The genetic differentiation of a cricket (Velarifictorus micado) with two modes of life cycle in East Asia after the middle Pleistocene

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Abstract

Cricket Velarifictorus micado is widely distributed in East Asia and colonized North America since 1959. It has been reported that they had two modes of life cycle and distributed in southern and northern Asia respectively. Aimed to investigate the biogeographic boundary between the two groups and the causes of differentiation, mitochondrial fragments including COI and CytB were used for phylogenetic analysis, time estimation and demographic analysis. The results showed that, (i) Haplotype network indicated that V. micado has diversified to three lineages based on COI. Individuals with egg diapause lived in northern Asia, whereas those with egg and nymph diapause lived in southern Asia, and the populations colonized North America belongs to the egg diapause group from both North and South Asia. (ii) The molecular chronograms indicated that the first diversification between individuals in the northern and southern Asia occurred during ~0.79 Ma BP in the Middle Pleistocene Transition. The second event occurred in southern individuals during ~0.49 Ma BP, when the glaciers developed in Yulong mountain (Yunnan province). (iii) V. micado has diversified to two main clades based on CytB. The individuals distributed in southern China have not been differentiated. Haplotype network indicated that the egg diapause lived in southern China most possibly originated from Yunnan, where lies at the foot of the Tibetan plateau. Our study suggested that the twice divergence of V. micado co-occurred with tendency of cooling climatic in Asia after the Mid-Pleistocene.

Questions the manuscript attempts to address

Cricket *Velarifictorus micado* was found that it had two modes of life cycle. However, there are still three questions unsolved.

1. Their distribution in most regions of East Asia

Method: Wide sampling

2. Gene markers that were diversified between two groups

Method: Amplify mitochondrial fragments

3. Divergence time and the possible reasons why they were differentiated.

Methods:

(i). The time estimation based on COI to reveal which important events result in the divergence of the *V. micado*. (ii). The evolutionary difference between two genes to speculate the origin of the ancient residents.

1. Introduction

The extant species in the planet have evolved for hundreds of millions of years. Evidence of climatic and environmental changes could be found through the adaptation of species and the historical development of the population within the species (Emerson *et al* ., 2001). Lucky individuals survived when the environment had changed dramatically. They migrated to the new habitat, and they settled, adapted and reproduced

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there. In the process of adaptation, new behavioral, physiological and genetic structure occurred (Avise *et al* ., 1998). Speciation duration is about 1-2 Myr, but morphological, physiological and behavioral changes may produce subspecies and species at almost any time (Hewitt *et al.* , 2000). The current distribution and the differentiation of species are compatible with the local geographical and climatic environment (Udvardy, 1981). Insects have the relatively rapid divergence rate (Orr *et al* ., 1998). *Velarifictorus micado* , selected in our study, is widely distributed in Asia, included China, Russia, Japan, Korea, Cambodia, Vietnam, Indonesia and nearby islands, covering the Palearctic and Oriental realm, and the distribution range of this cricket has gradually expanded since the introduction of *V. micado* to the United States in 1959 (Alexander and Walker, 1962; Bowles, 2018). *Velarifictorus micado* (Saussure, 1877) has been found to have two modes of life cycle. In a population that diapause as eggs, adults sing from August to October, and their eggs hatch after getting through the winter. In the other population that diapause as nymphs, adults sing in May-July, hatch quickly after mating and get through the winter as nymphs. Different adaptation types of *V. micado* lead to differentiation of reproductive modes and stimulate speciation among populations with different life cycle patterns (He and Takeda, 2013). Aimed to find out which gene markers can distinguish the two groups and the biogeographic boundary between them, figure out why *V. micado* differentiate into two modes of life cycle, mitochondrial fragments were extracted from the large sampling specimens and used in phylogenetic analysis, time estimation and demographic analysis.

2. Materials and methods

2.1.Sample collection and genomic DNA extraction

A total of 72 *Velarifictorus micado* populations contain 346 individuals were collected, 57 populations from China, one from Korea, eight from Japan, one from Vietnam, one from Cambodia and four from America (Fig. 1). All materials were presented in 100% ethanol and stored in a freezer at -20°C, genomic DNA was extracted from the leg of the cricket using AxyPrep^{TM} Multisource Genomic DNA Miniprep kit.

2.2.PCR amplification and sequencing

Universal primers for cytochrome c oxidase unit 1 (COI), and cytochrome b (CytB) genes were designed in the present study (Table 1). The PCR procedure for the three genes included an initial denaturation at 94degC for 4 min, followed by 35 cycles of 30s at 94degC, 30 s at 45degC and 30 s at 72degC, ending with a final extension at 72degC for 5 min. Sequencing was performed on 3730*xl* DNA Analyzer, sequencing was proofread and aligned in ATGC Ver 7.0.2 (Genetyx Corporation, Tokyo, Japan).

2.3.Genetic analysis

2.3.1. Genetic polymorphism

Base substitution saturation test was performed in DAMBE (Xia and Xie, 2001) to make sure ISS < ISS.cSym. Different haplotype, Haplotype diversity (Hd) and nucleotide diversity (π) were calculated in DNASP 5.10.01 (Librado and Rozas, 2009), A median joining (MJ) haplotype network was constructed in Popart (Leigh and Bryant, 2015), each population has its own color. Genetic differentiation among different populations and sets that defined groups was calculated in ARLEQUIN 3.5.2.2 (Excoffier and Lischer, 2010). Analyses of PairwiseF_{ST} were performed in two ways. The first comprising data sets divided by the different modes of life cycle mainly and the second sets divided by the main zoogeographic regions (Zhang, 1983) comprising NEC (northeastern China), NC (northern China), SWC (southwestern China), CC (central China), SC (southern China), VK (Vietnam and Cambodia), KJ (Korea and Japan) and USA (The United States) data (Table 2).

