Cicada minimum age tree: Cryptic speciation and exponentially increasing base substitution rates in recent geologic time

[version 4; peer review: 1 approved, 3 approved with reservations, 1 not approved]

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Abstract

We developed a new time-calibrated tree incorporating primarily endemic along with some cryptic Ryukyu islands cicada data, following the recent publication of global cicada data by Marshall \textit{et al.} (2018), Łukasik \textit{et al.} (2018), Simon \textit{et al.} (2019), Price \textit{et al.} (2019), and Hill \textit{et al.} (2021). A total of 352 specimens were analyzed using BEAST v1. X software with a relaxed clock model. Fossil calibrations as old as Triassic were adopted largely following Johnson \textit{et al.} (2018) and Moulds (2018), and a Quaternary geological event calibration was adopted following Osozawa \textit{et al.} (2012, 2021b) and input into BEAST v1. X. Our timetree suggests that Tettigarctidae had a cicada basal lineage as old as 200.63 Ma, with Derotettiginae the next oldest lineage at 99.2 Ma. Tibicininae is a sister of the remaining subfamilies of Tettigomyiinae, Cicadettinae, and Cicadidae, and their species level differentiation and radiation began at 40.57 Ma. The Cicadinae clade consists of specific tribes with paraphyletic relationship, and the vicariance and adaptive radiation generated many cryptic species in each tribe. We estimated base substitution rate as a function of age, and the result strongly indicates an exponential increase of base substitution rate in recent geologic time. The consequent increase in cicada biodiversity, including generation of cryptic species in the Ryukyu Islands and surroundings, may have been driven by the generation and spreading of C4 grasses and coeval Quaternary climate change.

Open Peer Review

Approval Status

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1. Sungsik Kong\textsuperscript{1}, University of Wisconsin-Madison, Madison, USA
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4. Jacek Szwedo\textsuperscript{1}, University of Gdańsk, Gdańsk, Poland
5. Christopher H Dietrich, University of Illinois
Amendments from Version 3

We have newly cited the following reference:

Urban, J., Bartlett, C., & Cryan, J. (2010). Evolution of Delphacidae (Hemiptera: Fulgoroidea): Combined-evidence phylogenetics reveals the importance of grass host shifts. Systematic Entomology, 35(4), 678-691.

Additionally, we have added the following sentence at the end of the main text:

The rapid diversification of Delphacini is linked to shifts in host plants, particularly from C3 to C4 grasses within the Poaceae family (Urban et al., 2010).

Any further responses from the reviewers can be found at the end of the article.

Introduction

A phylogenetic tree of worldwide cicada was recently constructed by Marshall et al. (2018) and Simon et al. (2019) applying five concatenated sequences of mitochondrial COI and COII, and nuclear ARD1, EF-1a, and 18S rRNA, and by Łukasik et al. (2019) applying whole mitochondrial sequences for representative species in Marshall et al. (2018), and family level phylogenetic relation has been clarified. Although Tettigarctinae is an old diverged lineage and Derotettiginae may be next, their worldwide phylogenetic trees were not dated trees. Price et al. (2019; restricted to Platycleurini) and Hill et al. (2021; restricted to Asian Cicadinae) built partial (not worldwide) dated trees using BEAST v.2.5 (Bouckaert et al. 2014) applying COI and other sequences, but much of global cicada evolution has not been tied to absolute time.

The latest version of BEAST (Bayesian Evolutionary Analysis Sampling Trees v1. X) released on 10 June 2018 has a clear and simple age calibration protocol and function, updated from BEAST v.1. 7 (v.1. X ≒ v1. 8). This calibration involves applying times of the most recent common ancestors (tMRCA) of the ingroup species, i.e., applying the node age of a specific clade as a minimum age, in the associated software of BEAUti (Bayesian Evolutionary Analysis Utility; BEAST is the platform software). The maximum age constraint normally considered in MCMCtree (4.9e 2017; Yang 2007) was not clearly defined (Benton & Donoghue 2007; Marshall 2008; Hill et al. 2021), and simply handled by ignoring the maximum age in BEAST v.1. X calibration (Osozawa & Wakabayashi 2021; Osozawa et al. 2021a). We sought the oldest fossil of the corresponding node of specific clade with an assumption that the oldest fossil age was equivalent to the minimum age and equivalent to “tMRCA” in BEAST v1. X. Moulds (2018) reviewed the ages of cicada fossils. These redefined ages, ranging from 16.45 ± 0.45 Ma to 244.5 ± 2.5 Ma, were available for our fossil-based time calibrations in BEAST v1. X.

Klopfeinstein (2021) suggested that recent node dating approaches including Misof et al. (2014) and Montagna et al. (2019) have a credibility problem: different studies using the same molecular data and even the same sets of fossils regularly arrive at drastically different age estimates. She showed that a major reason for these differences is well known: even well-dated and firmly placed fossils can only provide a minimum age for a particular node. Therefore our fossil calibration applying solely minimum age (= tMRCA) was credible.

As shown by Osozawa et al. (2017a), Platycleurina and some other endemic cicadas in Ryukyu Islands can be rigidly calibrated by a geological event calibration at 1.55 ± 0.15 Ma (Quaternary; Osozawa et al. 2012). As shown by Osozawa et al. (2021a), Meimuna opalifera and some other endemic cicadas on Hachijo-jima, Izu-Bonin islands, can be calibrated by a geological event calibration of emergent age at 0.24 Ma (Quaternary; Osozawa et al. 2021b).

Through these analyses, we corroborated the classification and some rearrangement of species into four subfamilies of Tibicininae, Tettigomyiinae, Cicadettinae, and Cicadinae included in a family Cicadidae by Marshall et al. (2018) and Łukasik et al. (2018), and then estimated the splitting dates of these subfamilies, tribes (especially Cicadinae tribes after Hill et al. 2021), and species (Figures 1–3). In the BEAST analyses, we included Derotettix, a relict species of new subfamily Derotettiginae with the oldest lineage in family Cicadidae (Simon et al. 2019), and attempted to estimate the crown age (Figure 2). Comparison to the entire Hemipteroid insect timetree (Johnson et al., 2015) and entire insect timetree (Misof et al. 2014) could be conducted as an extension of this analysis, by adding other Hemiptera species as outgroup (Figures 1–3).

Our primary goal was to present the precise evolutionary history of all cicadas by constructing the BEAST timetree, and also taxonomic reconsiderations for Cicadinae tribes after Hill et al. (2021) and for Ryukyu endemic cicadas. Another BEAST v1. X function facilitates additional evaluation of the time variability of base substitution rates. Recent dating
Figure 1. Simplified cicada timetree built by BEAST v1.X, applying a 1,534 bp in maximum COI sequence. Inserted figure: Base substitution rate (= rate median shown at each node; substitutions per site per million year; s/s/myr) vs age (= posterior age shown at each node) diagram. Red approximate curve with its formula was drawn by an Excel function, with the intersection for the curve = 0.0128 s/s/myr, the rate median shown on Tracer.
Figure 2. Cicada timetree built by BEAST v1.1X, applying 1,534 bp COI sequence. OUTs with isolate number: our own analyzed specimens shown in Table 1, and others: from GenBank/DDJB. In outgroup Hemiptera: #: analyzed family by Johnson et al. (2018); % analyzed family by Misof et al. (2014). Inserted figure: Base substitution rate (= rate median shown at each node; substitutions per site per million year; s/s/myr) vs age (= posterior age shown at each node) diagram. Red approximate curve with its formula was drawn by Excel function, with the intersection for the curve = 0.0128 s/s/myr, the rate median shown on Tracer.
analyses employ a relaxed clock model, which allows each branch of a phylogenetic tree to have its own evolutionary rate (Drummond et al. 2012). Although the relaxed distribution can be set to lognormal in BEAUti, the rate of variability has not been documented prior to this study. The output figure of BEAST v1. X presents the base substitution rate and age at each node, and shows the acceleration of base substitution rates through the time.

Methods

Ethical approval

The present study did not concern invertebrate experiments and did not involve endangered or protected species. We obtained permission of collection in the Taroko National Park, Taiwan, from the director (No. 0990012881; August 1 ~ 11, 2010), with a help of Bor-ming Jahn, and permission of collection in the Tokara islands, from the Toshima village headman, from August 29 ~ September 8, 2010. Collection in the Ryukyu islands was before the designation of National Park since 2016. No specific permission was required outside the national parks and private areas.

