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Cryptic Genetic Differentiation of the Cicada *Vagitanus terminalis* in the South Ryukyu Islands

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The biota of the southern part of the Ryukyu Archipelago (South Ryukyu) is considered to have been formed relatively recently by organisms that migrated from Taiwan Island and continental China. However, phylogeographic patterns of insect species in South Ryukyu remain largely unexplored. In this study, we elucidated the intraspecific phylogeny and divergence time of *Vagitanus terminalis*, a small cicada species distributed from continental China to Taiwan Island and South Ryukyu. We conducted phylogenetic analyses using two mitochondrial and two nuclear gene sequences, and genome-wide DNA sequences and SNPs obtained through MIG-seq. We found that *V. terminalis* populations from Sichuan in continental China and Taiwan Island were genetically closely related despite the large geographical distance between them. In contrast, in South Ryukyu substantial genetic differentiation was detected between Yaeyama Islands and Miyako Island. The Yaeyama Islands populations were estimated to have diverged from Miyako Island and Taiwan-continental China populations approximately 2.4 million years ago (confidence interval: 1.1–4.0), during Early Pleistocene when the formation of the Ryukyu Archipelago began. These results suggested that colonization to the two island areas in South Ryukyu occurred at different times and via different routes, although precise phylogenetic relationships and divergence times among these populations and Taiwan-continental China populations remain to be clarified through additional taxon sampling and sequence data. Overall, our findings revealed substantial genetic differentiation among non-endemic insect populations in South Ryukyu, including the presence of relict populations with an ancient origin.

Key words: Cicadidae, divergence time, relict distribution, MIG-seq, continental island

INTRODUCTION

The ocean acts as a powerful dispersal barrier for many terrestrial organisms. Consequently, factors enabling species to colonize islands include dispersal abilities such as flight assisted by air currents and rafting by ocean currents, and geohistorical factors such as land bridge formation (Avice, 2000; Whittaker and Fernandez-Palacios, 2007). For continental islands located near a continent and historically connected to it, the distance from the source continent is known to correlate with geographical genetic differentiation (MacArthur and Wilson, 1967, 2001; Emerson, 2002).

The Ryukyu Archipelago consists of continental islands located between continental China and the Pacific Ocean (Fig. 1). The Sakishima Islands (South Ryukyu) are located in the southwestern part of this archipelago and consist of the Yaeyama Islands, comprising Yonaguni Island, Ishigaki Island, and Iriomote Island, and the Miyako Islands, including

Miyako Island. Yonaguni Island, the westernmost island of the Yaeyama Islands, faces Taiwan across a strait approximately 100 km wide. The Ryukyu Archipelago is thought to have separated from the continent and formed since the Early Pleistocene due to the formation of the Okinawa Trough, which reaches a maximum depth of approximately 2000 m (Lu et al., 1981; Miki, 1995; Sibuet et al., 1998; Machida et al., 2001; Iryu et al., 2006). However, because this region has experienced significant crustal movements, the exact geological history that led to the formation of the present archipelago remains unclear. Within South Ryukyu, Ishigaki Island and Iriomote Island in the Yaeyama Islands are currently connected by shallow coral reefs and are thought to have formed a single large island during periods of sea level lowstands, such as glacial periods (Yonekura et al., 2001). Conversely, the straits between Taiwan and Yonaguni Island, Yonaguni Island and Ishigaki/Iriomote Islands, and Ishigaki/Iriomote Islands and Miyako Island are hundreds of meters deep. Even when measured using the 130 m isobath assumed to represent sea level during the Last Glacial Maximum (Lambeck et al., 2014; Yokoyama et al., 2018), these areas remain over

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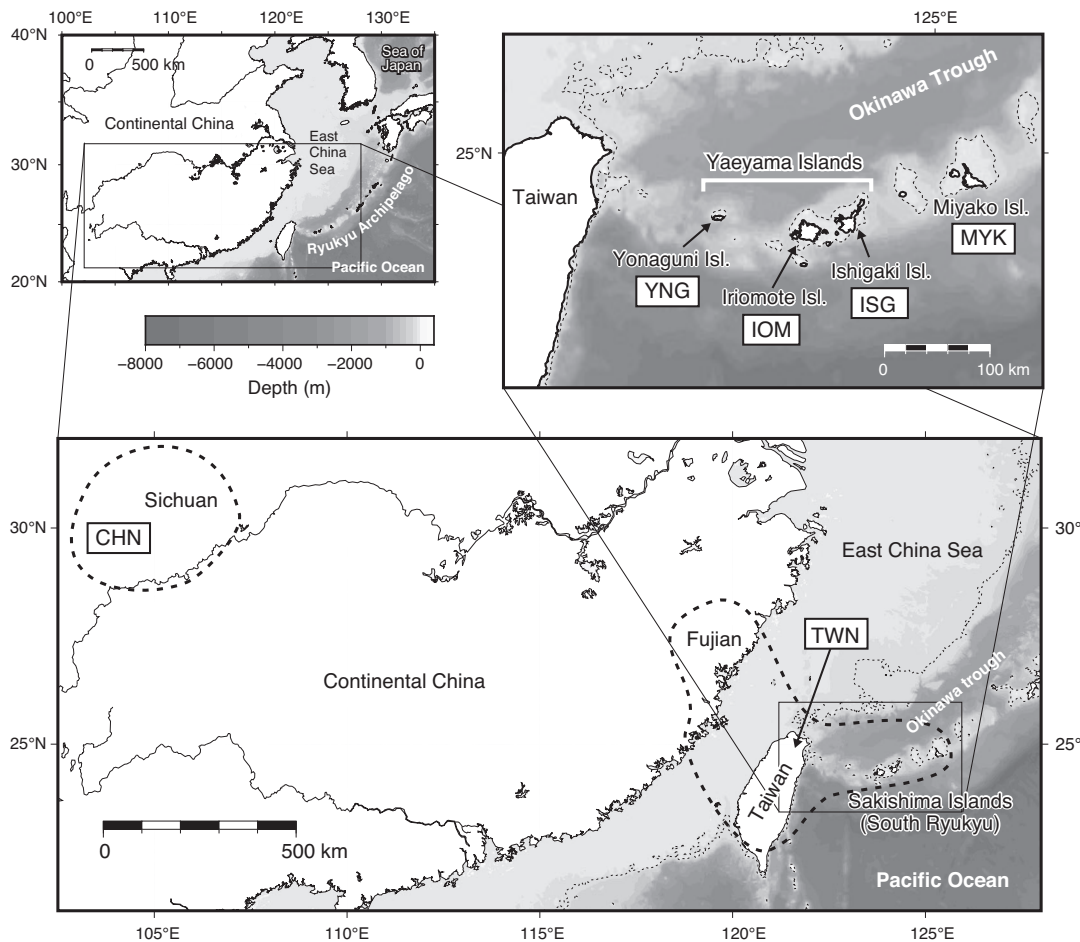