2.3.2.Historical demographic changes

Haplotype diversity, nucleotide diversity and neutrality test were used to speculate historical demographic change. Neutrality tests were implemented in ARLEQUIN 3.5.2 (Excoffier and Lischer, 2010), including Tajima' D (Tajiama, 1989) and Fu' Fs (Fu, 1997).

2.3.3.Divergence time estimation

Estimating divergence times were based on mitochondrial markers of COI in BEAST 2.5.0 (Bouckaert *et al* ., 2014). A set of 15 specimens comprising 6 *Velarifictorus* specimens and 9 ancient haplotypes of *V. micado* was employed for the estimation of divergence time (Table 3). The substitution models were selected under JModeltest v.2 (Darriba *et al* ., 2012). The Bayesian information criterion (BIC) was preferentially used to compare substitution models, and HKY+I+G model of sequence evolution was selected. An uncorrelated lognormal relaxed clock was applied with 1.7% per site per lineage per million years for COI (Pons and Vogler, 2005; Kiyoshi and Sota, 2006; Sharipo,*et al* ., 2006; Papadopoulou *et al* ., 2010; Allegrucci*et al* ., 2011; Kaya *et al* ., 2016). Divergence time was estimated using a Yule model. After running the chains for 500 million generations in BEAST, the stability on the log-likelihood curves and the split-frequencies were checked in Tracer v1.7.1. The parameter estimates with ESS > 200 were accepted. Discarding the first 20% as burn-in and summarize trees in Tree Annotator. Trees were visualized with the FigTree v1.1.2 (These software are included in the BEAST package).

3. Results

3.1.Genetic polymorphism and haplotype network

COI gene (658 bp) was successfully obtained from 346 individuals. The variable sites included 12 singleton variable sites and 33 parsimony information sites. 36 unique haplotypes were derived from 346 individuals. The distribution of different haplotypes based on COI was showed in Fig.2. The haplotype distributions were dividing three starry shapes in the network. According to the haplotype network, the geographical populations were further divided into 85 populations and this was done for further analysis of individuals from different lineages in the same collection site. 85 populations belonged to three groups, which included NE, SE and SN group. NE contained the individuals that produced with egg diapause and mainly lived in the northern regions. SE included the populations with egg diapause and mainly lived in the southern regions. SN group was consisted with individuals that produced with nymph diapause and mainly lived in the southern regions (Table 2). The location of three groups was showed in the Fig.1. There was no sharing haplotype among three groups. Hap24, Hap 2, Hap 4 and Hap 11 haplotypes were the most frequent haplotypes, characterizing 25.72%, 25.14%, 17.92%, 4.05% individuals, respectively. Hap 2 and Hap 11 were the ancestral haplotypes of NE group, and Hap11 was disjoint from Hap 2, which suggested was differentiated due to the long distance. Hap 4 and Hap 24 were the ancestral haplotypes of SE and SN groups, respectively. There were two lineages in populations of SDDY, but the haplotypes of the residents of SDDY belonged to the SE were the tips of Hap 2, thus the SE lineage may immigrate lately. In additional, the haplotypes from the United States were the tips of the gene tree, Hap 5 and Hap 8 were originated from the NE group. Populations of USAGA3 and USATN3 shared hap 5 with JPDB3, JPSH3 and JPSS3 in Japan. Hap 7 was from SE group, populations of USAMO1 and USAVA1 shared hap 7 with SDDY1, ZJTM1 and SHBS1 in China (Fig.2 and Table 2).

Pairwise F_{ST} among SE, NE and SN groups range from 0.87072 to 0.95084 (P < 0.001 Table 4), which suggested that the three groups were differentiated significantly. Isolations between SE, SN and NE group were consistent with results of AMOVA. The partitioning of total genetic variation in three clades using AMOVA indicated 93.23% diversity among groups, 2.78% within populations, and 4.00% among populations within groups (Table 5), suggesting that *V. micado* has significant genetic differentiation at the different mode of life cycle and geographical separation of northern and southern regions. According to the zoogeographic regions, 85 populations were divided into 8 regions, including NEC, NC, SWC, CC, VK, KJ and USA. The partitioning of total genetic variation in nine clades using AMOVA indicated 24.94% diversity among groups, 69.56% among populations within groups, and 5.49% within populations (Table 6). The results were consistent with the network based on COI, which meant the different modes of life cycle accounted for the most of genetic variation.

Based on the CytB gene, 579 bp fragments were successfully obtained from 162 individuals. The variable sites included 18 singleton variable sites and 31 parsimony information sites. 36 unique haplotypes were derived from 162 individuals. The distribution of different haplotypes was showed in Fig.3. All individuals were divided into two groups significantly. Hap 12 and Hap 13 were the ancestral haplotypes of northern

China (NC), and Hap 4 was the ancient haplotypes of southern China (SC). Coincidentally, all the individuals in the NC group were the members of the NE group defined by the COI, and others were in the SE and SN group. There were two lineages in SDDY and SHBS, which mainly including Hap1, Hap15 and Hap12. While Hap1 and Hap 15 were the tips of Hap 4, thus the NE lineage may be the native in SDDY and SHBS (Fig.3). This result was consisted with the assumption based on COI.

The partitioning of total genetic variation in different regions indicated 41.11% among populations within groups, 30.23% diversity among groups, and 28.66% within populations (Table 7). While the AMOVA result showed that the source of variation among groups categorized by three clades based on COI accounts 84.78% (Table 8).