Taxon sampling

Marshall et al. (2018), Simon et al. (2019), Łukasik et al. (2019) included comparatively few Asian cicada species in their analyses. We have previously published 70 isolate data from Platypelura primarily from the Japan, Ryukyu, and Taiwan islands (Osozawa et al. 2017a; our aim was the vicarince acted on each island population started at 1.55 Ma and the cryptic speciation), and 21 of these data were used in the present analyses by excluding duplicated sequence data.
| Isolate | Country | Species | Collection date | COI accession no. | 18SrRNA accession no. |
|---------|---------|---------|-----------------|------------------|----------------------|
| pp44a   | South Korea: Busan | Platypleura kempferi (Fabricius, 1794) | 13-07-2011 | Soichi Osozawa |
| pp53    | China: Zhejiang | Platypleura kempferi (Fabricius, 1794) | 31-07-2014 | Soichi Osozawa |
| pp26a   | Japan: Honshu, Tobi-shima | Platypleura kempferi (Fabricius, 1794) | 07-06-2012 | Akira Mihama |
| pp21    | Japan: Ryukyu, Ishigaki-jima | Platypleura yayeyamana Matsumura, 1917 | 05-07-2013 | Soichi Osozawa |
| pp27    | Japan: Ryukyu, Ishigaki-jima | Platypleura yayeyamana Matsumura, 1917 | 14-06-2014 | Soichi Osozawa |
| pp54    | Taiwan: Yangmingshan park | Platypleura kempferi (Fabricius, 1794) | 13-08-2014 | Akira Mihama |
| pp26     | Japan: Ryukyu, Ishigaki-jima | Platypleura yayeyamana Matsumura, 1917 | 05-07-2013 | Soichi Osozawa |
| pp54     | Japan: Ryukyu, Iriomote-jima | Platypleura yayeyamana Matsumura, 1917 | 14-06-2014 | Soichi Osozawa |
| pp9R     | Japan: Ryukyu, Miyako-jima | Platypleura miyakona Matsumura, 1917 | 03-07-2011 | Soichi Osozawa |
| pp40a    | Japan: Ryukyu, Amami-Oshima | Platypleura kempferi Matsumura, 1917 | 07-07-2014 | Soichi Osozawa |
| pp15d    | Japan: Ryukyu, Tokuno-shima | Platypleura kempferi Matsumura, 1917 | 05-06-2012 | Soichi Osozawa |
| pp32     | Japan: Ryukyu, Okinawa-jima | Platypleura kempferi Matsumura, 1917 | 20-06-2013 | Soichi Osozawa |
| pp10b    | Japan: Ryukyu, Kume-jima | Platypleura kempferi Matsumura, 1917 | 23-06-2011 | Soichi Osozawa |
| pp13b    | Japan: Ryukyu, Okinawa-jima | Platypleura kempferi Matsumura, 1917 | 22-06-2010 | Soichi Osozawa |
| pp23     | Japan: Ryukyu, Amami-Oshima | Platypleura kempferi Matsumura, 1917 | 23-06-2010 | Soichi Osozawa |
| pp35a    | Japan: Ryukyu, Okinoerabu-jima | Platypleura kempferi Matsumura, 1917 | 28-06-2013 | Soichi Osozawa |
| pp42     | Japan: Ryukyu, Amami-Oshima | Platypleura kempferi Matsumura, 1917 | 09-07-2013 | Soichi Osozawa |
| pp20     | Taiwan: Chiang Rai | Platypleura nobilis (Germar, 1830) | 05-08-2008 | Tetsuo Miyashita |
| pp1      | Philippines: Babuyan Island | Platypleura hilpa (Walker, 1850) | 05-07-2018 | Kei Nishiguro |
| pp56a    | Philippines: Luzon Island, Mt. Mayon | Platypleura hilpa (Walker, 1850) | 05-07-2014 | Soichi Osozawa |
| kum25    | Japan: Ryukyu, Amami-Oshima | Cryptotympana facialis (Walker, 1858) | 14-06-2016 | Soichi Osozawa |
| kum27A   | Japan: Ryukyu, Amami-Oshima | Cryptotympana facialis (Walker, 1858) | 26-06-2017 | Soichi Osozawa |
| kum15    | Japan: Ryukyu, Ishigaki-jima | Cryptotympana facialis (Walker, 1858) | 14-06-2017 | Soichi Osozawa |
| kum9     | Japan: Ryukyu, Kume-jima | Cryptotympana facialis (Walker, 1858) | 26-06-2017 | Soichi Osozawa |
| kum19    | Japan: Ryukyu, Ishigaki-jima | Cryptotympana facialis (Walker, 1858) | 14-06-2017 | Soichi Osozawa |
| kum33    | Japan: Ryukyu, Kume-jima | Cryptotympana facialis (Walker, 1858) | 14-06-2017 | Soichi Osozawa |
| isolate | species | collection date | collected by |
|---------|---------|----------------|--------------|
| kum36A  | Cryptotympana facialis (Walker, 1858) | 07/03/2018 | Hiroshi Irino |
| kum18   | Cryptotympana facialis (Walker, 1858) | 10/07/2018 | Minoru Saijo |
| kum34   | Cryptotympana facialis (Walker, 1858) | 21/07/2018 | Soichi Osozawa |
| kum12   | Cryptotympana facialis (Walker, 1858) | 04/07/2013 | Soichi Osozawa |
| kum2    | Cryptotympana facialis (Walker, 1858) | 29/08/2013 | Soichi Osozawa |
| kum3    | Cryptotympana facialis (Walker, 1858) | 12/07/2013 | Soichi Osozawa |
| kum13   | Cryptotympana atrata (Fabricius, 1775) | 07/03/2018 | Hiroshi Irino |
| kum14   | Cryptotympana atrata (Fabricius, 1775) | 10/07/2018 | Minoru Saijo |
| kum15   | Cryptotympana atrata (Fabricius, 1775) | 21/07/2018 | Soichi Osozawa |
| kum16   | Cryptotympana atrata (Fabricius, 1775) | 04/07/2013 | Soichi Osozawa |
| kum17   | Cryptotympana atrata (Fabricius, 1775) | 29/08/2013 | Soichi Osozawa |
| kum18   | Cryptotympana atrata (Fabricius, 1775) | 12/07/2013 | Soichi Osozawa |
| kum19   | Cryptotympana atrata (Fabricius, 1775) | 07/03/2018 | Hiroshi Irino |
| kum20   | Cryptotympana atrata (Fabricius, 1775) | 10/07/2018 | Minoru Saijo |
| kum21   | Cryptotympana atrata (Fabricius, 1775) | 21/07/2018 | Soichi Osozawa |
| kum22   | Cryptotympana atrata (Fabricius, 1775) | 04/07/2013 | Soichi Osozawa |
| kum23   | Cryptotympana atrata (Fabricius, 1775) | 29/08/2013 | Soichi Osozawa |
| kum24   | Cryptotympana atrata (Fabricius, 1775) | 12/07/2013 | Soichi Osozawa |

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**Note:**
- Table 1. Continued
- Each row represents a unique isolate with its associated species, accession numbers for COI and 18SrRNA, collection dates, and collectors.
- The table continues with similar entries for various species under different isolates.
| isolate | country | species | accession no. COI | accession no. BSRNA | collection date | collected by |
|---------|---------|---------|------------------|---------------------|-----------------|-------------|
| min-mn4 | Japan: Ryukyu, Iriomote-jima | Mogannia minuta (Matsumura, 1907) | LC5088130 | LC508905 | 30-04-2010 | Soichi Osozawa |
| min-mn5 | Japan: Ryukyu, Miyako-jima | Mogannia minuta (Matsumura, 1907) | LC5088131 | LC508906 | 25-04-2011 | Soichi Osozawa |
| min-mn6 | Japan: Ryukyu, Okinawa-jima, Tsuken-jima | Mogannia minuta (Matsumura, 1907) | LC5088132 | LC508907 | 11-05-2014 | Atsuko Nitta |
| min-mn7 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088133 | LC508908 | 18-06-2014 | Soichi Osozawa |
| min-mn8 | Japan: Ryukyu, Okinawa-jima, Tamagusuku | Mogannia minuta (Matsumura, 1907) | LC5088134 | LC508909 | 30-05-2014 | Satoru Nitta |
| min-mn9 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088135 | LC508910 | 06-07-2014 | Soichi Osozawa |
| min-mn10 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088136 | LC508911 | 18-06-2016 | Soichi Osozawa |
| min-mn11 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088137 | LC508912 | 06-07-2014 | Soichi Osozawa |
| min-mn12 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088138 | LC508913 | 18-06-2016 | Soichi Osozawa |
| min-mn13 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088139 | LC508914 | 28-08-2013 | Soichi Osozawa |
| min-mn14 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088140 | LC508915 | 07-06-2014 | Soichi Osozawa |
| min-mn15 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088141 | LC508916 | 09-06-2013 | Satoru Nitta |
| min-mn16 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088142 | LC508917 | 06-02-2014 | Soichi Osozawa |
| min-mn17 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088143 | LC508918 | 18-06-2014 | Soichi Osozawa |
| min-mn18 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088144 | LC508919 | 18-07-2013 | Soichi Osozawa |
| min-mn19 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088145 | LC508920 | 29-08-2013 | Soichi Osozawa |
| min-mn20 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088146 | LC508921 | 07-06-2014 | Soichi Osozawa |
| min-mn21 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088147 | LC508922 | 31-08-2010 | Soichi Osozawa |
| min-mn22 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088148 | LC508923 | 06-09-2014 | Soichi Osozawa |
| min-mn23 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088149 | LC508924 | 13-08-2014 | Soichi Osozawa |
| min-mn24 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088150 | LC508925 | 11-10-2017 | Haruo Fukuda |
| min-mn25 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088151 | LC508926 | 23-06-2010 | Soichi Osozawa |
| min-mn26 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088152 | LC508927 | 23-06-2010 | Soichi Osozawa |
| min-mn27 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088153 | LC508928 | 09-09-2014 | Soichi Osozawa |
| min-mn28 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088154 | LC508929 | 05-09-2014 | Soichi Osozawa |
| min-mn29 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088155 | LC508930 | 15-08-2014 | Soichi Osozawa |
| min-mn30 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088156 | LC508931 | 09-09-2015 | Soichi Osozawa |
| min-mn31 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088157 | LC508932 | 03-09-2010 | Soichi Osozawa |
| min-mn32 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088158 | LC508933 | 15-08-2014 | Soichi Osozawa |
| min-mn33 | Japan: Ryukyu, Okinawa-jima, Yagachi-jima | Mogannia minuta (Matsumura, 1907) | LC5088159 | LC508934 | 15-08-2014 | Soichi Osozawa |
| isolate | country | species | collection no. | accession no. | collection date | collector |
|---------|---------|---------|----------------|---------------|-----------------|------------|
| kur5    | Japan: Ryukyu, Okinawa-jima | Membrana kurikore (Matsumura, 1917) | LC508861 | LC508935 | 17-09-2015 | Soichi Osozawa |
| kur6    | Japan: Ryukyu, Okinawa-jima | Membrana kurikore (Matsumura, 1917) | LC508862 | LC508936 | 17-09-2015 | Soichi Osozawa |
| kur7    | Japan: Ryukyu, Okinawa-jima | Membrana kurikore (Matsumura, 1917) | LC508863 | LC508937 | 18-09-2015 | Soichi Osozawa |
| kur8    | Japan: Kyushu, Kagoshima, Cape Sata | Membrana kurikore (Matsumura, 1917) | LC508864 | LC508938 | 04-10-2017 | Haruo Fukuda |
| kur9    | Japan: Kyushu, Kagoshima, Cape Sata | Membrana kurikore (Matsumura, 1917) | LC508865 | LC508939 | 10-10-2017 | Haruo Fukuda |
| kur10   | Japan: Ryukyu, Kikai-jima | Membrana kurikore (Matsumura, 1917) | LC508866 | LC508940 | 10-11-2017 | Soichi Osozawa |
| TH-pomp1a | Taiwan: Hualien | Pomponia linearis Walker, 1850 | LC508867 | LC508941 | 04-06-2013 | Soichi Osozawa |
| TH-pomp11 | Japan: Ryukyu, Ishigaki-jima | Pomponia linearis Walker, 1850 | LC508868 | LC508942 | 21-08-2014 | Tadafumi Nakada |
| TH-pomp9a | China: Zhejiang | Pomponia linearis Walker, 1850 | LC508869 | LC508943 | 25-07-2014 | Akira Mishima |
| pomp3   | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508870 | LC508944 | 07-07-2013 | Soichi Osozawa |
| pomp5   | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508871 | LC508945 | 19-08-2014 | Akira Mishima |
| pomp7b  | China: Zhejiang | Tanna sp. | LC508872 | LC508946 | 24-08-2013 | Shusuke Osozawa |
| pomp10  | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508873 | LC508947 | 24-08-2013 | Shusuke Osozawa |
| pomp11  | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508874 | LC508948 | 24-08-2013 | Shusuke Osozawa |
| pomp12a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508875 | LC508949 | 26-06-2013 | Soichi Osozawa |
| pomp12b | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508876 | LC508950 | 26-06-2013 | Soichi Osozawa |
| pomp13a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508877 | LC508951 | 26-06-2013 | Soichi Osozawa |
| pomp14a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508878 | LC508952 | 05-07-2013 | Soichi Osozawa |
| pomp15a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508879 | LC508953 | 21-08-2014 | Soichi Osozawa |
| pomp16a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508880 | LC508954 | 16-06-2013 | Shusuke Osozawa |
| pomp17a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508881 | LC508955 | 16-06-2013 | Shusuke Osozawa |
| pomp18a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508882 | LC508956 | 16-06-2013 | Shusuke Osozawa |
| pomp19a | Japan: Honshu, Senkaido | Tanna japonensis Distant, 1892 | LC508883 | LC508957 | 20-08-2013 | Soichi Osozawa |
We also collected and analyzed cicada specimens, adding isolate data from 92 specimens. Accordingly, our own data total 21 + 92 = 113 specimens (Table 1). Note that we collected all the 35 species from Japan including the Ryukyu Islands, but excepting severely protected *Platyclea albivannata* (see Osozawa et al. 2017a; may be extinct without DNA sequence data) and *Meimuna boninensis* (see Osozawa et al. 2021a).