Fig. 1. Distribution range and sample localities of *V. terminalis*. The known distributional areas are enclosed by broken lines. Sample localities are indicated by the three-letter names within rectangles. The dotted lines indicate the 130-meter depth contours of the sea. The maps were generated using GMT v6.5.0 (Wessel et al., 2019).

100 km apart, suggesting that land bridges did not form during the last glacial period. Due to their geographical relationships, much of the biota of South Ryukyu consists of species that are shared with or closely related to those of continental China, Taiwan, and Southeast Asia, although the presence of multiple endemic species on each island suggests a considerable time has elapsed since their isolation as islands, leading to debate about the origin of the biota (Ota, 1998; Motokawa and Kajihara, 2017). While studies on interspecific and within-species phylogenetic relationships are advanced for amphibians and reptiles within the Ryukyu Archipelago (Tominaga et al., 2015, 2019; Kaito and Toda, 2016; Okamoto, 2017), such studies are largely absent for insects. Consequently, the genetic relationships within the insect fauna of the Ryukyu Archipelago and the process by which they formed remain unclear.

Vagitanus terminalis is a small cicada distributed in inland China (Sichuan Province and Chongqing), southeastern coastal China (Fujian Province), Taiwan Island, and South Ryukyu (Fig. 1) (Chou et al., 1997; Yang and Wei, 2013; Hayashi and Saisho, 2015). In South Ryukyu, it occurs in the Yaeyama Islands and Miyako Island (Hayashi and Saisho, 2015). On Miyako Island, the easternmost limit of its range, *V. terminalis* is confined to highly limited habitats, and its popula-

tions are extremely small. This population is highly dependent on the host tree *Distylium racemosum*, and the historical removal of this tree is the main cause of the severe decline in the *V. terminalis* population, placing it at an extremely high risk of local extinction. Consequently, the Miyako Island population is designated as a threatened Local Population (LP) on the Red List of Japan (Ministry of the Environment, Government of Japan, 2015). *Vagitanus terminalis* spans an approximately 2200 km east–west range from inland continental China to the Ryukyu Archipelago, yet no clear morphological differences have been identified across this range (Yang and Wei, 2013; Hayashi and Saisho, 2015). Because cicadas depend on trees throughout their life cycle (both as nymphs and adults), their dispersal ability is considered limited, and this limitation may be even more pronounced in small species such as *V. terminalis*. Therefore, dispersal among islands is expected to follow a stepping-stone model (i.e., gradual colonization between neighboring islands). Accordingly, genetic differentiation among island populations should reflect isolation by distance. Specifically, populations on islands closer to the continent (e.g., Yaeyama Islands) may show high genetic relatedness to the continental populations, whereas populations on more remote islands (e.g., Miyako Island) may be most genetically differentiated from the conti-

nent. Among *Meimuna* cicadas distributed in the Ryukyu Archipelago, some species show substantial genetic differentiation even within the archipelago. For example, *Meimuna iwaskii* exhibits genetic divergence between Ishigaki and Iriomote islands, although these islands are presumed to have been connected by land bridges during the Last Glacial Maximum (LGM) (Nagata et al., 2021). A similar pattern has been observed in the damselfly *Euphaea yaeyamana* (Kanke et al., 2021). Thus, genetic differentiation may also occur in *V. terminalis* between Ishigaki and Iriomote islands, despite their historical land-bridge connection during LGM. To examine these hypotheses, we reconstructed the biogeographic history of *V. terminalis* populations by elucidating the phylogenetic relationships and divergence times among populations in South Ryukyu, Taiwan and continental China, using two mitochondrial and two nuclear gene sequences, as well as genome-wide DNA sequences and SNPs obtained through MIG-seq.

MATERIALS AND METHODS

A total of 16 individuals of *Vagitanus terminalis* were collected from Miyako Island, the Yaeyama Islands (Yonaguni Island, Iriomote Island, and Ishigaki Island), and the northern part of Taiwan Island (Table 1). In addition, sequences of two *V. terminalis* individuals from continental China (Sichuan Province) and Taiwan Island were obtained from DDBJ/GenBank/EMBL (Table 1). As outgroups, we collected *Mogannia minuta* and *Muda kuroiwae* from Okinawa Island, Japan. Total genomic DNA was extracted from the flight muscle using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) or a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

We sequenced four gene regions by Sanger sequencing to construct a multi-locus dataset: mitochondrial cytochrome oxidase subunit I (*COI*, 658 bp), mitochondrial 16S ribosomal RNA (16S, 509 bp), nuclear elongation factor 1 alpha (*EF-1 α* , 677 bp), and

nuclear acetyltransferase (*ARD1*, 679 bp). Amplification and sequencing of these regions were performed following the method described by Nagata et al. (2021). All newly obtained sequences were deposited in DDBJ/GenBank/EMBL (accession numbers: LC645091–LC645156). Sequences of each region were aligned using MAFFT version 7 (Kato et al., 2019). The final multi-locus dataset consisted of 17 *V. terminalis* and one outgroup, excluding one individual in which PCR failed for nuclear genes.