3.2.Historical demographic changes

Based on COI, the negative of values of Tajima' D suggested a relative constant population size. In SHBS3, SDDY3 and SDTA3, the current population size is stable. The NE lineage may be the native in SDDY and SHBS, which is consistent to the results of haplotype network (Table 2). Based on CytB, the negative and significant values of Tajima' D indicated past population expansion in populations of GZXY (Guizhou province). Additionally, Hap 4 was the ancient haplotypes of populations of egg and nymph diapause. Individuals produced with nymph diapause in Zhejiang, Hainan and Guangxi (ZJTT, HNBS, HNJFL, HNWZ, HNCJ, HNXA, GXPM, GXSL and GXJX), whereas those were with egg and nymph diapause in Yunnan and Guizhou (YNKM and GZXY), which suggested the ancient haplotypes of SE group distributed there (Fig.3), and the positive Tajima' D value based on COI and CytB suggested there may be a colonization of SE group in Guizhou (Table 2 and 9). Thus, it is possibility that SE group was originated from Yunnan and colonize to the other regions.

3.3.Divergence time estimation

The chronogram reconstructed with BEAST was based on COI (Fig. 4). The most recent common ancestor for all *V. micado* was dated at approximately 0.79 Myr (95% HPD: 0.46-1.13Myr). The diversification time between SN and SE clades was 0.50 Myr (95% HPD: 0.25-0.71Myr).

4. Discussion

4.1 Life cycle and distribution

V. micado has two ways to get through the winter, one group prefer to nymph diapause, the other group choose to egg diapause (He and Takeda, 2013). Adults of egg diapause population were found from July to October (Shandong, Heilongjiang, Henan, Guizhou and Yunnan), and nymphs were from July to August (Shandong and Yunnan). The individuals with nymph diapause, as we found, were adults in March (Hainan), April (Guangxi), June (Hubei and Zhejiang) and July (Guizhou). The nymph were found in the spring or the later autumn, such as March (Hainan), April (Guangxi), August (Guizhou), September and October (Zhejiang).

Our results indicated that *V. micado* was transferred to America, which is consistent with this result (Bowles, 2018). *V. micado* was first found in the North America since 1959 (Alexander and Walker 1962), then they were widely distributed around eastern and southeastern America (Walker 1977; Peck *et al* . 1992). The recent research indicated the cricket had dispersed both northwards and westwards (Bowles, 2018). Our results revealed they might be immigrated from China and Japan, and belongs to the egg diapause populations. Their distribution range in the United States might continue to expanding due to their adaptation to dry and cold areas, for example, they were widely distributed in the northeastern China. Limited to sampling size, it is unknown if there are nymph populations in North America. The mitochondrial fragment COI could be easily used to check the origin of *V . micado* for prevention of invasion. The similar latitude and climate make the species orginated from North America easy to settle down to China, such as *Corythucha ciliata, Homalodisca coagulata and Anopheles quadrimaculatus* (Li *et al* ., 2007; Wang *et al* ., 2008; Zhang *et al* ., 2008) and vice versa. Similarly, *V. micado* was easy to adapt to the environment of North America. Although it has not been found that this cricket has destructive damage to human and nature, the impact

to system of the new colony is unknown. Besides, although sampling size was small, individuals in Vietnam and Cambodia might be native due to the ancient haplotype.

The wide sampling in Asia enabled us to find the distribution of two groups and compare differences between them. The egg diapause population lives in both the north and south of the Yangtze River. Based the COI, the NE group contained the populations lived in north of the Yangtze River regions as well as those in Shanghai and Zhejiang. These populations have the ancient haplotypes and have not migrated previously and recently through the values of Tajima' D. The SE group is distributed in the south of the Yangtze River regions as well as those in Hubei, Jiangsu and Shandong. The haplotypes were the tips of gene tree in Shandong and Jiangsu, maybe the SE group was introduced. However, there was ancient haplotype in Hubei. Limited to the sample size, it is uncertain whether the three populations migrated before. The SN group included the populations lived in the south of Yangtze River and HBWH (Hubei). The two individuals are the ancient haplotype in Hubei, but it is also uncertain whether there was a migration before. Based on CytB, all individuals were divided into two groups, NC (northern China) group contains the populations lived in the north of the Yangtze River regions as well as those in Shanghai and Zhejiang, in which there is ancient haplotype. SC (southern China) group contains the populations lived in the south of the Yangtze River regions as well as those in Hubei, Anhui, Shandong, in which there are both tips of gene tree, and these individuals may immigrate into the north of the Yangtze River artificially or naturally. This phenomenon with two life histories bounded by the Yangtze River could be seen between *Teleogryllus emma* and *T. occipitalis* (He *et al* ., 2017).

According to the distribution of species, there is a broad faunal transition zone in the Quaternary between the Palearctic and Oriental realm in China (Zhang, 2002) and no strict biogeographic division (Norton *et al* ., 2011). However, a map of zoogeographic regions generated by phylogenetic relationships of species shows that there are three realms containing Palearctic realm, Sino-Japanese realm and Oriental realm (Holt *et al.,* 2013). However, our results were basically consistent with an earlier suggestion of Wallace (Wallace, 1876). The Yangtze River is the biogeographic boundary between the Oriental and Palearctic. Although it is not strictly defined by the Yangtze River, Shandong, Jiangsu, Shanghai and Zhejiang regions are the intersection of groups. Those are the broad transition zone for *V. micado* . When the cool climate shift occurred in the mid-Pleistocene, the northern individuals immigrate southwards. When the glaciation in the southwestern mountain, the native residents immigrate eastwards the refuge, but some remained and scattered. The analysis is as following.

4.2 Divergence time and induced causes

Phylogenetic analysis and haplotype network have well supports that *V. micado* has diversified to two main lineages (NE and SE+SN) and two modes of life cycle (egg diapause and nymph diapause), and they are evolved independently. There are three main questions. The first question concerns the divergence of *V. micado* . Which important events triggered them dividing to two main clades (NE and SN+SE)? The second question is which climate shifts led them had developed two modes of life cycle? The third question is about the sympatric co-existence of two modes of life cycle of *V. micado* , what made them differentiate?