We incorporated representative sequence data from the GenBank/DDJBJ. This is because Tettigartciniae, Derotettiginae, Tibicininae, and Tettigomyiinae are not known from East Asia, and Cicadettinae has only two species of *Kosemia* in the Japan main islands. Thus to extend our analyses beyond East Asia, the Marshall et al. (2018) and Łukasik et al. (2019) data were essential for us. We combined our data from 113 East Asian specimens with data from 75 specimens from the studies of Marshall et al. (2018; including 20 East Asian specimens), and Łukasik et al. (2018; including 27 East Asia specimens). In addition, we incorporated data of 15 Platypleurini (other than *Platyclea*) from Price et al. (2019), and data of 149 Asian Cicadinae from recently published Hill et al. (2021). Accordingly we analyzed sequence data from 113 + 75 + 15 + 149 = 352 specimens.

*Platyclea* cicada (Osozawa et al. 2017a) experienced vicariance triggered by the 1.55 ± 0.15 Ma isolation of the Ryukyu, Japan, and Taiwan islands from Chinese continent (Osozawa et al. 2012), and we collected specimens from each island population for each *Platyclea* species. Similarly, we collected cicada specimens for the present analyses from each island population of *Mogannia* (Cicadettinae), and *Cryptotympana, Gruptopsaltria, Hylessa, Pomponia, Meimuna, Tanna, and Euterpnosia (Cicadinae)*. *Hylessa maculaticollis* was known to be affected by vicariance within China and Japan (Liu et al. 2018). Our 113 East Asian specimens consist primarily of these endemic and cryptic species inhabiting Japan, Taiwan, and the Ryukyu islands.

**DNA sequence**

COI and 18S rRNA sequence data from our collected 113 isolates, including *Platyclea* in Osozawa et al. (2017a), are shown in Table 1. Primers used, amplifications, and sequencing are given in Osozawa et al. (2017a). These sequence data were aligned by ClustalW in MEGA 5 (Tamura et al. 2011). The COI sequence data comprise 1,534 bp, and the 18S rRNA sequence 874 bp, with high enough resolution to construct a phylogenetic tree, as we showed previous analyses of *Platyclea* (Osozawa et al. 2017a). We did not analyze calmodulin in Osozawa et al. (2017a), because the resolution was insufficient. The COI data in Marshall et al. (2018) comprised 1,485 bp, comparable to ours. The COI data in Price et al. (2019) comprised 940 bp, and Hill et al. (2021) comprised 648 bp, comparable to ours, so we incorporated these data into our present analyses. Nuclear 18S rRNA shows less variation with much slower base substitution rate compared to mitochondrial COI (Osozawa et al. 2017a; COI: 0.0270 substitutions/site/myr, 18S rRNA: 0.000492 s/s/myr; strict clock model; solely calibrated by 1.55 ± 0.15 Ma following Osozawa et al. 2012). The tree topology was unaffected by 18S rRNA (Osozawa et al. 2017a), but 18S rRNA was included in the analyses in this paper.

We used COI and 18S rRNA sequence data, from 352 total specimens (239 from GenBank/DDJBJ + 113 of our own) for the COI timetree in Figures 1 and 2, and 155 total specimens (42 from GenBank/DDJBJ + 113 of our own) for the COI +18S rRNA timetree in Figure 3. The COI and 18S rRNA data in table 1 in Marshall et al. (2018) contain missing and incomparable data, so some of their GenBank/DDJBJ data were not applicable for our analyses. Whole mitochondrial sequence data by Łukasik et al. (2019) are included in our analyses as corresponding COI regions. Within COI sequence data in Marshall et al. (2018), 21 data for Cicadettinae and 14 data for Cicadinae were incorporated into our analyses. 18S rRNA sequence in Marshall et al. (2018) was used for only *Nabilistes heterochroma* (Tettigomyiinae) and *Platypedia putnamii* (Tibicininae). Only the COI sequence data in Price et al. (2019) for Platypleurini and in Hill et al. (2021) for Asian Cicadinae were applied to our study.

North American Cryptotympanini were analyzed by Hill et al. (2015), applying 1,467 bp of COI and 783 bp of nuclear EF-1a with sufficient resolution. Cicadettini, primarily from Australia, was analyzed by Marshall et al. (2016), applying 1,492 bp of COI and 1,047 bp of nuclear EF-1a also with sufficient resolution. Some of these COI sequence data were included in our analyses.

For our initial analysis, we constructed a minimum age tree solely applying COI sequence data (Figures 1 and 2; 352 specimens) that covers Tettigomyiinae and Tibicininae species. Following this analysis, we constructed a minimum age tree by applying both COI and 18S rRNA sequences (Figure 3; 155 specimens, i.e., 352 − 155 = 197 specimens lack 18S rRNA sequences). These analyses showed that topology and ages associated with the analyses were not impacted by inclusion or exclusion of 18S rRNA sequence data.
| Calibration point | Subfamily | Family | Infraorder | Order | Ingroup clade | Johnson et al. (2018) | Moulds (2018) | System | Stage | tMRCA (Ma) | Method | Paleontological reference | Geological reference |
|-------------------|-----------|--------|------------|-------|---------------|-----------------------|---------------------|--------|-------|-------------|--------|----------------------------|----------------------|
| A1                | † Vosegus triassicus | Aphidoidea | others | Aphidomorpha | Hemiptera | others (stem) | X | Bundsandstein | Triassic | Anisian | 244.5/2.5 | correlation | Szwedo & Nel (2011) | established |
| A2                | † Odrowazicoris polonicus | Belostomatidae | Nepomorpha | Lethocerus | Hemiptera | deyrollei (stem) | Z | Zagaje Formation | Jurassic | Hettangian | 200.3/1.0 | lacking | Popov (1996) | lacking |
| A3                | † Ctenopalinus sp. | Derotettiginae | Cicadomorpha | Ledra auditura | Hemiptera | (stem) | X | French amber | X | Green River | Palaeocene | Palaeocene | 61 ± 6 | dating | Zhang (1997) | Hong (1982) |
| A4                | † Cretogerris albianus | Gerridae | Gerromorpha | Aphis gossypii | Hemiptera | (stem) | X | French amber | X | French amber | Palaeocene | Palaeocene | 61 ± 6 | dating | Perrichot et al. (2005) | lacking |
| A5                | † Delphacidae | Fulgoromorpha | Hemiptera | Nilaparvata lugens | (stem) | X | Maifand Shand Formation | Jurassic | Hettangian | 203.1/1.0 | correlation | Whalley (1985) | established |
| B1                | † Liassocicada ignota | Tettigarctinae | Tettigarctidae | Epiophlebia superstes | Hemiptera | (stem) | Y | Dorset Jurassic | Hettangian | X | 244.5/2.5 | correlation | Whalley (1985) | established |
| C1                | † Burmacicada protera | Derotettiginae | Cicadomorpha | Derotettix mendosensis | Hemiptera | (stem) | Y | Colima Formation | Cretaceous | Hauterivian | 130.7/1.4 | correlation | Zhang (1997) | Hong (1982) |
| C2                | † Davispia bearcreekensis | Derotettiginae | Cicadomorpha | Tibicininae | Hemiptera | (stem) | Y | Fort Union Formation | Paleocene | Palaeocene | 57.6/1.6 | correlation | Cooper (1941) | Flores & Bader (1999) |
| D1                | † Lyristes sp. | Cicadinae | Cicadomorpha | Lyristes plebejus | Hemiptera | (stem) | Y | Seifhennersdorf Jurassic | Tithonian | 30.4/1.52 | K-Ar dating | Tietz et al. (1998) | Walther & Kryscy (2012) |
| D2                | † Cryptotympana incasa | Cicadinae | Cicadomorpha | Cryptotympana spp. | Hemiptera | (stem) | Y | Shanwang Miocene | Miocene | Langhian | 16.45/0.45 | correlation | Roček et al. (2011) | established |
| D3                | † Paracicadetta sp. | Cicadettinae | Cicadomorpha | Paracicadetta sp. | Hemiptera | (stem) | Y | Shanwang Miocene | Miocene | Langhian | 16.45/0.45 | correlation | Roček et al. (2011) | established |

**Table 2. Hemiptera, mostly Cicadomorpha calibrations. These are primarily fossil calibrations but include geological event calibrations. See main text and Figures 1–3.**
| Calibration point | Fossil | Subfamily | Family | Infraorder-suborder | Order | Ingroup clade | Formation | System | Stage | tMRCA (Ma) | Method | Paleontological reference | Geological reference |
|-------------------|--------|-----------|--------|--------------------|-------|---------------|-----------|--------|-------|-------------|--------|--------------------------|---------------------|
| G2†               | Hyalessa lapidescens | Cicadinae | Cicadidae | Cicadomorpha | Hemiptera | Hyalosa maculaticollis | Shanwang | Neogene | Langhian | 16.45 ± 0.45 | correlation | Zhang (1989) | Roček et al. (2011) |
| H†                | Meimuna protopalifera | Cicadinae | Cicadidae | Cicadomorpha | Hemiptera | Meimuna spp. | Zhirkinde | Neogene | Messinian | 6.4 ± 0.4 | fission track | Fujiyama (1969) | Fujiwara et al. (2003) |
| Q7                | geological event | Cicadinae | Cicadidae | Cicadomorpha | Hemiptera | Meimuna opalifera | Hachijo-jima | Quaternary | Chibanian | 0.024 ± 0.0024 | U-Pb dating | Osozawa et al. (2021b) | Osozawa et al. (2021b) |
| Q1-6, Q8-12       | geological event | Cicadinae | Cicadidae | Cicadomorpha | Hemiptera | Meimuna opalifera and others | Ryukyu | Quaternary | Calabrian | 1.55 ± 0.15 | biostratigraphy | Osozawa et al. (2012) | Osozawa et al. (2012) |
**Why was BEAST2 not used?**

Regarding BEAST2 (= *BEAST2, StarBEAST2), our approach diverged from previous studies such as Osozawa et al. (2016), Price et al. (2019), and Hill et al. (2021), who utilized BEAST v2.5 (Bouckaert et al., 2014). Instead, we opted for BEAST v1.8 and subsequently v1. X. While the calibration function in BEAUti of BEAST v2.5 bears similarities to BEAST v1. X, there are notable differences. In BEAST v2.5, the “Partition” tab only permits the input of individual sequence data. Consequently, if the sequence data are not concatenated, separate BEAST runs must be conducted for each set of applied sequence data (e.g., mitochondrial COI and nuclear 28S rRNA), as demonstrated by Osozawa et al. (2016).

The resulting tree files from these runs must then be combined into a single file using LogCombiner. However, when merging these tree files, the branches in the resultant tree become folded, reflecting the incongruent topology arising from different sequence data sources, such as mitochondrial COI and nuclear 28S rRNA. To mitigate this issue, Osozawa et al. (2016) employed DensiTree to obscure the foldings. Consequently, we discourage the usage of BEAST v2.5 due to the inconvenience and potential confusion caused by folded branches in the combined tree.

In the case of BEAST v2.6, which was released in May 2019, and BEAST v2.7, released in 2023 (Bouckaert et al., 2019), significant changes were made to the protocols. A tutorial for these versions can be found in [https://taming-the-beast.org/tutorials/starbeast2-tutorial](https://taming-the-beast.org/tutorials/starbeast2-tutorial). Notably, the inclusion of cladistic data alongside molecular data became possible with the implementation of total-evidence dating (Zhang et al., 2016). For extinct species, tip dating is set at their youngest fossil age, while for extant species, it is set at zero age. However, the fossil age is often poorly constrained, with minimum and maximum age ranges typically used. In BEAST v2.6 and v2.7, the calibration and node dating function that was implemented in BEAST v2.5 was abandoned, and node dating for extant species is solely based on applying and assuming the base substitution rate.