We also generated genome-wide DNA sequence and SNP datasets using MIG-seq (Suyama and Matsuki, 2015) for 16 individuals of *V. terminalis* collected by the authors. Library preparation followed Suyama and Matsuki (2015), except that the annealing temperature for the first PCR was set to 38°C following the improved protocol (Suyama et al., 2022). The prepared libraries were quantified using a TapeStation 4150 system (Agilent Technologies, Santa Clara, CA, USA) with D1000 ScreenTape and sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (150 cycles; Illumina, San Diego, CA, USA). The obtained sequences were quality-filtered using fastp v0.23.4 (Chen, 2025) to remove adapters and low-quality reads, followed by assembly and SNP calling using ipyrad v0.9.104 (Eaton and Overcast, 2020). Assembly was performed under multiple parameter settings to account for the trade-off between the number of loci and the extent of missing data, both of which can affect the robustness of phylogenetic inferences (Roure et al., 2013; Takahashi et al., 2014; Huang and Knowles, 2016). We tested four settings for the minimum number of samples per locus (min_samples_locus): four, eight, 10, and 12; while other parameters were set to defaults, except for min_depth_statistical = 5, min_depth_majrule = 3, and clust_threshold = 0.85. For each of assembled datasets, we collected one SNP randomly from each locus to generate a dataset of unlinked SNPs.

For both the multi-locus and MIG-seq datasets, phylogenetic trees were constructed using Bayesian inference (BI) and maximum-likelihood (ML) methods. BI analyses were conducted using MrBayes v3.2.2 (Ronquist et al., 2012). Four Markov Chain Monte Carlo (MCMC) chains were run for 10 million generations, sampling every 1000 generations. Convergence of the runs was

Table 1. Specimen ID, locality, and accession numbers for each gene and MIG-seq data.

Taxon	ID	Locality	<i>COI</i>	16S	<i>ARD1</i>	<i>EF-1α</i>	MIG-seq (raw reads)
<i>Vagitanus terminalis</i>	MYK01	Miyako Island, Okinawa, Japan	LC645093	LC645111	LC645128	LC645144	SAMN55892508
	MYK02	Miyako Island, Okinawa, Japan	LC645094	LC645112	LC645129	LC645145	–
	MYK03	Miyako Island, Okinawa, Japan	LC645095	LC645113	LC645130	LC645146	SAMN55892509
	MYK04	Miyako Island, Okinawa, Japan	LC645096	LC645114	LC645131	LC645147	SAMN55892510
	MYK05	Miyako Island, Okinawa, Japan	LC645097	LC645115	LC645132	LC645148	SAMN55892511
	YNG01	Yonaguni Island, Okinawa, Japan	LC645098	LC645116	LC645133	LC645149	SAMN55892512
	YNG02	Yonaguni Island, Okinawa, Japan	LC645099	LC645117	LC645134	LC645150	SAMN55892513
	ISG01	Ishigaki Island, Okinawa, Japan	LC645101	LC645118	LC645135	LC645151	SAMN55892514
	ISG02	Ishigaki Island, Okinawa, Japan	LC645102	LC645119	LC645136	LC645152	SAMN55892515
	ISG03	Ishigaki Island, Okinawa, Japan	LC645103	LC645120	LC645137	LC645153	SAMN55892516
	ISG04	Ishigaki Island, Okinawa, Japan	LC645100	–	–	–	SAMN55892517
	IOM01	Iriomote Island, Okinawa, Japan	LC645104	LC645121	LC645138	LC645154	SAMN55892518
	TWN01	Yilan, New Taipei, Taiwan Island	LC645105	LC645122	LC645139	LC645155	SAMN55892519
	TWN02	Yilan, New Taipei, Taiwan Island	LC645106	LC645123	LC645140	LC645156	SAMN55892520
	TWN03	Yilan, New Taipei, Taiwan Island	LC645107	LC645124	–	–	–
	TWN04	Yilan, New Taipei, Taiwan Island	LC645108	LC645125	LC645141	–	SAMN55892521
	TWN05	Yilan, New Taipei, Taiwan Island	MG737734	MG737734	–	–	–
	CHN01	Mt. Emeishan, Sichuan, continental China	JX966342	JX966348	–	–	–
<i>Muda kuroiwae</i>		Okinawa Island, Okinawa, Japan	LC645091	LC645109	LC645126	LC645142	–
<i>Mogannia minuta</i>		Okinawa Island, Okinawa, Japan	LC645092	LC645110	LC645127	LC645143	–

confirmed when the average standard deviation of split frequencies fell below 0.01. Additionally, the effective sample sizes (ESS > 200) of parameters were checked using Tracer v1.7.0 (Rambaut et al., 2018). After discarding the first 25% of trees as burn-in, a 50% majority-rule consensus tree was constructed. Node support was evaluated based on posterior probabilities (PP). ML analyses were performed using IQ-TREE 3 (Nguyen et al., 2015; Wong et al., 2026). For the multi-locus dataset, the optimal substitution model for each gene was selected using ModelFinder (Kalyaanamoorthy et al., 2017) based on the Bayesian Information Criterion (BIC): HKY+I for *COI* and *ARD1*, HKY+G for 16S, and F81 for *EF-1 α* . Similarly, for the MIG-seq datasets, substitution models were selected using ModelFinder with the option to merge partitions. Node support was evaluated using 1000 replicates of both SH-aLRT (Shimodaira–Hasegawa approximate likelihood ratio test; Guindon et al., 2010) and ultrafast bootstrapping (UF-boot; Hoang et al., 2018).