The time estimation analysis is the most reliable criterion to confirm which important events or climate shifts result in the divergence of the *V. micado* . Although the rate of CytB is bigger than COI (Brower and DeSalle, 1998; Gray *et al* ., 2006), the individuals lived in south Asia have not diversified based on CytB. Thus, the divergence in COI occurred early than CytB after the climate shifts, and COI was selected to estimate the time of diversification.

Climate changes during the Quaternary have great effects on species, for example, climate oscillation occurred during Pleistocene has affected the distribution of species *Gymnocarpos przewalskii* in Northwestern China (Jia and Zhang, 2019), the genetic divergence in species as *Petrogale penicillata* in southeast Australia appear to date to the mid-Pleistocene (Hazlitt *et al* ., 2014) and even contributed to speciation of arctic-alpine *Campanula* occurred in mid-Pleistocene in western North America (DeChaine *et al* ., 2014). According to the researches about Quaternary glaciations in Asia, the climatic transformation of Asia occurred in 0.8

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Ma BP was closely related to Mid-Pleistocene Transition (MPT), which was called due to glacial-interglacial cycles from a 41 ka to 100ka dominant frequency (Pisias *et al* ., 1981; Ruddiman *et al* ., 1989; Raymo*et al* ., 1998), and the Kunhuang (Kunlun Huanghe) movement of 0.9 Ma BP, (Li *et al* ., 2001; Li *et al* ., 2004). Since 800 ka BP, there were 8 complete glacial-interglacial cycles with a 100-ka dominant frequency (Shackleton and Opdyke, 1977). During these global glacials after the Kunhuang movement, glaciers developed gradually in the various part of the Qinghai-Tibet plateau (Wu *et al* ., 1999; Yao *et al* ., 2000; Zheng, 2000; Zhou *et al* ., 2002). Mountains lower than 2000 m in the East China were no glaciers during the middle Pleistocene (Shi *et al* ., 1987). The deposit sediment and the current distribution of the extant land species were related to the geographical and climatic shifts since the Quaternary. The most recent Vermiculated Red Soil (VSR) in southern China, an indication of extremely warm and humid conditions, was formed about 0.85 Ma BP due to the strengthen summer monsoon in the middle Pleistocene (Yang *et al* ., 1996; Qiao *et al* ., 2003) and the summer monsoon also strengthened in northern China (Guo *et al* ., 1998). However, subtropical zone reached at 42°N before the middle Pleistocene, and retreated southwards after the mid-Pleistocene (Liu and Ding, 1983). Many species immigrated southwards, including boreal, thermophilic and humid-preferring fauna. The species adapted to the new environment, and some remained and scattered in the locality during the Quaternary glaciation (Zhang, 2004). The current distribution of these species revealed the overall trend after the climate oscillations. The divergence time between northern and southern individuals was dated to 0.79 Ma, which was basically consistent with this time, thus, it is possible that the life history of the individuals lived in northern China changed under the tendency of change towards cooling climate. During the MPT, mid-Pleistocene *Homo* (MPH) (Bae, 2010; Wu and Poirier, 1995) in East China adapted to the diverse and various climate, distributing from the temperate to subtropical zone (Guo *et al* ., 2018; Kong *et al* ., 2019). The climate shifts in MPT induced the diversification and distribution of species, and we observed that this cricket is common around the habitats of human, but not in the wild. It is possible that human behavior influenced the distribution of the cricket to some extent after the mid-Pleistocene.

The earliest Yunshanping moraine in Yulong mountain (Yunnan province) was dated to 592.6 ± 118.5 ka BP (Zheng, 2000). Yao conducted dating studies on the red moraine in the same mountain, the ages of several samples range from 500 to 600 ka BP (Yao *et al* ., 2000). The second differentiation occurred during ˜ 0.50 Ma between southern individuals with egg and nymph diapause populations, and our results showed the egg diapause population might originated from Yunnan. Thus, the individuals with nymph diapause changed to get through the winter with egg due to the dry and cool climate.

The behavioral and genetic changes in the southern residents correspond to the environmental transitions. The genetic diversity indices and the phylogenetic analysis are the reliable evidence to support that the differentiation among sympatric co-existence of *V. micado* with two modes of life cycle has occurred for a long time. The divergence between them is maintained in the absence of obvious environmental difference and barriers to gene flow, such as the same photoperiod, temperature, humidity and habitats, while the genomic underpinning of ecological speciation often appear to have been found to be the result of a long period of allopatry (Bernatchez and Dodson, 1990; Feder*et al.* , 2003; Gray *et al* ., 2006; Kuehne *et al* . 2007; Foote and Morin 2015; Lucek *et al* ., 2018). The egg diapause population might origin from Yunnan colonized the new habitat. The individuals lived in the same locality may choose differently suitable habitats to their own life cycle.

It should be noted that there are still parts to be improved in our research. First, the range of the sampling time in the tropical region of China should be increased to further figure out if there was the egg diapause population. Second, are genes (COI and CytB) and behavior differentiation (different modes of life cycle) just two independent responses to the dramatic environment? Has the efficiency of the function of COI changed in individuals with two modes of life cycle? Or maybe it's just due to the founder effect. As this research shows, a large of proportion of genetic variation is originated from the founder effect rather than natural selection in human populations (Ramachandran*et al* ., 2005).

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

Authors' contribution

B. W., K. L., and Z. H. designed the research. B. W. and Z. H. performed the experiments. B. W. analyzed the data and wrote the paper. K. L. reviewed the paper.