In the context of BEAST v2.6 and v2.7, it is important to clarify that the term “tip” does not refer to terminal nodes for extant species. Instead, it refers to the tip node representing extinct fossil species from ancient times (c.f., [https://beast.community/first_tutorial](https://beast.community/first_tutorial)). The tip date for fossil species is inferred from the fossil age, and it is worth noting that the age assigned is not necessarily the minimum age for the oldest fossil, but rather the youngest fossil, which is often poorly constrained. Additionally, it is crucial to ensure that these fossil species are indeed extinct, and determining their relative placement in relation to the lineage of extant species can be problematic, as it involves the concept of ghost lineage. It is important to understand that tip dating does not contribute to the quality of node dating.

**Phylogenetic analyses by BEAST v1. X**

A Bayesian inference (BI) tree (Figures 1–3) was constructed using the software BEAST v1. X, running BEAUti, BEAST, TreeAnnotator, and FigTree, in ascending order. Before operating the BEAST software, the BEAGLE Library must be downloaded. Tracer v.1.6 was applied for checking the calculation status and estimating the median base substitution rate.

For graphic explanation of the operation of this software, see Osozawa (2021a; BEAST v1.X tutorial, in a case of four cicada genera) at: dx.doi.org/10.17504/protocols.io.bq6mmz6.

In BEAUti, the following software settings were used (Appling Appendix BEAUti file, readers may run the platform software BEAST and check the protocol and reliability).

Partitions: Loading fasta files was by using the Import Data or plus button. Partitions defined by the COI and 18S rRNA gene sequences appeared in the Partition box (For Figure 3; COI file only for Figures 1 and 2). Note that COI and 18S rRNA partitions automatically appear in Partitions without employing PartitionFinder, and the partitioning is performed simply by applying each COI and 18S rRNA sequence, instead of the concatenating of genes by SeaView (Gouy et al., 2010) as done by Price et al. (2019) and Hill et al. (2021). Additional partitioning by PartitionFinder 2 (Lanfear et al., 2016) in MCMCTree and BEAST2 analyses is not required in the present BEAST1 analyses.

Taxa: Loading of taxa as ingroup was by using the plus button. The left screen: Taxon Set (monophyletic boxes were checked for all, and stem box were checked in case by case; see Table 2), and the right screen: Included monophyletic Taxa (= specific clade) and the resting Excluded Taxa in the central screen. As input in Figures 1–3, calibration dates were set in Priors bellow.

Tips and Traits: Default.

Sites: Substitution Model: HKY (Hasegawa, Kishino and Yano) model, Base frequencies: Empirical, Site Heterogenety Model: Gamma, Number of Gamma Categories: 4, Partition into codon positions: Off. The GTR model generates similar topology.
Clocks: Clock Type: Uncorrected relaxed clock, Relaxed Distribution: Lognormal. Uncorrelated relaxed clocks allow each branch of a phylogenetic tree to have its own evolutionary rate under log-normal distribution, and the node rate is the rate median of three branches (Drummond et al. 2006).

Trees: Tree Prior: Speciation: Yule Process.

Priors: tMRCA (time of MRCA) was input from the calibration point date as Prior Distribution: Normal, and as the Mean and Standard deviation. See below for Priors as detailed setting of age calibration.

Operators: Default.

MCMC: Length of chain: 10,000,000.

Running BEAST was done by incorporating xml input file made by BEAUti. The consequent tree was drawn by FigTree v1.4.2, for that, the tree files were input into TreeAnnotator. The 95% highest posterior density for confidence intervals of ages can be output in FigTree, but not shown in Figures 1–3 to avoid visual complexity. In FigTree, posterior probability (“posterior”), posterior age (“Node ages”), and “rate median” (not constant) can be output, and these are shown at each node in Figures 2 and 3. This rate related function was not used in any previous paper, and we found in this paper variable base substitution rates the time as suggested by the relaxed clock model of BEAST (Drummond et al. 2012). Consequently, we made base substitution rate (“rate median” shown at each node in FigTree) vs age (“Node age” shown at each node in FigTree) diagram (Figures 1–3 inset) using a function of Excel.

The inset in Figures 1–3 shows that the base substitution rate was relatively slow until the Quaternary higher rate. To evaluate whether the slow rate reflected saturation, we examined the relation between pairwise distance and number of transition or transversion for each gene, using the MEGA5 function (Tamura et al. 2011; Figure 4).

Fossil and geological event calibrations by BEAST v1. X
Calibrations points are shown on minimum age trees in Figures 1–3, and these dates were input in “Priors” in BEAUti as noted above; they are summarized below (Table 2). As noted above, corresponding ingroup species were included in ingroup taxa (= leaf node taxa in a specific clade) by Taxon Set on the Taxa screen in BEAUti.

Fossil calibrations are after Johnson et al. (2018) and Moulds (2018) (Table 2; Figures 1–3). For these fossil calibrations, some are based on radio-isotopic dating of the fossil-bearing strata, whereas others are based on biostratigraphy assigned to an age/stage on the geologic time scale, for which absolute age ranges are generally based on radio-isotopic dates of associated strata in key global localities. This time scale has been standardized by the International Commission on Stratigraphy (ICS) (www.stratigraphy.org) and the most recent version of the time scale is available at http://www.stratigraphy.org/index.php/scs-chart-timescale, and the explanatory paper related to the generation of the time scale is Cohen et al. (2013).
Calibration points Q1 to Q6 and Q8 to Q12 are after our geological event calibration that adopts a 1.55 Ma date (Osozawa et al. 2012). This geologic event calibration was used in previous studies of Platypleuridae cicadas (Osozawa et al. 2017a) and four cicada groups (Osozawa et al. 2021a).

The specific calibration points are as follows: tMRCA of Mogannia minuta (Q1), M. hebes (Q2), Cryptotympana facialis (Q3), dark winged Platypleura (Q4) and right winged Platypleura (Q5; Osozawa et al. 2017a), Graptosphaeria nigrofusca + G. bimaculata (Q6), Meimuna kauvoiae (Q7), M. oshimaensis + M. iwakii + M. goshizana (Q9), Tanna japonensis + T. japonensis ishigakiana + T. sozanesis + T. sp. (Q10) Euterpnosia chibensis + E. chibensis daitoensis + E. chibensis okinawana (Q11), E. iwakii + E. viridifrons + E. olivacea + E. gina + E. sp. (Q12): The date of the geological event, which records the isolation of the Ryukyu Islands from the Chinese mainland by the opening of the Okinawa trough that began (i.e., islands had separated from mainland and each other by this time) at 1.55 ± 0.15 Ma (Osozawa et al. 2012). The age assignment is from multiple biostratigraphic and radio-isotopic ages from the oldest marine strata on the landward side of the islands as well as the sides facing other islands, so that the age of such strata constrains the physical separation of the islands from the mainland and each other. There is no geologic evidence for land bridges that could have aided dispersal in the Ryukyu Islands.

Calibration point Q7 (Meimuna opalifera) is distinct from the above 1.55 ± 0.15 Ma event calibration. Hachijo oceanic island is a part of the Izu Trench, and we recently estimated the emergence time of Hachijo-jima as an island at 0.24 Ma (Osozawa et al. 2021b). This date is applicable for crown Meimuna opalifera on the Hachijo-jima + the Japan-Tokara islands (= Stem Meimuna opalifera on Hachijo-jima).

With the assumption that the oldest fossil age is equivalent tMRCA (= minimum age), the specific fossil calibration points and associated dates are as follows:

Calibration point A1: Crown Hemiptera: Fossils of Aphidoidea were reported from the French Bundsandstein (Szwedo & Nel 2011; Bashkuev et al. 2012) of Anisian age (244.5 ± 2.5 Ma).

A2: The oldest fossil Belostomatidae was reported from the Zagaje Formation, Poland (Popov 1996) of Hettangian age (200.3 ± 1.0 Ma).

A3: Fossil Ledridae (Zhang 1997) and fossil Cercopidae (Hong 1982) were recovered from the Jehol Biota of northern China. The Jehol Biota horizon has been dated by the Ar-Ar method on associated silicic tuff at 130.7 ± 1.0 Ma (He et al. 2006).

A4: Fossil Gerridae were recovered from French amber (Perrichot et al. 2005) of Albian age (107 ± 6 Ma).

A5: Fossil Delphacidae were found in the Green River Formation, USA (Grande 1980). Ar-Ar dating on silicic tuff within the formation yields ages of 53.5 – 48.5 Ma (weighted average age of 51.25 ± 0.31 Ma; Smith et al. 2003).

A6: Fossil Flatidae were found in the Maiz Gordo Formation, northwest Argentina (Petrulevičius 2011) of Paleocene age (61 ± 5 Ma).

Calibration point B: Stem Tettigarcinia: Oldest fossil of Tettigarcininae were found in strata Dorset, England (Whalley 1985) of Hettangian age (203.1 ± 1.0 Ma).

Calibration point C: Stem Derotettiginae: The preferred food of Derotettix mendozaensis is Amaranthaceae in Argentina (Simon et al. 2019), and this worldwide C4 plant was phylogenetically studied by Pirainen et al. (2017). This plant fossil was reported by Zucol et al. (2018), and the fossil-bearing horizon was dated by the Ar-Ar method at 49.512 ± 0.019 Ma (Eocene; Woodburne et al. 2014). However, fossil Burmacicada protera were found from Burmese amber (Poinar & Krisky 2011). Detrital zircons from the amber bearing matrix yielded a maximum depositional age U-Pb age of 98.79 ± 0.62 Ma, that was interpreted to closely approximate the actual depositional age on the basis of geologic relationships and associated fossils (Shi et al. 2012). We applied this older date of Burmese amber for stem Derotettiginae or crown Cicadidae.

Calibration point D: Stem Platypedia putnami (= crown Tibicina): Fossil Platypedia primigenia were found in the Florissant Formation, Colorado, USA, and the associated strata was dated by the Ar-Ar method at 35.15 ± 1.65 Ma (Mcintosh et al. 1992). However, we used an older crown date for crown Tibicina based on fossil Davispia bearcreekensis that were found in the Fort Union Formation, Montana, USA (Cooper 1941). The age of the enclosing
strata has been considered Thanetian in age (57.6 ± 1.6 Ma) (Flores & Bader 1999). Crown Cryptotympanini: Fossil *Hadoa grandioides* were also found in the Florissant Formation, Colorado, USA, but this calibration generated an unreasonable tree and was not adopted.

Calibration point E: Crown Cicadettinae: *Paracacideta oligocenica* (Boulard & Nel 1990) were recovered from deposits of Céreste, France, and this famous fossil locality was considered to be of Rupelian age (31 ± 2.9 Ma; Ducrœux et al. 1985).

Calibration point F: Stem *Lyristes plebejus*: Fossil *Lyristes* sp. were reported from Seifhennersdorf, Germany (Tietz et al. 1998), and associated strata was dated by the K-Ar method as 30.44 ± 1.52 Ma (Walther & Kvacek 2007).

Calibration point G: Crown *Cryptotympana*: Fossil *Cryptotympana incasa* and *C. miocenica* (G1), and also *Hyalella lapidescens* (G2) were found in Shanwang, Shandong, China (Zhang 1989; Zhang et al. 1994), and these strata are considered to be time correlative to the European MN5 mammalian unit (16.45 ± 0.45 Ma; Roček et al. 2011).

Calibration point H: Crown *Meimuna* spp.: Fossil *Meimuna protopalifera* were found in the Itamuro Formation, Tochigi, Japan (Fujitaya 1969; Yoshikawa 2005), and the zircon fission track age of correlative terrestrial strata of the Nashino Formation of the Sendai area is 6.4 ± 0.4 Ma (Fujitwara et al. 2008).