Divergence time estimation was performed using BEAST v2.6.3 (Bouckaert et al., 2019) based on the multi-locus dataset. Only *M. minuta* was used as outgroup. Due to the lack of fossil records for *Vagitanus* or its sister genus (Moulds, 2018), we used an uncorrelated lognormal relaxed molecular clock model for the *COI* partition, with a lognormal prior on the mean substitution rate (ucldMean) centered at a median of 0.0177 substitution per site per million years (Papadopoulou et al., 2010), with a log-space standard deviation of 0.5. This rate has been widely used for estimating divergence times in several hemipteran lineages (Sota et al., 2016;

Wang et al., 2018; Nagata et al., 2021). For the other partitions, we used an unlinked uncorrelated lognormal relaxed clock model, allowing their relative clock rates to be estimated. The substitution models selected using ModelFinder were applied to each partition in BEAST2, except that F81 for *EF-1 α* was implemented as GTR with all substitution rate parameters fixed to 1.0. A Yule process was adopted as the tree prior. MCMC iterations were performed for 200 million generations, with sampling every 10,000 generations. Random trees were used as starting trees. MCMC convergence was confirmed using Tracer based on ESS values (all > 1000). Node support was evaluated based on posterior probabilities (PP).

To elucidate population genetic structure, we performed haplotype network analysis using 18 *COI* gene sequences and population structure analysis based on the MIG-seq datasets. A statistical parsimony network (Templeton et al., 1992) for *COI* haplotypes was constructed using TCS version 1.21 (Clement et al., 2000). TCS subdivides networks by determining connection limits (the threshold number of substitutions) to connect sub-networks (Templeton et al., 1992). To infer population structure, Bayesian clustering analysis was performed using STRUCTURE v2.3.4 based on unlinked SNPs. Ten independent runs were performed for each number of clusters (*K*) from one to 10. MCMC analysis was conducted with a burn-in period of 200,000 steps followed by 1,000,000 iterations. Optimal group number (*K*) was determined using the delta *K* method (Evanno et al., 2005).

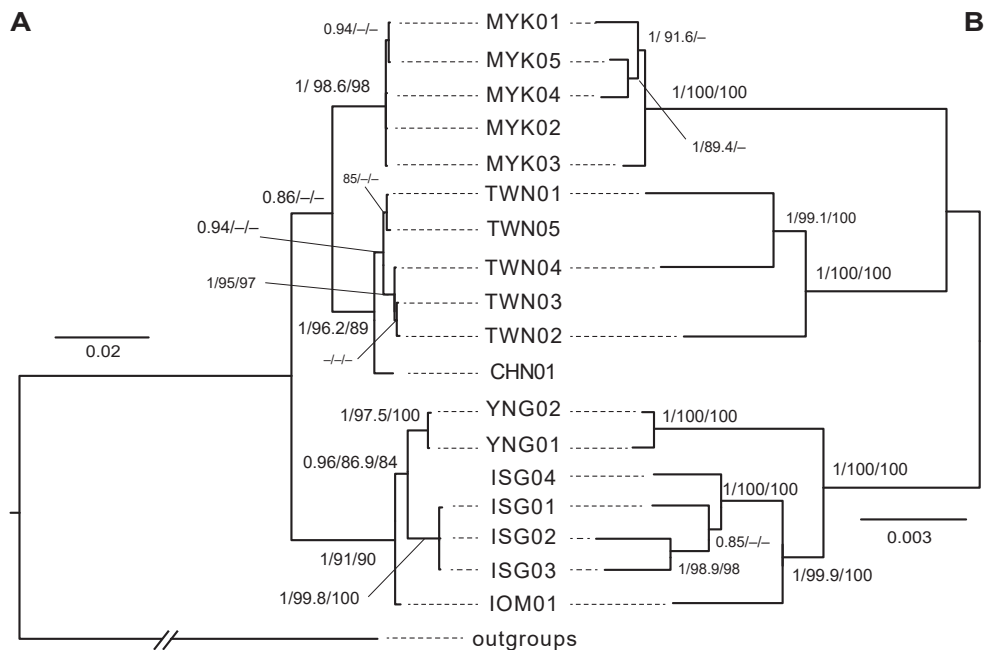


Fig. 2. Bayesian inference trees based on the multi-locus dataset (A) and the MIG-seq dataset (B). Numerals at each node indicate Bayesian posterior probability (shown when > 0.85), followed by ML ultrafast bootstrap percentage (shown when > 85%), and ML SH-aLRT support percentage (shown when > 80%).

Table 2. Summary statistics of assembled MIG-seq datasets assembled under different minimum taxon coverage thresholds (min_samples_locus). The number of polymorphic loci corresponds to the number of unlinked SNPs used in STRUCTURE analysis (one SNP per locus).

min_samples_locus	Loci (polymorphic loci)	SNPs	Alignment length (bp)	Missing (alignment)
4	4,348 (3,377)	12,114	319,238	49.56%
8	1,566 (1,363)	5,183	117,873	25.28%
10	1,003 (881)	3,260	75,591	16.49%
12	558 (496)	1,764	42,126	8.11%

RESULTS

Phylogenetic analyses based on the multi-locus dataset (four genes totaling 2523 bp) yielded largely identical topologies between the ML and BI analyses, with all island populations recovered as monophyletic (Fig. 2A, and see Supplementary Figure S1). Three major clades were identified. One clade comprised the Yaeyama Islands (YNG, ISG, IOM), which diverged first; another included TWN (along with CHN in the multi-locus analysis); and the third comprised MYK. Although the MYK and TWN+CHN clades formed a sister group, this relationship received low statistical support (UF-boot < 95, aLRT < 80, $PP < 0.9$). Within the Yaeyama clade, IOM diverged first, with YNG and ISG forming a sister group (UF-boot = 100, aLRT = 100, $PP = 1.0$).

