; AC, ALB male by CLB female; PP, putative hybrid male by putative hybrid female; PA, putative hybrid male by ALB female; AP, ALB male by putative hybrid female; PC, putative hybrid male by CLB female; and CP, CLB male by putative hybrid female.

hybridization, especially the hybridization potential of invasive alien species. This is particularly concerning when invasion of currently isolated species or subspecies into the same areas may promote potential interspecific or intraspecific hybridization.

ALB and CLB live in overlapping area in China

Based on our analyses, the putative hybrids have SNP profiles that place them within the ALB; therefore, the difference in elytra in the punitive hybrids can be regarded as morphological variation within the ALB population. In China, ALB is mainly located in the north, while CLB is distributed in southern China. The ALB population in this area likely recently invaded this southern location and the very limited genetic variation we observed within the population is indicative of either a small founding population or strong selection for viable haplotypes in this part of the expanded range. In addition to Cixi, Zhejiang province, we also found that ALB and CLB overlapped and maintained sympatric populations in the Bengbu region, Anhui province. The 2 cities are both in southern China, suggesting that the ALB population is expanding southward in China. In addition, putative hybrids were also found when ALB and CLB occupied the same host in Bengbu. It is possible that the punitive hybrids with bumpy bases of the elytra of some ALB adults might be associated with the haplotypes that were able to successfully colonize southern regions, which will be addressed in a future study.

We demonstrated that the putative hybrid is morphologically different from the previous descriptions published for ALB and CLB populations. Given our findings, keys to distinguish ALB form CLB adults should be updated and the potential for ALB to have bumps on the anterior portion of the elytra should be included. This is particularly important for border inspections so that intercepted *Anoplophora* are classified as the correct species because their ecology (part of the tree they use and thermal responses) and host range are somewhat different. The differences between the species require the employment of slightly different management or eradication techniques.

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Disclosure

The authors declare no competing interest.

Data availability statement

Raw sequence data are available at the Sequence Read Archive (SRA) with the BioProject ID PRJNA902116.

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