**Results**

**Hemiptera minimum age tree (Figure 1)**

Our timetree spans a range as old as ca. 250 Ma, and there is no evidence of saturation of mutations (Figure 4), suggesting our minimum age tree is robust and reliable.

Because the topology is concordant between Figures 1 and 2 (COI) and Figure 3 (COI + 16S rRNA), the following description follows Figure 2 with 352 specimens. Our analyses was concordant to the subfamily classification of Marshall et al. (2018), Lukasik et al. (2019), and Simon et al. (2019). Figures 1 and 2 also include data in Price et al. (2019) and Hill et al. (2021).

Hemiptera, including Cicadoidea, has a single common ancestor of 242.96 Ma, as calibrated by the 244.5 ± 2.5 Ma age reviewed above as A1. The dated tree of the outgroup Hemiptera calibrated by A1 to A6 was concordant to Johnson et al. (2018) and Misof et al. (2014).

In the Cicadoidea ingroup, Tettigarctidae was an old lineage that differentiated from Cicadidae at 200.63 Ma, as calibrated by 200.3 Ma (calibration point B), so Tettigarctidae is essentially a living fossil that has persisted since 200.63 Ma. We estimated a date of the common ancestor of two extant species of *Tettigarcta tomentosa* (Tasmania) and *T. crinita* (southeast Australia) at 13.96 Ma, and the youngest fossil of Tettigarctinae was reported from the Aquitanian (21.735 ± 1.295 Ma), southern New Zealand (Kaufuss & Moulds 2015). However, Tettigarctidae includes 19 extinct genera according to Kaufuss and Moulds (2015) and with many more genera according to Moulds (2018).

Simon et al. (2019) proposed a new subfamily Derotettiginae consisting of a single species of *Derotettix mendoensis*, which is a sister of the remaining Cicadinae species and the oldest lineage species in Cicadidae dated at 99.2 Ma, as calibrated by point C at 98.79 ± 0.62 Ma. Lukasik et al. (2018) showed such a basal lineage of *D. mendoensis* in Cicadidae.

Our timetree showed that Tibicininae is a sister of Tettigomyiinae + Cicadettinae + Cicadinae and differentiated at 66.15 Ma, and Tibicininae started differentiation at 57.31 Ma, as calibrated by point D at 57.6 ± 1.6 Ma. Tettigomyiinae is a sister of Cicadettinae and differentiated at 35.46 Ma, Tettigomyiinae + Cicadettinae is a sister of Cicadinae differentiated at 40.57 Ma. Cicadettinae started differentiation at 30.85 Ma, as calibrated by point E at 31 ± 2.9 Ma. Cicadinae started differentiation at 38.25 Ma. Differentiation of Tettigomyiinae + Cicadettinae took place simultaneously after 35.46 Ma.

A single common ancestor of Cicadidae except Derotettiginae started differentiation and speciation into Tibicininae, Tettigomyiinae, Cicadettinae, and Cicadinae at 66.15 Ma. Although the pre-Miocene fossil Cicadidae collectively include ten extinct genera, comprising *Davispsia* and *Lithiocicada* for Tobicininae, *Paracacidetta*, *Paleopsalta*, *Minyscaphus*, and *Miocenoprisa* for Cicadettinae, and *Burmacicada*, *Camuracicada*, *Tymocicada*, *Dominicicada* for Cicadinae, the remaining 23 genera post-Oligocene fossil cicadas are extant (Moulds 2018). Cicadidae, consisted of only one species but coexisted with a Tettigarctidae species between 200.63 and 66.15 Ma, and cicada biodiversity was extremely low during this period except for extinct species and *D. mendoensis*. 
In the Cicadettinae major clade, each tribe constitutes a distinct clade. In the Cicadinae major clade, apart from older five tribe clades containing only one specimen, six tribe clades of Platycleurini, Cryptotympanini, Psithyriristiini, Dundubini + Cosmopsaltriini, Polynoeurini + Sonatini, and Leptopsaltriini + Gaeanini are recognized. Discrepancies are addressed by reconsideration of taxonomy in the discussion.

The geologic calibration points Q1 to Q12 at 1.55 ± 0.15 Ma (and 0.24 Ma) apply to multi furcations that were recognized for Mogannia minuta and other cicadas endemic to in the Ryuku Islands and Taiwan (and in Hachijo-jima) as noted above. Each island or island group population was mostly genetically distinct, endemic, and cryptic, as shown for Platycleura in Osozawa et al. (2017a). This also applies to Meimuna opalifera on Hachijo oceanic island (Osozawa et al. 2021a,b). However, note that some cicadas were accidentally dispersed by super typhoons up to 1,000 km in modern and ancient times including Meimuna boninensis (Osozawa et al. 2021a).

Inconsistent cicada base substitution rate (Figures 1–3 insets)
Comparing base substitution rate vs age shows that the rate has not been constant; the rate appears to have exponentially increased into the Holocene. The data points, approximate curve, and associated equation are shown on the insets of Figures 1–3. The curves and associated rates are similar for analyses based on COI alone (Figures 1 and 2 insets), and combined COI + 18S rRNA (Figure 3 inset).

Figure 4 shows that even mitochondrial COI gene with rapid base substitution rate (Osozawa et al. 2017a) is never saturated toward the ancient time up to ca. 250 Ma.

Discussion
Taxonomic implications from the dated tree
Tibicininae is solely from North and South America with an exceptional occurrence from the Mediterranean region, but absent from Asia and Africa (+ Australia). The stem age is estimated at 66.15 Ma (Figures 1 and 2), and if we assume that Tibicininae was generated by vicariance its differentiation may have been influenced by the formation of the Atlantic Ocean. Marine magnetic anomalies on the Atlantic Ocean floor can be used to ascertain spreading history and separation of continents that resulted from this spreading. The configuration at Chron34 (84 Ma) after the Cretaceous magnetic quiet zone (long normal polarity epoch; superchron K-T at 118-84 Ma) was shown by Moulin et al. (2010), and the south Atlantic Ocean spread over 500 km (minimum distance between Africa and South America) at Chron 34 (84 Ma). The date of 84 Ma can be considered to be a starting date of continent level vicariance, which may have triggered the Tibicininae differentiations relative to especially Cicadinae shown in Figures 1 and 2.

In Cicadettinae, Prasini is a sister of Cicadettini. Muda karoivae in Prasini (Hayashi & Saisho 2011) is endemic and restricted to Okinawa-jima and Kume-jima, and represents as a sister of the similar species of Katoa taibaiensis on the Chinese mainland.

In the Moganniini clade, Nipponosemia terminalis (Matsumura 1913) (synonym: Vagitanus terminalis) is a sister of Mogannia spp. N. terminalis has been documented from the Yaeyama islands and Miyako-jima (endangered and protected), Ryukyu, and Taiwan (Figure 2 from the Taiwan specimen; another species of N. virecens is known from the Kaoshun peninsula, southern most Taiwan; Lee & Hayashi 2004), but Yang & Wei (2013) reported N. terminalis and other three Nipponosemia from China. A detailed phylogenetic study for these Nipponosemia species would be useful. The genitalia and morphological character are similar to Mogannia (Hayashi & Saisho 2011), concordant to the sister relationship with Mogannia. See Osozawa et al. (2021a) for the Mogannia minuta vicariant speciation on the Ryuku Islands and the accidental typhoon dispersals in recent and also ancient times. Mogannia hebes in northern Taiwan and in southern China has sister relationship reflecting vicariance by the Taiwan strait (Osozawa et al. 2011), and this species in southern Taiwan was differentiated relative to the northern Taiwan species reflecting vicariance triggered by the physical barriers of the Yilan basin and Lanyang valley (Osozawa et al. 2017b; http://kawasombg1.livedoor.blog/?p=26 and others).

We combined East Asian Platycleura data after Osozawa et al. (2017a) with mostly African Platycleurini data excluding Platycleura after Price et al. (2019), and the terminal node of the East Asian Platycleura in the Platycleurini clade suggests the possibility of a Gondwanan origin and dispersal to Far East of Japan and Ryukyu, and Taiwan (Price et al. 2019). See Osozawa et al. (2017a) for the Platycleura vicariant speciation (see below for the cryptic speciation) on the Ryuku Islands.

Tacua speciosa (Tacuini) represents the basal lineage of Cryptotympanini concordant with Marshall et al. (2018). In the Cryptotympanini clade, Auritibicen in Japan is the basal lineage, and Lyristes plebejus (synonym: Tibicen plebejus) in Croatia is the next. Asian Cryptotympana species is a sister of North American Noetibicen species (Hill et al. 2015).
Intercontinental dispersal by way of a Bering land bridge during Oligocene to Miocene climatic optima (Wu et al. 2015) was proposed for Papilionoidea butterflies feeding on Magnoliidae.

_Zammarra smaragdina_, Costa Rica, represents the basal lineage of the remaining major clades that are paraphyletic each other. _Distantialna splendidida_, renamed from _Tosena splendidida_ by Boulard (2009; referred in Hill et al. 2015), is represented by Tosenini, as a next basal lineage, distinct from another Tosenini of _Tosena melanopteryx_ in the Ptythyrstrini composite clade. _Tosena_ (Tosenini) and _Pomponia_ (Ptythyrstrini) has a sister relationship, and these are similar tribes (species level transfer may be needed; Duffels & Hayashi, 2006). _Pomponia backanensis_, northern Vietnam, was described by Pham et al. (2015). _Pomponia linearis_ on the Yaeyama islands and Taiwan, mildly differentiated each other as cryptic species, was renamed _P. yayeyamana_ based on Kato (1932). The original _P. linearis_ was reported from primarily Indochina, and has been treated as the _P. linearis_ complex, including cryptic Chinese and Indian populations (Hayashi & Saisho 2011). _Unipomponia decem_ (Ptythyrstrini?) was renamed _Pomponia decem_ (Lee & Sanborn 2010).

_Megapomponia_ (Lee & Sanborn 2010) was associated with the genera from Dundubiina (Hill et al. 2021), and included in the Dundubiini clade. Oceanian Cosmopsaltriini is a sister of Asian Dundubiini, reflecting large scale vicariance driven by bio-geographic barrier of the Wallacea line, as well as endemism within the islands by Oceanian arc fragmentations (Boer & Duffels 1996). In the Dundubiini clade, see Osozawa et al. (2021a) for the vicariant and cryptic speciation of _Meimuna kuroiwae_ on the Ryukyu islands and the accidental typhoon dispersals in recent and ancient times (including _Meimuna boniensis_ on the oceanic Bonin islands). See Osozawa et al. (2021a) for the vicariant and cryptic speciation of _Meimuna opalifera_ on the oceanic Hachijo-jima island (Osozawa et al. 2021b) by the accidental typhoon dispersal from the Japan continental islands. _Meimuna mongolica_ in Korea and China is the basal lineage relative to the sympatric _M. opalifera_. _Meimuna oshimensis_ endemic on the Amami and Okinawa islands (cryptic species), _Meimuna iwasaki_ endemic on the Yaeyama islands and Taiwan (no specimen collected from Taiwan specimen; cryptic species), and _Meimuna goshizana_ and _M. gakokizana_, other endemic species on Taiwan, were vicariously speciated or adaptively radiated in Taiwan.