The assembled MiG-seq datasets ranged from 558 loci with a total length of 42,126 bp and 1764 SNPs (min_samples_locus = 12) to 4348 loci with 319,238 bp and 12,114 SNPs (min_samples_locus = four) (Table 2). Phylogenetic trees inferred from these datasets were consistent across all min_samples_locus values and between ML and BI analyses, with each island clade recovered as monophyletic with high support values (see Supplementary Figure S2). In Fig. 2B, we present the phylogenetic tree based on the dataset with min_samples_locus = eight, which showed the best-balanced combination of locus number and proportion of missing data (1566 loci, 117,873 bp in total length, 25.28% of

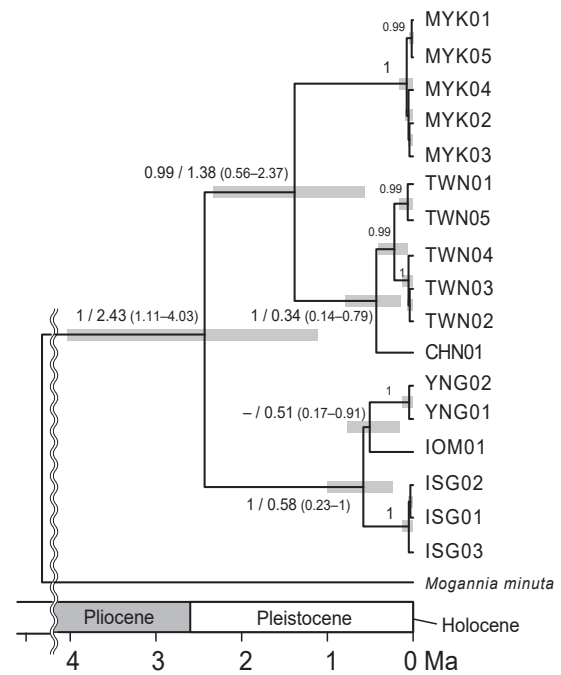


Fig. 3. Divergence time estimation based on the multi-locus dataset. Grey bars at the nodes indicate 95% HPD intervals. For the major clades, posterior probabilities (shown when > 0.95) and estimated dates (with 95% HPD intervals in parentheses) are shown.

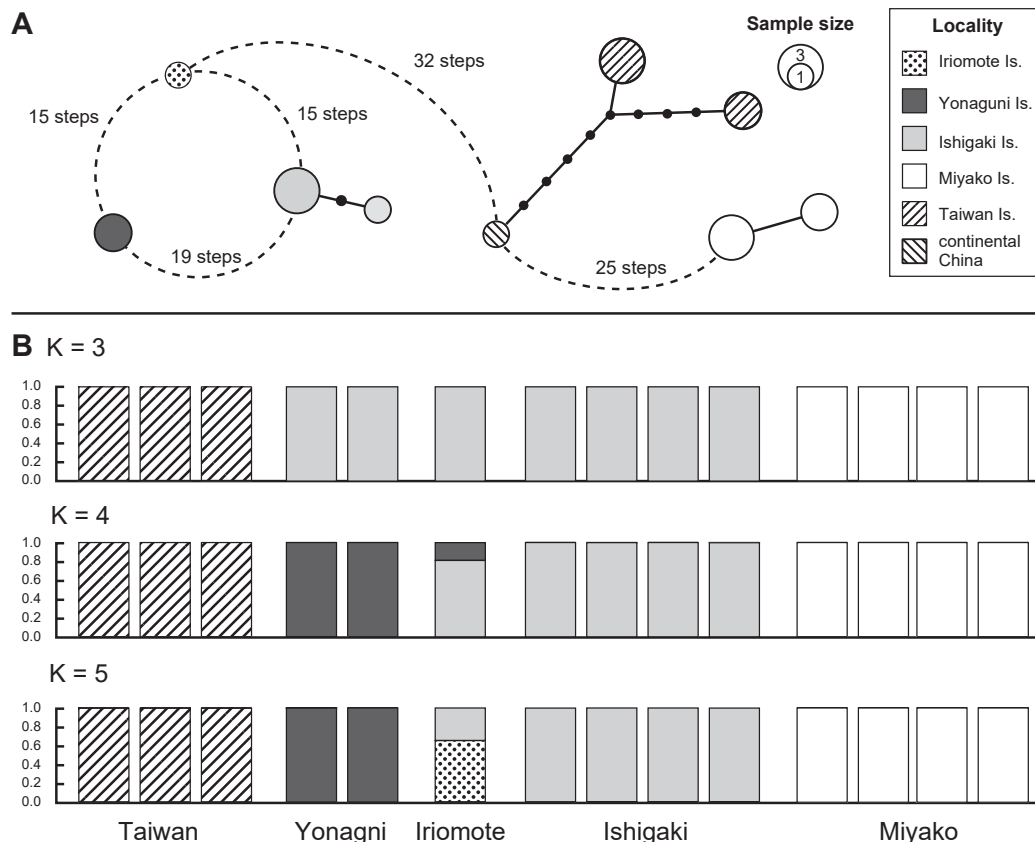


Fig. 4. Genetic structure of *V. terminalis* populations. **(A)** Statistical parsimony network of 658-bp *COI* haplotypes. Small black circles indicate inferred missing haplotypes. The dashed line indicates mutation steps exceeding the connection limit. **(B)** Population structure revealed by ancestry probabilities based on 1363 unlinked SNPs for $K =$ three, four, and five groups.

missing data). Although midpoint rooting was applied to this MIG-seq tree due to the absence of outgroups, its overall topology was consistent with that of the multi-locus tree. However, the phylogenetic relationships within the Yaeyama Islands differed: the MIG-seq tree strongly supported the early divergence of YNG and a sister relationship between the geographically proximate IOM and ISG.

Divergence time estimates (Fig. 3) revealed that the Yaeyama clade diverged from the other lineages 2.43 million years ago (Ma) (95% HPD: 1.11–4.03 Ma). Subsequently, the MYK population diverged at 1.38 Ma (0.56–2.37 Ma), while the CHN and TWN populations diverged at 0.43 Ma (0.13–0.79 Ma). Although the branching order among the three islands of Yaeyama was not fully resolved, the divergence among these island populations was estimated to have occurred almost simultaneously, around 0.55 Ma (0.17–1.0 Ma).