Data availability statement

DNA sequences are depositing to Genbank.

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Table 1 Primer sequences used in this study.

Gene region	Primer name	Sequence (5'-3')	
COI	LCO	GGTCAACAAATCATAAAGATATTGG	Simon *et al.*, 1994
	HCO	TAAACTTTCAGGGTGACCAAAAAATCA	
CytB	REVCB2H	TGAGGACAAATATCATTTTGAGGW	Simmons *et al.*, 2001
	REVCBJ	ACTGGTCGAGCTCCAATTCATGT	

Table 2 Geographic information, nucleotide polymorphism and neutrality tests of 85 populations. S, number of segregating sites; Hap, haplotype distribution; Hd, haplotype diversity; π, nucleotide diversity; The number after the population name represents the groups divided by genetic marker COI which it belongs to, 1 represents it belongs to NE group. 2 and 3 represents SN group and SE group, respectively. The bold black font represents the groups divided by zoogeographic regions.

population	Lat.	Long.	Sampling time	phase	Sample size	S	Hap
NEC							
HLJ3	N46°02'57.67"	E125°57'22.18"	2019.08.18	adult	11	0	h 11
LN3	N41°40'30.64"	E123°27'30.98"			1	0	h11
JL3	N43°50'45.16"	E126°33'35.93"			2	1	h11, h 34
NC							
BJ3	N39°54'11.46"	E116°24'2.71"			1	0	h 2
HNJY3	N35°04'42.16"	E112°42'26.30"	2019.08.15	adult	22	1	h 2(21), h 28
HNPY3	N35°51'7.02"	E115°29'53.63"	2019.10.03	adult	1	0	h 2
SXXA3	N34°20'33.29"	E108°56'7.28"			1	0	h 36
SXNS3	N33°18'44.97"	E108°18'33.70"			1	0	h 2
SCCD3	N30°39'8.18"	E104°04'20.98"			1	0	h 2
SDDY1	N37°26'53.41"	E118°34'35.12"	2019.07.25	adult	2	3	h 1, h 7
SDDY3	N37°26'53.41"	E118°34'35.12"	2019.08.14	adult	12	1	h 2(11), h 23(1)
SDTA3	N36°0'3.16"	E116°43'12.42"	2019.08.15	adult	16	0	h 2
CC							
HBXY1	N32°0'39.61"	E112°06'57.21"	2019.09.25	adult	4	0	h 24
HBWH2	N32°0'39.61"	E112°06'57.21"	2019.08.04	adult	2	0	h 4
ANNF3	N31°49'20.30"	E117°13'17.39"	2019.10.01		1	0	h 2
JSNJ3	N32°03'57.43"	E118°46'56.02"			2	0	h 2
JSYZ1	N32°23'46.68"	E119°24'27.01"			1	0	h 1
JSZJ1	N32°11'25.78"	E119°25'6.73"			1	0	h 1
GZXY1	N25°05'41.85"	E104°53'36.79"	2019.07.25	adult	6	1	h 24(3), h 25(3)
GZXY2	N25°05'41.85"	E104°53'36.79"	2019.07.25	nymph	4	0	h 4(4)
GZGY1	N26°35'33.05"	E106°43'7.60"	2019.10.08	adult	1	0	h 24
GZRH1	N27°50'48.13"	E106°20'51.17"	2019.10.02	adult	2	0	h 24
GZZY1	N28°35'24.74"	E107°35'55.73"	2019.08.09	adult	1	0	h 24
GXTM2	N24°11'31.18"	E109°58'44.90"			1	0	h 4