_Hyalessa_ is in Sonatini was renamed from _Onoctympana_ in the distinct Oncotympanini. _Hyalessa maculaticollis_ in Japan and China (Liu et al. 2018) is deeply differentiated. Sonatini is a sister of Polyneurini (once included in Tosenini; noted in Hayashi & Saisho 2011), and these constitute the Polyneurini + Sonatini clade. _Graptopsaltria nigrofusca_ in Japan and _G. bimaculata_ on the Amami and Okinawa islands were vicariously speciated.

_Terpnosia_ was synonymised with _Leptopsaltria_ (discussed in Hill et al. 2021) constitutes the basal lineage with Thailand Leptopsaltriini in the Leptopsaltriini-Gaeanini major clade. _Kalabita operculata_, Malaysia, was a member of above-mentioned Platyleurini known to have diversified in Africa (Price et al. 2019), but this Asian species is included in the Leptopsaltriini + Gaeannini clade. _Trilar_ (2006) presented an adult photo of _K. operculata_ showing that it lacks the pronotum that characterizes Platyleurini. Furthermore, the spectrogram - oscillogram of its song is similar to those of _Euterpnosia_ spp., Leptopsaltriini, shown in Hayashi and Saisho (2011). Platyleurini both in African and Asia are monophyletic as noted above, and an exception is unreasonable. Hill et al. (2021) transferred _Kalabita_ Moulton, 1923 from Platyleurini to Leptopsaltriini. _Tosena paviei_ is a member of Tosenini, but is a sister of _Callogaeana guansiensis_ in Gaeannini, and renamed as _Vittagaeana paviei_ in Gaeannini by Hill et al. (2021). Both the species are from Vietnam, and constitute a Gaeannini clade with another Vietnam species of _Balinta cf. tenebricosa_ of Gaeannini. An interesting result is that Gaeannini including _T. paviei_ is not monophyletic, and paraphyletic in the Leptopsaltriini major clade. Hill et al. (2021) showed wing phenotypes of Gaeannini- Tosenini, which are distinct from phenotypes of Leptopsaltriini. Note that _Gaeana_ (Gaeannini) is also a sister of _Tanna_ (Leptopsaltriini) with different wing and chest phenotypes. _Tanna japonensis_ is differentiated between Japan and the isolated population on the Amami-Oshima island, and further differentiated from the isolated _Tanna japonensis ishigakiana_ population on Ishigaki-jima island. Taiwan yields _Tanna sozanensis_ (sister of _T. japonensis ishigakiana_ and the other seven _Tanna_ species (Chen 2011), might have adaptively radiated within the island. _Tanna_ in China is a sister of _T. sozanensis_. According to Hill et al. (2021), _Cicadmallus micheli_ is characterised by an unusual ‘hammer-head’ morphology but otherwise bears morphological relationships to Leptopsaltriini, and represents the basal lineage of Indochina _Terpnosia_ (Thai & Yang 2009) and East Asian _Euterpnosia_ of the Leptopsaltriini clade. See Osozawa et al. (2021a) for the vicariant and cryptic speciation for _Euterpnosia_ for the northern population on Japan-Amami-Okinawa and the southern population on Yaeyama-Taiwan, as well as accidental typhoon dispersal in ancient times (_Euterpnosia chibensis datiostens_ on the oceanic Daito islands from Tokuno-shima continental island). Taiwan yields _Euterpnosia gina, E. olivacea, E. viridifrons_, and other 12 _Euterpnosia_ species (Chen 2011), and may have adaptively radiated within the island.
Recently increased cicada biodiversity

Hemipteroid insects of Psocodea, Thysanoptera, and the subject of this study, Hemiptera, include 120,000 described species which comprise over 10% of known insect diversity; they date back to 400 Ma (Hemiptera: 300 Ma; Johnson et al. 2018). Johnson et al. (2018) estimated that differentiation into species took place primarily in the Cretaceous, including Cercopoidea, Gerridae, Flatidae, and Cicadoidea, which are common to our analyses. However, they analyzed only two to nine taxa, in contrast to the 344 taxa of Cicadoidea and 8 taxa for other Hemiptera of our analyses. Misof et al. (2014) estimated mostly pre-Paleogene dates of differentiation into species including Cercopoidea, Aphididae, and Delphacidae, concordant with our analyses, with less than 13 taxa analyzed. Their higher-level phylogeny suggested long branches and an old lineage of each super family species concordant with ours, but did not suggest the geologically recent increase in insect diversity apparent from our analyses of 352 Hemiptera taxa (Figure 2).

In Figure 2, ingroup Cicadidae, excluding Derotettiginae, underwent extensive differentiation into 341 taxa after 66.15 Ma, mostly after 40.57 Ma, leading to increasing biodiversity of Cicadidae, although Price et al. (2019) suggested that number of lineages saturated in the Pleistocene. Cicadidae consisted of only two species including D. mendosensis between 99.2 and 66.15 Ma, although Cicadidae contains many extinct species that remain to be identified as fossils (Moulds 2018).

Cryptic species on each island of Ryukyu chain are typical examples of increased biodiversity. For example, Platypleura kaempferi in the Amami and Okinawa islands has light colored wings, contrasting with dark colored wings in Japan-Korea-China and Taiwan, and the clades are distinct from each other (Osozawa et al. 2017a). P. kaempferi is not a single species but includes at least two cryptic species of light or dark winged Platypleura. Cicadas calibrated by other Quaternary calibration points include cryptic species, which also contributed to increasing biodiversity. The Okinawa trough is currently spreading (widening) and the Ryukyu islands are separating from the Chinese mainland. Accordingly, vicariant speciation and radiation is in progress, which is also contributing increasing biodiversity. On the Chinese mainland, Hyalessa maculaticollis and Platypleura hilpa extensively radiated to form cryptic species (Liu et al. 2018, 2020).

Exponentially increased base substitution rate as a factor of Hemiptera diversity, and their possible causes

Figures 1–3 insets show a large range of base substitution rates for different time periods, at variance with the constant molecular clock hypothesis (relatively constant rate over time; Ho 2008). The trend in base substitution rates shows an exponential increase into the Holocene.

Such an increase in base substitution rate was first shown for taxa such as primates by Ho et al. (2005) who showed that a Quaternary calibration date resulted in a more a rapid base substitution rate than that associated with an older calibration date. They employed an older version of BEAST (v1. 3; Drummond & Rambaut 2003) that required repeated runs, applying a date at each calibration point. In contrast, BEAST v1. X, used in our analyses, can simultaneously apply multiple calibration points, as we have done using dates ranging from the Triassic to the Quaternary. As a result, the calculated increasing rate of base substitution in our analyses is not an artifact of a Quaternary calibration, but is constrained by multiple age calibrations across a wide range of geologic time. Therefore, although the base substitution rate trendlines and associated equations of Ho et al. (2005) are similar to ours, their timetrees do not reflect the changing of base substitution rates through time, but rather reflect a constant base substitution rate as if constrained by a strict molecular clock. A similar analysis was done for beetles in the Aegean region by Papadopoulo et al. (2010).

The increasing base substitution rate is apparently associated with the recently increasing cicada diversity, expansion, and radiation (in Figure 2 timetree) that started at 40.57 Ma. The rapid diversification of Delphacini is linked to shifts in host plants, particularly from C3 to C4 grasses within the Poaceae family (Urban et al. 2010). The timing of the most rapid diversification coincides with Quaternary environmental change, marked by the start of glacial-interglacial cycles. The initiation of Quaternary glaciations may have been triggered by rapid expansion of land grasses (Poales), that led to increased carbon fixation that decreased atmospheric CO2 concentrations, because of the high efficiency of CO2 fixation of such C4 plants (Sage 2004; Taira 2007). C4 Poales appeared and began diversification during the Oligocene (23 – 33.9 Ma) based on molecular clock approach, and after 14.5 Ma based on fossil evidence (Sage 2004). We estimated 20.35 Ma by our Angiospermae timetree, that also employed BEAST v1. X with robust plant fossil calibrations (Osozawa et al. 2021c).

Food plants of D. mendosensis are, however, C4 dicots of Amaranthaceae (see figure 9 in Sage 2004) and Chenopodiaceae in degraded salt-plain habitats in arid regions of central Argentina (Simón et al. 2019). These dicot fossils and C4 monocot fossils of Poales grass (Chloridoideae) were reported from the Eocene in Patagonia by Zucol et al. (2018), and the
fossil horizon was dated by the Ar-Ar method at 49.512 ± 0.019 Ma (Woodburne et al. 2014). The C4 photosynthetic pathway began at ca. 50 Ma in South America, earlier than elsewhere. For Chloridoideae, however, transition from C3 to C4 photosynthesis occurred in the Oligocene (23–33.9 Ma) as reported by Christin et al. (2008), consistent with our estimate for C4 dicots at 31.92 Ma (Osozawa et al. 2021c), whereas Sage (2004) suggested a fossil date at 14.5 Ma as noted above.

The trigger of increasing biodiversity may have been the generation and radiation of C4 plants and development of grasslands on the Earth since the Oligocene or perhaps more definitively since middle Miocene, by decreasing atmospheric CO2 concentrations. This may have led to the start of Quaternary ice ages and consequent adaptive radiation and increasing base substitution (θ) mutation rates. Thus, biologic activity, including spreading C4 grasses may have significantly impacted Earth’s environment.

Data availability
Sequence data in Table 1 are found in GenBank/DDBJ by incorporating the accession number. The xml file generated by BEAUti for running the BEAST platform software contains all the utilized sequence data and can be obtained upon email request to the senior author.

Acknowledgements
We thank Chris Simon and David Marshall for privately offering Tetrigarcta sequence data (later released in GenBank/DDBJ). David Marshall also privately offered Asian Cicadinae sequence data of Hill et al. (2021) and reprint of Price et al. (2019). We pay our respects to their extensive and perfect taxon samplings including in protected countries with necessary permissions. We thank the collectors shown in Table 1. Bor-ming Jahn (Taiwan University; deceased 1 December, 2016), Ping-Shih Yang (Taiwan University), Chin-Ho Tsai (National Dong Hwa University), and Jen-Zon Ho (deceased 2018) and Hua-Tf Feng (Endemic Species Research Institute) supported sample collections and obtained permission to collect in Taiwan. This project was partly financed through the Osozawa Fund (former), Tohoku University. We thank Keiji Nunohara (Nunohara Office for Geological Survey), Kohei Sugawara (Ecofarm GSK), Atsushi Momose (Mitsubishi Material Techno Corporation), CTI Engineering Co., Ltd., and NEWJEC, Inc. for contributing to this fund. This work was supported by Japan Society for the Promotion of Science, “Extrusion Wedge of the Sambagawa High P-T Metamorphic Rocks,” in the form of a grant awarded to the senior author (20540441).

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Open Peer Review

This interesting paper provides a detailed timetree for cicadas, combining several new minimum age calibrations for recent speciation events in Asia to some calibrations for some older divergences published previously. The methods are described in detail and the conclusions seem justified. Of particular interest is the exponential increase in nucleotide substitution rates for the gene regions used to estimate the timetree. The main remaining problem with the manuscript is that it needs considerable editing to improve the English and clarify some points that are not currently clear as written, e.g., in the abstract it is stated "The Cicadinae clade consists of specific tribes with paraphyletic relationship"; does this mean that the tribes are each paraphyletic, some tribes gave rise to others, or something else? Earlier in the abstract the statement "Tettigarctidae had a cicada basal lineage as old as 200.63 Ma"; the meaning is again unclear but presumably this could be stated more precisely as "Tettigarctidae are sister to the remaining cicadas..."