The network analysis yielded five networks with a connection limit of 11 steps (Fig. 4A). Populations from the four islands in the Ryukyu Archipelago formed distinct, non-overlapping networks, whereas the TWN and CHN populations were grouped into the same network despite possessing different haplotypes. STRUCTURE analysis using 1363 unlinked SNPs (min_samples_locus = eight; Table 2) identified groups corresponding to TWN, MYK, and the Yaeyama Islands at $K = 3$ (Fig. 4B), which was determined to be the optimal K . At $K = 4$, the YNG population was distinguished as a separate cluster from the remaining Yaeyama Island populations. At $K = 5$, an additional genetic cluster emerged within the Iriomote Island population. Beyond this value, the cluster assignments remained largely stable.

DISCUSSION

It was unexpected that insular populations of *V. terminalis* in the South Ryukyu Islands exhibit complex phylogenetic relationships, despite occurring at the easternmost edge of the wide distribution range and being situated close to Taiwan and continental China, which are likely to be source areas for colonizers. Our phylogenetic analyses consistently revealed that *V. terminalis* comprises three lineages, two of which are distributed within the South Ryukyu Islands. It should be noted that our divergence-time estimation is based on an evolutionary rate of a mitochondrial gene without fossil calibrations, and thus contains uncertainty due to confounding factors including the time-dependent variation in the evolutionary rates (e.g., Ho et al., 2011, 2015). Caution is therefore required when interpreting inferred divergence times. The earliest divergence among *V. terminalis* populations occurred between the populations of the Yaeyama Islands (YNG, ISG, and IOM) and the remaining lineages (Miyako Island and Taiwan/continental China), with an estimated divergence time of approximately 2.4 Ma (95% HPD: 1.11–4.03 Ma) during the Early Pleistocene. This estimate overlaps with the onset of the major rifting phase of the Okinawa Trough (~2 Ma) and the subsequent marine isolation of the Ryukyu Islands, as evidenced by coral reef initiation at 1.45–1.65 Ma (Lu et al., 1981; Miki, 1995; Sibuet et al., 1998; Iryu et al., 2006). The Miyako Island (MYK) population diverged from the Taiwan and continental China (Emeishan, Sichuan Province) populations at 1.38 Ma, whereas the latter two populations diverged more recently, 0.43 Ma, despite

the considerable geographic distance (~1800 km) between the two localities. Thus, the South Ryukyu populations of *V. terminalis* are not recent colonizers from continental China or Taiwan Island, but rather relict populations that diverged from continental Chinese or Taiwanese populations at a relatively ancient time.

Among cicadas distributed on the Japanese main islands, which were connected to continental East Asia during the Late Pleistocene, no significant inter-island regional genetic differentiation has been reported for *Hyalessa maculaticollis* (Liu et al., 2018) or species of the genus *Lyristes* (Sota et al., 2016). In contrast, on the relatively older continental islands of the Ryukyus, substantial genetic differentiation among islands has been reported for species of *Meimuna* (Nagata et al., 2021) and *Platypleura* (Osozawa et al., 2017). In *V. terminalis*, significant genetic differentiation has occurred between populations of the South Ryukyu Islands and those of continental China and Taiwan, and the degree of genetic differentiation tends to increase with longer isolation times between island populations. While such relictual differentiation in the Ryukyu Islands has been reported for reptiles and amphibians (Ota, 1998; Kaito and Toda, 2016), no such cases have been reported for insects, to our knowledge. Unfortunately, this study included only a single published sequence from Sichuan in continental China, and no data were available from other continental populations, including those in Fujian. There may be populations genetically closer to the South Ryukyu populations across the vast area between Sichuan and Fujian. Therefore, additional studies using specimens from multiple localities in continental China and Taiwan are needed to corroborate the findings of the present study. Furthermore, since regional differences in morphological characters such as body coloration and acoustic signals are recognized within *V. terminalis*, a more detailed evaluation of morphological traits and a taxonomic revision of genetically diverged populations may be necessary.

It is unclear why the easternmost Miyako population was genetically closer to the Taiwan populations than to the geographically closer Yaeyama Islands populations. The paleogeography involved in the formation of Miyako Island's biota remains largely unclear, with recent studies suggesting a connection to the more easterly Okinawa Island (Watanabe et al., 2023). However, *V. terminalis* is not distributed on Okinawa Island. Furthermore, Pleistocene sea-level fluctuations caused the East China Sea to become shallower and narrower during glacial periods, raising the possibility that ancient topography could have facilitated genetic exchange between Miyako Island and the continent. However, no such topography has been identified at the 130-meter depth contour (Austermann et al., 2013; Yokoyama et al., 2018), which corresponds to the sea level during the Last Glacial Maximum (Fig. 1). Alternatively, long-distance dispersal may have occurred, as in *Meimuna boninensis* endemic to the oceanic Ogasawara Islands in the northwest Pacific, which is thought to have colonized the islands via long-distance dispersal after the island formation period (Nagata et al., 2021).

The Miyako Island population of *V. terminalis* is designated as “regionally endangered” in the Ministry of the Environment's Red List and has been protected as a Natural Monument by the Miyakojima City government. Populations

exhibiting mitochondrial DNA monophyly and significant nuclear genome differentiation are recognized as evolutionarily significant units (ESUs) or management units (MUs) (Moritz, 1994, 1999). This study showed that the Miyako Island population of *V. terminalis* is genetically highly distinct and should be considered to be a unique MU or ESU. Thus, this study provides genetic support for the “regionally endangered” designation of *V. terminalis*.

Despite their geographical proximity, the populations on the three islands of the Yaeyama group did not share mitochondrial COI haplotypes. Notably, YNG was clearly distinguishable from ISG and IOM in the MIG-seq analysis, indicating advanced differentiation within the Yaeyama group. Ishigaki and Iriomote Islands are separated by the shallow Sekisei Lagoon, where reef-building corals thrive, an area that was likely exposed as dry land during Pleistocene sea-level lowstands. Although the population structure analysis using SNPs generally placed ISG and IOM in the same cluster, a unique genetic signature emerged for IOM at $K = \text{five}$. However, because only one individual was available from Iriomote Island, the true extent of this differentiation remains unclear. Genetic differentiation has been observed between Ishigaki and Iriomote islands in other insect species such as the damselfly *Euphaea yaeyamana* (Kanke et al., 2021) and the scarabaeid beetle *Dasylepida ishigakiensis* (Muraji et al., 2008). To determine whether genetic differentiation has also occurred between these two islands in *V. terminalis*, further analyses using more specimens and DNA markers are needed.