population	Lat.	Long.	Sampling time	phase	Sample size	S	Hap
GXLG2	N25°15'3.64"	E110°11'20.83"			1	0	h 4
GXPM2	N22°56'40.47"	E105°59'35.62"	2019.04.20	adult	7	2	h 4(5), h 17(1), h 18(1)
GXSL2	N23°36'42.50"	E108°33'25.47"	2019.04.18	adult	3	3	h 4(1), h 19(1), h 20(1)
GXJX2	N23°04'14.23"	E106°27'20.61"	2019.04.19	adult	4	0	h 4(4)
ZJJD2	N29°28'38.51"	E119°16'33.40"			1	0	h 4
ZJTT1	N30°08'9.03"	E119°01'14.75"	2018.09	adult	11	1	h 1(9), h 13(2)
ZJTT2	N30°08'9.03"	E119°01'14.75"	2018.09	nymph	7	2	h 4(5), h14 (1), h 21 (1)
ZJFY1	N27°53' 28.88"	E119°10' 15.09"	2019.10.02	adult	1	0	h 24
ZJBMS1	N28°38' 38.96"	E119°09' 0.31"			2	0	h 24
ZJTM1	N30°21'0.63"	E119°25'28.82"	2019.08.16	nymph	5	3	h 1(3), h 7(2),
ZJTM2	N30°21'0.63"	E119°25'28.82"			4	0	h 4(4)
ZJTM3	N30°21'0.63"	E119°25'28.82"	2019.09.01	nymph	1	0	h 2
ZJBSZ1	N27°45' 16.60"	E119°12' 21.25"	2019.10.02	adult	25	0	h 24
ZJBSZ2	N27°45' 16.60"	E119°12' 21.25"			1	0	h 4
ZJQY2	N27°37'20.59"	E119°03'28.56"			1	0	h 4
ZJGT2	N29°15'29.68"	E118°08'59.26"	2018.10.03	nymph	4	1	h 4(3), h 15(1)
ZJWYL1	N27°42'50.78"	E119°39'29.97"	2019.10.03	adult	1	0	h 29
ZJWYL2	N27°42'50.78"	E119°39'29.97"	2019.10.03	nymph	3	0	h 4
ZJDP1	N30°0'7.75"	E120°04'8.21"	2019.10.04	adult	32	7	h 1(1), h 24(20), h 30(8), h 3
ZJDP2	N30°0'7.75"	E120°04'8.21"	2019.10.04	nymph	9	2	h 4(8), h 15(1)
ZJSX1	N30°01'57.90"	E120°34'29.75"	2019.10.01	adult	1	0	h 1
ZJSX3	N30°01'57.90"	E120°34'29.75"	2019.10.01	adult	17	0	h 2
ZJJL2	N30°35'49.36"	E121°05'46.26"			9	1	h 4(8), h 12
SHCJ3	N30°47'54.27"	E121°24'6.62"			1	0	h 2
SHCM1	N31°37'28.83"	E121°23'33.41"			1	0	h 1
SHJS3	N30°43'2.21"	E121°19'33.99"			1	0	h 2
SHMH3	N31°01'58.20"	E121°26'59.25"	2019.09	adult	3	0	h 2
SHBS1	N31°24'25.97"	E121°29'5.70"	2019.07.25	adult	14	1	h 1(5), h 7(6), h 22(2), h 24(
SHBS3	N31°24'25.97"	E121°29'5.70"	2019.07.25	adult	10	1	h 2(9), h 26(1)
SWC							
YNKM1	N24°52'58.97"	E102°49'53.66"	2019.08.18	nymph	28	0	h 24
YNBS1	N25°07'16.90"	E99°09'5.34"			1	0	h 24
YNMK1	N25°26'40.25"	E98°51'38.72"	2019.08.20	nymph	3	0	h 27
YNHK2	N22°30'26.16"	E103°57'48.80"	2019.08.19	nymph	1	0	h 4
SC							
SZ1	N22°32'43.85"	E114°03'10.40"			2	0	h 9
FJWY2	N27°52'27.58"	E117°51'22.43"			2	0	h 4
GDHY2	N22°47'28.70"	E114°27'7.57"			1	0	h 4
HNTGL2	N19°39'15.29"	E111°01'15.55"			2	0	h 4
HNJFL2	N18°42'57.91"	E108°52'18.65"	2019.03	nymph	5	1	h 4(1), h 16(4)
HNWZ2	N18°46'37.03"	E109°30'46.33"	2019.03	adult	1	0	h 4
HNXA2	N19°40'50.21"	E110°21'52.01"	2019.03	adult	1	0	h 16
HNCJ2	N19°17'59.51"	E109°03'5.28"	2019.03	adult	1	0	h 16
TWNT2	N23°55'10.63"	E120°40'12.03"			1	0	h 6
TWWL2	N24°52'21.08"	E121°32'51.11"			1	0	h 6
KJ							
KR2	N35°52'17.17"	E128°36'5.20"			1	0	h 3
JPSH2	N34°39'4.63"	E135°10'32.81"			1	0	h 3
JPSH3	N34°39'4.63"	E135°10'32.81"			1	0	h 5
JPJG2	N34°58'37.63"	E138°22'59.11"			1	0	h 6

population	Lat.	Long.	Sampling time	phase	Sample size	S	Hap
JPDB2	N34°41'37.46"	E135°30'7.79"			1	0	h 6
JPDB3	N34°41'37.46"	E135°30'7.79"			1	0	h 5
JPBHD3	N43°13'13.18"	E142°51'48.51"			1	0	h 10
JPBK3	N35°07'44.02"	E 134°29'1.11"			1	0	h 11
JPQS3	N40°49'19.46"	E140°44'50.51"			1	0	h 10
JPXX3	N37°55'36.72"	E139°20'22.56"			1	0	h 10
JPSS3	N33°50'20.97"	E132°45'56.07"			1	0	h 5
VK							
VN2	N21°02'36.57"	E105°51'51.33"			1	0	h 4
KH2	N12°43'33.90"	E104°45'56.25"			2	0	h 4
USA							
USAGA3	N33°74'83.04"	W84°39'11.13"			1	0	h 5
USATN3	N37°09'37.14"	W86°18'30.94"			1	0	h 5
USAMO1	N38°32'53.40"	W90°21'5.63"			1	0	h 7
USAMO3	N38°32'53.40"	W90°21'5.63"			1	0	h 8
USAVA1	N37°75' 88.48"	W77°47' 93.41"			1	0	h 7
Total					346	32	

Table 3 Sampling details of the genus *Velarifictorus* .

	Coordinates			
Species (population)	Lat.	Long.	GenBank No.	Reference
Velarifictorus_dianxiensis	N24°44' 58.9"	E97°33' 45.54"	MH992037	Chen *et al.*, 2018
Velarifictorus_beybienkoi	N37°25' 58.84"	E118°40' 8.54"	MH992030	Chen *et al.*, 2018
Velarifictorus_aspersus	N27°21' 16.49"	E119°55' 57.38"	MH992029	Chen *et al.*, 2018
Velarifictorus_flavifrons	N21°55' 37.21"	E101°15' 22.31"	MH992033	Chen *et al.*, 2018
Velarifictorus_ornatus	N25°37' 19.37"	E110°25' 32.11"	MH992024	Chen *et al.*, 2018
Velarifictorus_agitatus	N22°09' 4.00"	E100°41' 29.04"	in submitting	this study
Velarifictorus_micado				
HLJ	N46°02'57.67"	E125°57'22.18"	in submitting	this study
LN	N41°40'30.64"	E123°27'30.98"	in submitting	this study
JL	N43°50'45.16"	E126°33'35.93"	in submitting	this study
BJ	N39°54'11.46"	E116°24'2.71"	in submitting	this study
HNJY	N35°04'42.16"	E112°42'26.30"	in submitting	this study
HNPY	N35°51'7.02"	E115°29'53.63"	in submitting	this study
SXXA	N34°20'33.29"	E108°56'7.28"	in submitting	this study
SXNS	N33°18'44.97"	E108°18'33.70"	in submitting	this study
SCCD	N30°39'8.18"	E104°04'20.98"	in submitting	this study
SDDY	N37°26'53.41"	E118°34'35.12"	in submitting	this study
SDTA	N36°0'3.16"	E116°43'12.42"	in submitting	this study
HBXY	N32°0'39.61"	E112°06'57.21"	in submitting	this study
HBWH	N32°0'39.61"	E112°06'57.21"	in submitting	this study
ANNF	N31°49'20.30"	E117°13'17.39"	in submitting	this study
JSNJ	N32°03'57.43"	E118°46'56.02"	in submitting	this study
JSYZ	N32°23'46.68"	E119°24'27.01"	in submitting	this study
JSZJ	N32°11'25.78"	E119°25'6.73"	in submitting	this study
GZXY	N25°05'41.85"	E104°53'36.79"	in submitting	this study
GZGY	N26°35'33.05"	E106°43'7.60"	in submitting	this study
GZRH	N27°50'48.13"	E106°20'51.17"	in submitting	this study