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Systematics, taxonomy, paleontology and phylogenomics of Auchenorrhyncha.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 14 November 2024
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Jacek Szwedo
University of Gdańsk, Gdańsk, Poland

The aims of research and methods are adequate. The results are presented accurately and with justified conclusions. These are well supported by the provided data, selected calibration points and results, and provided tables and figures.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Yes

**Competing Interests:** No competing interests were disclosed.
Reviewer Expertise: fossils, phylogeny, palaeoecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 3

Reviewer Report 25 July 2024

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Jacek Szwedo
University of Gdańsk, Gdańsk, Poland

There are several new data and interpretations concerning the classification and fossil record of cicadas and Hemiptera s whole - see attached file. Main issue is the selection and estimation of age taken for taxa used for calibration, these are to be revised and updated according to current data, classification proposals or if other than the oldest available taxa selected the reasons should be clearly explained. In my opinion the stem and crown groups should be clearly stated and pointed which are analysed and then discussed. The text is not easy to read, especially for those not familiar with numerous existing phylogenetic, classifications and relationships hypotheses. The better calibration points selection, and use of new data (e.g. Bucher et al. 2023, Jiang et al. 2024) can change the received topologies. There are several taxonomic decisions which are not clearly explained or references, e.g. transfer of Burmacicada protera from Cicadinae to Derotettiginae. Interpretation and discussion of data and results must be more detailed - the Clypeata is the only extant clade of extremely diversified Cicadomorpha, with most of the lineages gone extinct, this leads to misunderstandings of relationships based exclusively on molecular data, as these are available only for crown, extant, groups. This reservation must be taken for higher taxa especially. A comparison with evolutionary traits observed in Delphacidae planthoppers (Urban et al 2010 [Ref-11]) closely related to Poaceae can enrich the discussion and maybe similar phaenomena can be found and discussed.

Please refer to the following attachment for my additional comments on your article submission:
https://f1000research.s3.amazonaws.com/linked/665193.Jaeck-Reviewer_attachment.pdf

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Are the conclusions drawn adequately supported by the results? 
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*Competing Interests*: No competing interests were disclosed.

*Reviewer Expertise*: fossils, phylogeny, palaeoecology
I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

There are several new data and interpretations concerning the classification and fossil record of cicadas and Hemiptera s whole - see attached file. --NOT readable.

Main issue is the selection and estimation of age taken for taxa used for calibration, these are to be revised and updated according to current data, classification proposals or if other than the oldest available taxa selected the reasons should be clearly explained. In my opinion the stem and crown groups should be clearly stated and pointed which are analysed and then discussed. The text is not easy to read, especially for those not familiar with numerous existing phylogenetic, classifications and relationships hypotheses. The better calibration points selection, and use of new data (e.g. Bucher et al. 2023, Jiang et al. 2024) can change the received topologies. There are several taxonomic decisions which are not clearly explained or references, e.g. transfer of Burmacicada protera from Cicadinae to Derotettiginae. Interpretation and discussion of data and results must be more detailed - the Clypeata is the only extant clade of extremely diversified Cicadomorpha, with most of the lineages gone extinct, this leads to misunderstandings of relationships based exclusively on molecular data, as these are available only for crown, extant, groups. This reservation must be taken for higher taxa especially. A comparison with evolutionary traits observed in Delphacidae planthoppers (Urban et al 2010 [Ref-11]) closely related to Poaceae can enrich the discussion and maybe similar phaenomena can be found and discussed.

Thank you for review.

Bucher et al. (2023) provided the age of Cicadoidea fossils in Burmage amber as 98.79 Ma. I have used this age as calibration point C, representing the stem age of Derotettiginae (i.e., the stem of Cicadoidea).
Bucher et al. (2023) and other studies provide estimated ages based on node dating, which are not suitable for the present fossil age calibration. Stem and crown ages should be determined using fossil evidence. Jiang et al. (2024) and other recommended articles may include such indirect ages, but I used geologically robust fossil ages, including those from Burmese amber, for calibration.

Transfer of Burmacicada protera from Cicadinae to Derotettiginae.
--- The transfer of Burmacicada protera from Cicadinae to Derotettiginae was originally noted as Derotettiginae in Table 2.

the Clypeata is the only extant clade of extremely diversified Cicadomorpha, with most of the lineages gone extinct, this leads to misunderstandings of relationships based exclusively on molecular data, as these are available only for crown, extant, groups.
--- I have already included Cercopidae (Cicadomorpha, which comprises extant species other than those in Cicadoidea).
“A3: Fossil Ledridae (Zhang 1997) and fossil Cercopidae (Hong 1982) were recovered from the Jehol Biota of northern China. The Jehol Biota horizon has been dated by the Ar-Ar method on associated silicic tuff at 130.7 ± 1.4 Ma (He et al. 2006).”

I cited Urban et al (2010) at the end of discussion.
“The rapid diversification of Delphacini is linked to shifts in host plants, particularly from C3 to C4 grasses within the Poaceae family (Urban et al., 2010).”

Please refer to the following attachment for my additional comments on your article submission: https://f1000research.s3.amazonaws.com/linked/665193.Jaeck-Reviewer_attachment.pdf
Note that the data in the attachment may be lost or unreadable.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

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Competing Interests
No competing interests were disclosed.
This study primarily focused on estimating the divergence times of Cicadoidea. Compared to previous studies, the strength of this research lies in its inclusion of local data and data from enigmatic cicadas of the Ryukyu Islands. Exploring cicada biodiversity in the Ryukyu Islands and surrounding areas, as well as their historical evolution, is quite intriguing. A highlight of the paper is the addition of 92 new data points. Additionally, the discussion includes extensive content on cicada biodiversity and evolutionary history, which is very impressive. However, this manuscript still has some issues, such as poor readability of the figures.

I think some of the reasonable suggestions from the previous reviewers haven't been addressed by the authors. Such as "A typo in Abstract: parapheletic -> paraphyletic.", "It would be better to replace "country" with "sampling locality" in the title of Table 1". I haven't seen evidence of them being addressed in the revisions.

In addition to the above, I suggest the public sharing and presentation of intermediate data, such as alignment matrices, input files and output tree for BEAST. I appreciate and agree with the suggestion to provide a detailed description of the software analysis procedures. The materials and methods section in this manuscript reads more like a step-by-step tutorial rather than a description typically found in research articles. If a thorough description is desired, perhaps it would be more suitable to include it in the supplementary materials.

The following comments elaborate some places where the improvements can be made.

1. There's confusion in the description. The DNA sequences used are COI and 18S rRNA. However, in both the ‘Materials and Methods’ and ‘Results sections’, the description of Figure 3 mentions COI and 16S rRNA as well as COI and 18S rRNA simultaneously.
2. The captions for Figures 1 and 3 lack explanations for the different coloured asterisks, especially the red pentagon and the meaning of the small branches on the right side of the images. 
3. In Figures 1–3, the scatter plot of base substitution rate needs to specify the data used for the X-axis and Y-axis. In the Materials and Methods, I believe it’s important to explain the conceptual rationale behind the design of base substitution rate. 
4. The estimated divergence time ranges mentioned in the manuscript should ideally maintain the same number of decimal places for consistency.

Is the work clearly and accurately presented and does it cite the current literature? 
Partly

Is the study design appropriate and is the work technically sound? 
Yes

Are sufficient details of methods and analysis provided to allow replication by others? 
Partly

If applicable, is the statistical analysis and its interpretation appropriate? 
Yes

Are all the source data underlying the results available to ensure full reproducibility? 
Partly

Are the conclusions drawn adequately supported by the results? 
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Insect Phylogeny; Genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
In this study, Osozawa and Wakabayashi examine the timing of the splits within the Cicadas using a Bayesian framework for phylogenetic analysis. The motivation for the study and data are well presented. However, the methods have large amounts of superfluous information that makes them appear confusing. Overall, the manuscript is going in a good direction, but requires substantial streamlining.

My main comment is on the description of BEAST. For the purposes of this study, BEAST v1 and 2 should make very little difference. In fact, it would be surprising to find any difference in results whatsoever, unless the analyses failed to converge to stationarity. The differences highlighted by the authors, such as in the BEAUti interface, are not meaningful to the analyses. Therefore, the authors should consider removing any comparison between the software, and only mention BEAST once and very briefly in the methods.

Far more important is the models chosen for analysis using BEAST. At the moment these details seem buried in between the discussion about the software versions. I suggest the authors replace any sections about the BEAST versions, and exclude BEAST from section headings, leaving simply a section on "Molecular dating". Importantly, the authors do not mention how they assessed convergence of the MCMC chain to stationarity (e.g., effective sample sizes, multiple runs, and visual assessment, or similar).

There is substantial redundancy in the figures, which is confusing to the untrained eye. The authors should consider using a single main tree figure with the primary results and icons to indicate which analyses were congruent. This would make the study far more succinct and intuitive.

The excessive emphasis on the software versions obscures the true emphasis of the study: Cicada evolution. The authors should consider re-framing the Abstract and Introduction to focus on the previous biological findings on the topic. The methodological considerations are secondary and should be left to the methods. Otherwise, the study appears to primarily focus on a debate about methods, which is not the case. The emphasis should be on the novel molecular and fossil data, which are the true major source of novel insights in the study.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes
Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Phylogenetic modelling, molecular evolution, molecular dating.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

**Author Response 06 Aug 2023**

**Soichi Osozawa**

copyright
02 Aug 2023 | for Version 1
David A Duchêne
Center for Evolutionary Hologenomics, University of Copenhagen, Copenhagen, Capital Region of Denmark, Denmark

In this study, Osozawa and Wakabayashi examine the timing of the splits within the Cicadas using a Bayesian framework for phylogenetic analysis. The motivation for the study and data are well presented. However, the methods have large amounts of superfluous information that makes them appear confusing. Overall, the manuscript is going in a good direction, but requires substantial streamlining.

Thank you for your review and approval. I plan to compare MCMCTree to BEAST v1 and eagerly await the MCMCTree GUI for Mac. If you are involved in the development of MCMCTree, I appreciate your contributions to the field.

My main comment is on the description of BEAST. For the purposes of this study, BEAST v1 and 2 should make very little difference. In fact, it would be surprising to find any difference in results whatsoever, unless the analyses failed to converge to stationarity. The differences highlighted by the authors, such as in the BEAUti interface, are not meaningful to the analyses. Therefore, the authors should consider removing any comparison between the software, and only mention BEAST once and very briefly in the methods.

Regarding the comparison to BEAST v2, I understand that it has undergone significant modifications and is distinct from v1, even though they share some developers. If you prefer, you may choose to use BEAST v1 for this study.

Far more important is the models chosen for analysis using BEAST. At the moment these details seem buried in between the discussion about the software versions. I suggest the authors replace any sections about the BEAST versions, and exclude BEAST from section headings, leaving simply a section on "Molecular dating". Importantly, the authors do not
mention how they assessed convergence of the MCMC chain to stationarity (e.g., effective sample sizes, multiple runs, and visual assessment, or similar).

Title "Molecular dating" may be a suggestion to avoid negative review for us, but I would like to describe details.

It is important to emphasize that a larger sample size and genome size, along with an increased MCMC chain, is unrelated to dating and calibrating the tree. The MCMC chain is retained at its default settings.

There is substantial redundancy in the figures, which is confusing to the untrained eye. The authors should consider using a single main tree figure with the primary results and icons to indicate which analyses were congruent. This would make the study far more succinct and intuitive.

I have simplified Fig. 1 to the best of my ability. The node data, including base substitution rates, may be visualized in FigTree, created also by MCMCTree.