In this study, we revealed unexpectedly that *V. terminalis* populations in the South Ryukyu Islands are ancient colonizers and exhibit remarkable genetic differentiation from probable source populations and inter-island divergences. These findings highlight the evolutionary distinctiveness and conservation value of populations in the South Ryukyu Islands, despite their geographical proximity to Taiwan and continental China, in contrast to the Central Ryukyu Islands, which are known for higher faunal endemism. To understand whether the differentiation pattern observed in *V. terminalis* is common among other taxa within the South Ryukyu Islands, further phylogeographic studies of various organisms inhabiting this region are necessary.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

NN designed the study, and conducted molecular analysis. NN, TO, and CPL collected the samples. NN and TS wrote the manuscript. All authors reviewed the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: <https://doi.org/10.2108/zs260028>)

Supplementary Figure S1. Maximum likelihood tree based on the MIG-seq datasets.

Supplementary Figure S2. Bayesian inference trees based on four MIG-seq data matrices with various minimum-taxon-coverage thresholds (min_samples_locus).

REFERENCES

- Austermann J, Mitrovica JX, Latychev K, Milne GA (2013) Barbados-based estimate of ice volume at Last Glacial Maximum affected by subducted plate. *Nat Geosci* 6: 553–557
- Avice JC (2000) *Phylogeography: The history and formation of species*. Harvard University Press, Cambridge MA
- Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, et al. (2019) BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLOS Comput Biol* 15: e1006650
- Chen S (2025) fastp 1.0: An ultra-fast all-round tool for FASTQ data quality control and preprocessing. *iMeta* 4: e70078
- Chou I, Lei ZR, Li L, Lu XL, Yao W (1997) The Cicadidae of China: (Homoptera: Cicadoidea). Tianze Eldoneio, Hong Kong
- Clement M, Posada D, Crandall KA (2000) TCS: A computer program to estimate gene genealogies. *Mol Ecol* 9: 1657–1659
- Eaton DAR, Overcast I (2020) ipyrad: Interactive assembly and analysis of RADseq datasets. *Bioinformatics* 36: 2592–2594
- Emerson BC (2002) Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. *Mol Ecol* 11: 951–966
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Mol Ecol* 14: 2611–2620
- Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New Algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59: 307–321
- Hayashi M, Saisho Y (2015) *The Cicadidae of Japan*. 2nd ed, Seibundo-shinko-sha, Tokyo
- Ho SYW, Lanfear R, Bromham L, Phillips MJ, Soubrier J, Rodrigo AG, et al. (2011) Time-dependent rates of molecular evolution. *Mol Ecol* 20: 3087–3101
- Ho SYW, Duchêne S, Molak M, Shapiro B (2015) Time-dependent estimates of molecular evolutionary rates: evidence and causes. *Mol Ecol* 24: 6007–6012
- Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS (2018) UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol* 35: 518–522
- Huang H, Knowles LL (2016) Unforeseen consequences of excluding missing data from next-generation sequences: simulation study of RAD sequences. *Syst Biol* 65: 357–365
- Iryu Y, Matsuda H, Machiyama H, Piller WE, Quinn TM, Mutti M (2006) Introductory perspective on the COREF Project. *Island Arc* 15: 393–406
- Kaito T, Toda M (2016) The biogeographical history of Asian keel-back snakes of the genus *Hebius* (Squamata: Colubridae: Natricinae) in the Ryukyu Archipelago, Japan. *Biol J Linn Soc* 118: 187–199
- Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermini LS (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* 14: 587–589
- Kanke E, Suzuki K, Sekiné K, Suzuki T, Hatta K, Yang M-M, et al. (2021) Unexpected population genetic structure in two closely related euphaeid damselflies from the Yaeyama and Taiwan Islands (Odonata: Euphaeidae). *Biol J Linn Soc* 134: 214–228
- Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform* 20: 1160–1166
- Lambeck K, Rouby H, Purcell A, Sun Y, Sambridge M (2014) Sea level and global ice volumes from the Last Glacial Maximum to