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	Coordinates			
GZZY	N28°35'24.74"	E107°35'55.73"	in submitting	this study
GXTM	N24°11'31.18"	E109°58'44.90"	in submitting	this study
GXLG	N25°15'3.64"	E110°11'20.83"	in submitting	this study
GXPM	N22°56'40.47"	E105°59'35.62"	in submitting	this study
GXSL	N23°36'42.50"	E108°33'25.47"	in submitting	this study
GXJX	N23°04'14.23"	E106°27'20.61"	in submitting	this study
ZJJD	N29°28'38.51"	E119°16'33.40"	in submitting	this study
ZJTT	N30°08'9.03"	E119°01'14.75"	in submitting	this study
ZJFY	N27°53' 28.88"	E119°10' 15.09"	in submitting	this study
ZJBMS	N28°38' 38.96"	E119°09' 0.31"	in submitting	this study
ZJTM	N30°21'0.63"	E119°25'28.82"	in submitting	this study
ZJBSZ	N27°45' 16.60"	E119°12' 21.25"	in submitting	this study
ZJQY	N27°37'20.59"	E119°03'28.56"	in submitting	this study
ZJGT	N29°15'29.68"	E118°08'59.26"	in submitting	this study
ZJWYL	N27°42'50.78"	E119°39'29.97"	in submitting	this study
ZJDP	N30°0'7.75"	E120°04'8.21"	in submitting	this study
ZJSX	N30°01'57.90"	E120°34'29.75"	in submitting	this study
ZJJL	N30°35'49.36"	E121°05'46.26"	in submitting	this study
SHCJ	N30°47'54.27"	E121°24'6.62"	in submitting	this study
SHCM	N31°37'28.83"	E121°23'33.41"	in submitting	this study
SHJS	N30°43'2.21"	E121°19'33.99"	in submitting	this study
SHMH	N31°01'58.20"	E121°26'59.25"	in submitting	this study
SHBS	N31°24'25.97"	E121°29'5.70"	in submitting	this study
YNKM	N24°52'58.97"	E102°49'53.66"	in submitting	this study
YNBS	N25°07'16.90"	E99°09'5.34"	in submitting	this study
YNMK	N25°26'40.25"	E98°51'38.72"	in submitting	this study
YNHK	N22°30'26.16"	E103°57'48.80"	in submitting	this study
SZ	N22°32'43.85"	E114°03'10.40"	in submitting	this study
FJWY	N27°52'27.58"	E117°51'22.43"	in submitting	this study
GDHY	N22°47'28.70"	E114°27'7.57"	in submitting	this study
HNTGL	N19°39'15.29"	E111°01'15.55"	in submitting	this study
HNJFL	N18°42'57.91"	E108°52'18.65"	in submitting	this study
HNWZ	N18°46'37.03"	E109°30'46.33"	in submitting	this study
HNXA	N19°40'50.21"	E110°21'52.01"	in submitting	this study
HNCJ	N19°17'59.51"	E109°03'5.28"	in submitting	this study
TWNT	N23°55'10.63"	E120°40'12.03"	in submitting	this study
TWWL	N24°52'21.08"	E121°32'51.11"	in submitting	this study
KR	N35°52'17.17"	E128°36'5.20"	in submitting	this study
JPSH	N34°39'4.63"	E135°10'32.81"	in submitting	this study
JPJG	N34°58'37.63"	E138°22'59.11"	in submitting	this study
JPDB	N34°41'37.46"	E135°30'7.79"	in submitting	this study
JPBHD	N43°13'13.18"	E142°51'48.51"	in submitting	this study
JPBK	N35°07'44.02"	E 134°29'1.11"	in submitting	this study
JPQS	N40°49'19.46"	E140°44'50.51"	in submitting	this study
JPXX	N37°55'36.72"	E139°20'22.56"	in submitting	this study
JPSS	N33°50'20.97"	E132°45'56.07"	in submitting	this study
VN	N21°02'36.57"	E105°51'51.33"	in submitting	this study
KH	N12°43'33.90"	E104°45'56.25"	in submitting	this study
USAGA	N33°74'83.04"	W84°39'11.13"	in submitting	this study
USATN	N37°09'37.14"	W86°18'30.94"	in submitting	this study

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	Coordinates			
USAMO	N38°32'53.40"	W90°21'5.63"	in submitting	this study
USAVA	N37°75' 88.48"	W77°47' 93.41"	in submitting	this study

Table 4 Pairwise F_{ST} values for the three defined groups of *Velarifictorus micado* based on COI. ***P ¡ 0.001

	NE	SE	SN
NE	0.00000		
SE	0.95061***	0.00000	
SN	0.95084***	0.87072***	0.00000

Table 5 AMOVA in *Velarifictorus micado* categorized by different life cycles and corresponding fixation indices based on COI marker. d.f., Degree of freedom; SS, sum of squares; VC, variance components; %V, percentage of variation; F, multilocus F-statistic; F_{CT}, Variation among groups; F_{SC}, Variation among populations within groups; F_{ST}, Variation within populations.