The excessive emphasis on the software versions obscures the true emphasis of the study: Cicada evolution. The authors should consider re-framing the Abstract and Introduction to focus on the previous biological findings on the topic. The methodological considerations are secondary and should be left to the methods. Otherwise, the study appears to primarily focus on a debate about methods, which is not the case. The emphasis should be on the novel molecular and fossil data, which are the true major source of novel insights in the study.

I am emphasizing the increase in base substitution rate towards the present. To achieve this, the fossil dating process in BEAST v1 is thoroughly described. However, it is important to note that dating by MCMCTree and BEAST v2 does not align with our results, possibly due to the impact of maximum age assumptions.

Competing Interests: No competing interest.
existing data presented in the previous studies. The authors show improved taxonomy of some of the major cicada groups. Moreover the authors suggest an exponential increase of base substitution rate in recent geologic time. While the content of the manuscript is interesting, it requires substantial improvement and is not ready for indexing. The following comments elaborate some places where the improvements can be made.

- In general, the manuscript is not easy to understand. It is understandable that English may not be the authors' first language, however, an academic manuscript must be written clearly. For example, the last sentence of Abstract seems to be not connecting with the previous sentences. The first sentence of Introduction is excessively long.

- In figure 1, is is not clear what it means by "maximum" COI sequence. It is not clear what is the inserted figure. I assume it is the plot on the left side with white background. These are also called as 'incets' later in the manuscript. How about labeling these as a) and b)? Here, y-axis is not labeled. It is not clear what it means by the intersection for the curve and what it represents biologically. Green or red starts and the values corresponding to it is never explained in the caption.

- Basically figure 2 is an identical tree as what is presented in figure 1. Again, the caption is very difficult to understand. Red and green stars and the values associated with them are never explained. The three values at the node in the tree are never explained.

- Figure 3 has similar problems. More work is needed in presenting the trees. Also, the authors mention in the caption of Figure 3 that the rate is a 'little' slower than that of in Figures 1 and 2, but it is what it means by 'a little', which is a subjective description.

- The authors mention that the tree topology (based on COI) was unaffected by 18S rRNA. In other words, Figures 1, 2, and 3 are the same trees. If this is true, I am not sure presenting three identical trees is a good practice. Moreover, the authors must explore if the congruence occurred because they actually are congruent or simply the signals in 18S rRNA is too small to override the signals in COI. It would be interesting to explore the tree based on 18S rRNA.

- typo {Abstract} parapheletic -> paraphyletic.

- typo {Figure 2} OUTs -> OTUs.

- substitutions/site/myr and s/s/myr are used interchangeably. It would be a good idea to define for the first time and stick to the abbreviated version.

- Table 1 has a header labeled as 'country', but in fact, 'sampling locality' might be a better description of the content. Also, it might be a better to specify what accession numbers the authors are referring to, GenBank?

- Table 2 caption must be improved.

- It is unclear how the authors decided to use HKY model for the BEAST analysis.

- The authors mentioned that the 95\% highest posterior density for confidence intervals of
ages are not shown in Figure 1--3 to avoid visual complexity. Is it going to be available in supplementary material? This is important information to verify authors' claims regarding the node ages in the manuscript.

- Figure 4 mentions the number of base changes for whole mitochondrial gene, but the two plots are based only on COI and 18S rRNA. It's confusing which is correct. The caption needs improvement.

- In results, the authors refer to Figure 3 as COI + 16S rRNA. It seemed like the authors were working on 18S rRNA. It's confusing which is correct.

- The authors mentioned that the rate has exponentially increased into Holocene based on the incets of Figure 1--3. But no Holocene was labeled anywhere in these figures. Also, the authors stated the curves and rates are similar for the incets in Figure 1, 2 and 3, but it is hard to understand how the authors concluded that they are 'similar', which is a very subjective description.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Phylogenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 03 Jul 2023
Soichi Osozawa
The main objective of this study is to present a time-calibrated tree of cicada species reconstructed using BEAST v1.X. The trees contain cryptic cicada species from Ryukyu islands in addition to the existing data presented in the previous studies. The authors show improved taxonomy of some of the major cicada groups. Moreover, the authors suggest an exponential increase of base substitution rate in recent geologic time. While the content of the manuscript is interesting, it requires substantial improvement and is not ready for indexing. The following comments elaborate some places where the improvements can be made.

Thank you for your review, and we appreciate your attention to our main insight and new finding regarding the exponential increase of base substitution rate in recent geologic time. Although we acknowledge that our experience with BEAST v1.X and the calibration method is limited, we recommend that you become a BEAST v1.X user, as it will provide you with the tools and resources to address your queries effectively. Utilizing BEAST v1.X will enable you to delve deeper into the subject matter and gain a better understanding of the findings we presented.

In general, the manuscript is not easy to understand. It is understandable that English may not be the authors’ first language, however, an academic manuscript must be written clearly.

We appreciate your confidence in the writing quality of the present manuscript. Considering that John Wakabayashi is a native English speaker, it is likely that the manuscript is indeed well-written. However, we understand your desire for a thorough review of the English language in the R1 manuscripts.

For example, the last sentence of Abstract seems to be not connecting with the previous sentences.

“We estimated base substitution rate as a function of age, and the result strongly indicates an exponential increase of base substitution rate in recent geologic time.” Is connected to next sentence “The consequent increase in cicada biodiversity, including generation of cryptic species in the Ryukyu Islands and surroundings, may have been driven by the generation and spreading of C4 grasses and coeval Quaternary climate change.”

The first sentence of Introduction is excessively long.

It is crucial to emphasize the specific type of gene used in the phylogenetic analyses, and our current COI and 18S rRNA sequence data may not be sufficient for our study. Addressing these concerns, we reviewed here the significance of utilizing an appropriate and comprehensive set of genes for the recent trend of phylogenetic analyses for cicadas.

In figure 1, it is not clear what it means by “maximum” COI sequence.
“1,534 bp in maximum”, not “maximum COI sequence”. We will revise in R1 manuscript. It is not clear what is the inserted figure. I assume it is the plot on the left side with white background. These are also called as ‘inserts’ later in the manuscript. How about labeling these as a) and b)? Here, y-axis is not labeled. The inset was made by node data shown in Fig. 2. In R1, we will improve the expression. Y axis is Ma, or you questioned no species name?

It is not clear what is the intersection for the curve and what it represents biologically. To draw the approximate curve, better to apply the intersection data, and we applied rate median obtained from Tracer. Green or red starts and the values corresponding to it is never explained in the caption. You will find the captions within Fig. 1, but we will enlarge the caption in R1.

Basically figure 2 is an identical tree as what is presented in figure 1. We acknowledge the feedback provided by Jessica Ware, a reviewer of our dragonfly manuscript, regarding Figure 2 being overly crowded. To address this concern, we have made revisions by dividing the scientific content of Figure 2 into simplified and full version figures, namely Figures 1 and 2, respectively. It is important to note that both figures are identical, as you correctly pointed out.

By providing simplified and full versions of the figure, we aim to improve the clarity and readability of the scientific information presented. This division allows readers to quickly grasp the main concepts in the simplified version, while still having access to the more detailed information in the full version if desired.

We appreciate the valuable input from Jessica Ware and are grateful for her suggestion to enhance the visual presentation of our manuscript. Thank you for highlighting this issue, and we hope these revisions address the concern effectively.

Again, the caption is very difficult to understand. Red and green stars and the values associated with them are never explained. The three values at the node in the tree are never explained. You will find the captions within Fig. 2, but we will enlarge the caption in R1. Probably see also main text.

Figure 3 has similar problems. More work is needed in presenting the trees. Fig. 3 was presented to compare COI + 18S rRNA tree vs solely COI tree in Figs. 1 and 3. Allow us here too busy figure without simplified figure.

Also, the authors mention in the caption of Figure 3 that the rate is a 'little' slower than that of in Figures 1 and 2, but it is what it means by 'a little', which is a subjective description. Rate median 0.0114 in Fig. 3 inset is a little slower than 0.0128 in Figs. 1 and 2, reflecting Fig. 3 was considered slower rated (not sensitive) 18S rRNA data.

The authors mention that the tree topology (based on COI) was unaffected by 18S rRNA. In other words, Figures 1, 2, and 3 are the same trees. If this is true, I am not sure presenting three identical trees is a good practice. Moreover, the authors must explore if the congruence occurred because they actually are congruent or simply the signals in 18S rRNA
is too small to override the signals in COI. It would be interesting to explore the tree based on 18S rRNA.

*Note that COI tree is for 352 specimens and tips, and COI + 18S rRNA tree is for 1492 specimens and tips, and not strictly identical and congruent.*

18S rRNA tree is “low resolution” and discordant COI tree. See also “Why was BEAST2 not used”.

typo (Abstract) paraphetic -> paraphyletic.

*In R1, we revise.*

typo (Figure 2) OUTs -> OTUs.

*In R1, we revise.*

substrituions/site/myr and s/s/myr are used interchangeably. It would be a good idea to define for the first time and stick to the abbreviated version.

*We will delete later appeared “substrituions/site/myr”.*

*Some journal ask to offer abbreviations, but now not a case.*

Table 1 has a header labeled as ‘country’, but in fact, ‘sampling locality’ might be a better description of the content. Also, it might be a better to specify what accession numbers the authors are referring to, GenBank?

*In GenBank/DDJB “country” is requested to use.*

*Also our data with accession numbers are found in GenBank/DDBJ.*

Table 2 caption must be improved.

*In R1, we will check English.*

It is unclear how the authors decided to use HKY model for the BEAST analysis.

*Default setting.*

The authors mentioned that the 95\% highest posterior density for confidence intervals of ages are not shown in Figure 1--3 to avoid visual complexity. Is it going to be available in supplementary material? This is important information to verify authors’ claims regarding the node ages in the manuscript.

*Please consider adding 95% highest posterior density (HPD) bars to represent confidence intervals for ages on each node in Figure 2. The figure becomes further busy. We believe they may not add significant meaning.*

Figure 4 mentions the number of base changes for whole mitochondrial gene, but the two plots are based only on COI and 18S rRNA. It’s confusing which is correct. The caption needs improvement.

*Vertical axis is COI numbers of base changes and 18S rRNA numbers of base changes, respectively in left sided COI and right sided 18S rRNA figures.*

In results, the authors refer to Figure 3 as COI + 16S rRNA. It seemed like the authors were working on 18S rRNA. It’s confusing which is correct.
We found four mistakes of 16S rRNA in page 11 including Osozawa et al. (2016), and we collect as 18S rRNA and Osozawa et al. (2017a) in R1.

The authors mentioned that the rate has exponentially increased into Holocene based on the incets of Figure 1--3. But no Holocene was labeled anywhere in these figures. Also, the authors stated the curves and rates are similar for the incets in Figure 1, 2 and 3, but it is hard to understand how the authors concluded that they are 'similar', which is a very subjective description.

The Holocene is the most recent epoch of the Quaternary period, following the boundary at 0.0117 Ma. We acknowledge the difficulty in accurately labeling the time point at 0.0117 Ma in Figures 1 to 3.

Compare formula in the same one in Fig. 1 and 2, and the distinct one in Fig. 3.

We were hoping your constructive review about increasing rate, factor, and affect.

Is the work clearly and accurately presented and does it cite the current literature?  
Partly

Is the study design appropriate and is the work technically sound?  
Partly

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Partly

Are all the source data underlying the results available to ensure full reproducibility?  
Partly

Are the conclusions drawn adequately supported by the results?  
Partly

Competing Interests
No competing interests were disclosed.

Reviewer Expertise
Phylogenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

**Competing Interests:** No competing interests.
The benefits of publishing with F1000Research:

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