- the Holocene. *Proc Natl Acad Sci U S A* 111: 15296–15303
- Liu Y, Qiu Y, Wang X, Yang H, Hayashi M, Wei C (2018) Morphological variation, genetic differentiation and phylogeography of the East Asia cicada *Hyalessa maculaticollis* (Hemiptera: Cicadidae). *Syst Entomol* 43: 308–329
- Lu RS, Pan JJ, Lee TC (1981) Heat flow in the southwestern Okinawa Trough. *Earth Planet Sci Lett* 55: 299–310
- MacArthur RH, Wilson EO (1967) *The Theory of Island Biogeography*. REV-Revised, Princeton University Press
- MacArthur RH, Wilson EO (2001) *The Theory of Island Biogeography*. Reprint ed, Princeton University Press, Princeton
- Machida H, Ota Y, Kawana T, Moriaki H, Nagaoka S (Eds.) (2001) *Regional geomorphology of the Japanese islands, Vol. 7, Geomorphology of Kyushu and the Ryukyus*. University of Tokyo Press, Tokyo
- Miki M (1995) Two-phase opening model for the Okinawa Trough inferred from paleomagnetic study of the Ryukyu arc. *J Geophys Res Solid Earth* 100: 8169–8184
- Ministry of the Environment, Government of Japan (2015) *Red Data Book 2014: Threatened Wildlife of Japan, Insecta*. Gyosei Corporation, Tokyo
- Moritz C (1994) Defining 'Evolutionarily Significant Units' for conservation. *Trends Ecol Evol* 9: 373–375
- Moritz C (1999) Conservation units and translocations: strategies for conserving evolutionary processes. *Hereditas* 130: 217–228
- Motokawa M, Kajihara H (Eds.) (2017) *Species Diversity of Animals in Japan*. Springer Japan, Tokyo
- Moulds MS (2018) Cicada fossils (Cicadoidea: Tettigarctidae and Cicadidae) with a review of the named fossilised Cicadidae. *Zootaxa* 4438: 443–470
- Muraji M, Hirai Y, Akino T, Wakamura S, Arakaki N (2008) Genetic divergence among populations of the white grub beetle, *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae), distributed in the southern part of the Ryukyu Islands of Japan, detected from mitochondrial DNA sequences. *Appl Entomol Zool* 43: 287–292
- Nagata N, Toda M, Ohbayashi T, Hayashi M, Sota T (2021) Phylogeography of cicadas on continental and oceanic islands in the northwestern Pacific region. *J Biogeogr* 48: 3060–3071
- Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 32: 268–274
- Okamoto T (2017) Historical biogeography of the terrestrial reptiles of Japan: A comparative analysis of geographic ranges and molecular phylogenies. In "Species Diversity of Animals in Japan" Ed by M Motokawa, H Kajihara, Springer Japan, Tokyo, pp 135–163
- Osozawa S, Shiyake S, Fukuda H, Wakabayashi J (2017) Quaternary vicariance of *Platypleura* (Hemiptera: Cicadidae) in Japan, Ryukyu, and Taiwan islands. *Biol J Linn Soc* 121: 185–199
- Ota H (1998) Geographic patterns of endemism and speciation in amphibians and reptiles of the Ryukyu Archipelago, Japan, with special reference to their paleogeographical implications. *Popul Ecol* 40: 189–204
- Papadopolou A, Anastasiou I, Vogler AP (2010) Revisiting the insect mitochondrial molecular clock: the mid-Aegean trench calibration. *Mol Biol Evol* 27: 1659–1672
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol* 67: 901–904
- Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61: 539–542
- Roure B, Baurain D, Philippe H (2013) Impact of missing data on phylogenies inferred from empirical phylogenomic data sets. *Mol Biol Evol* 30: 197–214
- Sibuet J-C, Deffontaines B, Hsu S-K, Thareau N, Formal J-PL, Liu C-S (1998) Okinawa trough backarc basin: Early tectonic and magmatic evolution. *J Geophys Res Solid Earth* 103: 30245–30267
- Sota T, Kojima T, Lee YJ, Lin C-P (2016) Phylogenetic relationships of Japanese *Auritibicen* species (Hemiptera: Cicadidae: Cryptotympanini) inferred from mitochondrial and nuclear gene sequences. *Zool Sci* 33: 401–406
- Suyama Y, Matsuki Y (2015) MIG-seq: an effective PCR-based method for genome-wide single-nucleotide polymorphism genotyping using the next-generation sequencing platform. *Sci Rep* 5: 16963
- Suyama Y, Hirota SK, Matsuo A, Tsunamoto Y, Mitsuyuki C, Shimura A, et al. (2022) Complementary combination of multiplex high-throughput DNA sequencing for molecular phylogeny. *Ecol Res* 37: 171–181
- Takahashi T, Nagata N, Sota T (2014) Application of RAD-based phylogenetics to complex relationships among variously related taxa in a species flock. *Mol Phylogenet Evol* 80: 137–144
- Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132: 619–633
- Tominaga A, Matsui M, Eto K, Ota H (2015) Phylogeny and differentiation of wide-ranging Ryukyu Kajika frog *Buergeria japonica* (Amphibia: Rhacophoridae): geographic genetic pattern not simply explained by vicariance through strait formation. *Zool Sci* 32: 240–247
- Tominaga A, Matsui M, Shimoji N, Khonsue W, Wu C-S, Toda M, et al. (2019) Relict distribution of *Microhyla* (Amphibia: Microhylidae) in the Ryukyu Archipelago: High diversity in East Asia maintained by insularization. *Zool Scr* 48: 440–453
- Wang X, Hayashi M, Wei C (2018) Revision, phylogeny and phylogeography of the cicada genus *Auritibicen* (Hemiptera: Cicadidae), with descriptions of ten new species. *Eur J Entomol* 115: 53–103
- Watanabe N, Arai K, Otsubo M, Toda M, Tominaga A, Chiyonobu S, et al. (2023) Geological history of the land area between Okinawa Jima and Miyako Jima of the Ryukyu Islands, Japan, and its phylogeographical significance for the terrestrial organisms of these and adjacent islands. *Prog Earth Planet Sci* 10: 40
- Wessel P, Luis JF, Uieda L, Scharroo R, Wobbe F, Smith WHF, et al. (2019) The generic mapping tools version 6. *Geochem Geophys Geosyst* 20: 5556–5564
- Whittaker RJ, Fernandez-Palacios JM (2007) *Island Biogeography: Ecology, Evolution, and Conservation*. 2nd ed, Oxford University Press, Oxford ; New York
- Wong TKF, Ly-Trong N, Ren H, Demotte P, Baños H, Roger AJ, et al. (2026) IQ-TREE 3: phylogenomic inference software using complex evolutionary models. *Mol Biol Evol* (in press)
- Yang M, Wei C (2013) On the cicada genus *Nipponosemia* Kato, with description of one new species from China (Hemiptera, Cicadidae). *ZooKeys* 293: 19–39
- Yokoyama Y, Esat TM, Thompson WG, Thomas AL, Webster JM, Miyairi Y, et al. (2018) Rapid glaciation and a two-step sea level plunge into the Last Glacial Maximum. *Nature* 559: 603–607
- Yonekura N, Kaizuka S, Nogami M, Chinzei K (2001) *Regional Geomorphology of the Japanese Islands, Vol 1. Introduction to Japanese Geomorphology*. University of Tokyo Press, Tokyo

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