Source of variation	d.f.	SS	VC	%V	F	P
Among groups	2	1329.952	5.88524	93.23	F_{CT}=0.93227	0.00000
Among populations within groups	82	92.395	0.25222	4.00	F_{SC}=0.58988	0.00000
Within populations	261	45.768	0.17536	2.78	F_{ST}=0.97222	0.00000

Table 6 AMOVA in *Velarifictorus micado* categorized by zoogeographic regions and corresponding fixation indices based on COI marker. d.f., Degree of freedom; SS, sum of squares; VC, variance components; %V, percentage of variation; F, multilocus F-statistic; F_{CT}, Variation among groups; F_{SC}, Variation among populations within groups; F_{ST}, Variation within populations.

Source of variation	d.f.	SS	VC	%V	F	P
Among groups	7.00	424.959	1.24315	24.94	F_{CT}=0.24943	0.00000
Among populations within groups	74.00	970.888	3.46700	69.56	F_{SC}=0.92682	0.00000
Within populations	264.00	72.268	0.27374	5.49	F_{ST}=0.94507	0.00684

Table 7 AMOVA in *Velarifictorus micado* categorized by different regions and corresponding fixation indices based on CytB marker. d.f., Degree of freedom; SS, sum of squares; VC, variance components; %V, percentage of variation; F, multilocus F-statistic; F_{CT}, Variation among groups; F_{SC}, Variation among populations within groups; F_{ST}, Variation within populations.

Source of variation	d.f.	SS	VC	%V	F	P
Among groups	10.00	418.250	1.87379	41.11	F_{CT}=0.41111	0.03030
Among populations within groups	12.00	85.679	1.37792	30.23	F_{SC}=0.51336	0.00000
Within populations	139.00	181.565	1.30622	28.66	F_{ST}=0.71342	0.00000

Table 8 AMOVA in *Velarifictorus micado* based on CytB categorized three clades according COI and corresponding fixation indices based on CytB marker d.f., Degree of freedom; SS, sum of squares; VC, variance components; %V, percentage of variation; F, multilocus F-statistic; F_{CT}, Variation among groups; F_{SC},

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Variation among populations within groups; F_{ST}, Variation within populations.

Source of variation	d.f.	SS	VC	%V	F	P
Among groups	2.00	551.278	5.10265	84.78	F_{CT}=0.84778	0.00000
Among populations within groups	25.00	72.514	0.45573	7.57	F_{SC}=0.49742	0.00000
Within populations	134.00	61.702	0.46046	7.65	F_{ST}=0.92350	0.00293

Table 9 Neutrality tests in each geographic population based on CytB. S, number of segregating sites; Hap, haplotype distribution.

population	Hap	Sample size	S	Tajima' D	Fu' Fs
HLJ	h 13(9), h 14	10	1	-1.11173	-0.33931
HNJY	h 12(11), h17, h 32, h 33, h34	15	4	-1.81594*	-3.228*
GZXY	h 4(7), h 27, h 28, h29, h 31	11	5	-1.21975*	-1.68362*
YNKM	h 4(6), h 25(14), h 26,	21	2	-0.13298	-0.09012
HBXY	h 1(2), h 35(2)	4	3	-0.07339	1.77498
HBWH	h 2(1)	1			
ANHF	h 1, h 17	2	17		
ZJTT	h 1(7), h 2(3), h 3, h 4, h 11	13	6	-0.75444	-0.69700
ZJGT	h 2(4)	4	0		
ZJTM	h 1	1			
ZJSX	h 12(1), h 16(1), h17(2), h 36(1)	5	4	-0.41017	-1.19500
SDDY	h 1, h 12(6), h 15, h 18, h 19, h 20, h 21	12	22	-0.89013	0.37100
SDTA	h 12(7), h 17(2), h 19, h 22, h 23, h 24	13	5	-1.57943	-3.489*
SHMH	h 12(1), h 17(2)	3	1		
SHBS	h 1(7), h 12(5), h 15(5), h 16, h 17(2), h 30	21	21	2.23545*	6.159*
GXPM	h 4(5), h 8(2)	7	2	0.68731	1.70200
GXSL	h 4(2), h 9	3	1		
GXJX	h 4(1), h 8(2),h 10(2)	5	3	1.57274	0.46900
HNBS	h 4	1			
HNJFL	h 4(1), h 5(1), h 6(2)	4	2		
HNWZ	h 4	1			
HNXA	h 4(2), h 5(1)	3	1		
HNCJ	h 5(1), h 7(1)	2	2		

Fig.1 Sampling localities of *Velarifictorus micado.*

Fig.2 Median-joining haplotype network based on COI construced using DNASP and Popart, circle size represented the number of the haplotype, the different color represented the different populations.

Fig.3 Median-joining haplotype network based on CytB construced using DNASP and Popart, circle size represented the number of the haplotype, the different color represented the different populations.

Fig.4 Time estimation of *Velarifictorus micado* based on COI constructed using BEAST. The BEAST run was calibrated using the substitution rate of 0.017 subs/s/Myr, purple bars represent the node age 95%credible intervals. The numbers of the nodes were the estimated node ages. Green circles and blue circles on nodes indicate posterior probability values over 95 and 90, respectively.