Odonata Timetree; Exponentially Increased Base Substitution Rate Toward The Recent and Within the Carboniferous

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Abstract

Using BEAST v1.X, we constructed a credible timetree of 115 specimens of Odonata and five species of Ephemeroptera (Paleoptera; Pterygota) and two species of Archaeognatha and three species of Zygentoma (Apterygota). 88 specimens we ourselves analyzed were collected from the Ryukyu islands, Taiwan, Japan, and China, and the resting sequence data were mostly from whole mitochondrial data found in GenBank / DDJB. The combined gene (not concatenated gene) analysis of the mitochondrial COI (795 bp), COII (548 bp), and 16S rRNA (517 bp), and the nuclear 28S rRNA (825 bp) were performed. Using the calibration function of BEAST v1.X, the timetree was constructed by applying a 1.55 Ma geological event (isolation of the Ryukyu islands from China), in addition to chronologically robust fossil dates ranging from 400 Ma for Archaeognatha, 300 Ma for Ephemeroptera, and 200 Ma for Odonata and to 1.76 Ma for Calopterygidae, for a total of 13 calibration points (event: 6, fossil: 12; Quaternary 7, pre Quaternary 11). The resultant timetree showed that molecular clock was not uniformly progressed, and the base substitution rate has exponentially increased from ca. 20 Ma to the Recent by over an order of magnitude. Our new and attractive finding indicates that the Quaternary severe climatic change including a start of glacial and interglacial cycle might have resulted in the extensive radiation and speciation of Odonata, and consequently increased the biodiversity. C4 pores generated in the Miocene effectively decreased atmospheric CO₂, and triggered the Quaternary glaciation. Another peak of base substitution rate was found in the Carboniferous time around 320 Ma, and this may be analogous to the late Paleozoic icehouse. This glaciation has been triggered by the development of terrestrial plants to form thick coal layers, because this process also reduced the atmospheric CO₂.

Introduction

A reliable timetree (dated phylogeny) for the entirety of Odonata (dragonfly and damselfly) have not yet been reported to date, except for the very recent report in bioRxiv (Kohli et al., 2020). We have presented the timetree of the east Asian *Coeliccia* damselfly (details of the vicariance in Osozawa et al., 2017c) calibrated solely by the Quaternary date of geological event (1.55 ± 0.15 Ma; isolation of the Ryukyu-Japan-Taiwan islands from China; triggered the vicariant speciation onto the most recent common ancestor of the present each island and Chinese endemic species; no geological evidence of land bridge for dispersal; Osozawa et al. 2012). A problem with that analysis was that calculated basal node age of several million years was younger than what would be considered reasonable. This chronological problem also existed in analyses of other East Asian Odonata timetrees (*Anotogaster* dragonfly, Osozawa et al. 2013; *Chlorogomphus* dragonfly, Osozawa and Wakabayashi, 2015), and other east Asian insects (*Papilio* butterflies, Osozawa et al., 2013; *Mycalesis* butterfly, Osozawa et al., 2015a; *Pyrocoelia* firefly, Osozawa et al., 2015b; *Cicindela* tiger beetle, Osozawa et al., 2016; *Ypthima* butterfly, Osozawa et al., 2017a; *Platypleura* cicada, Osozawa et al., 2017b; Carabid ground beetle, Osozawa K. et al., 2016). Note that extensive vicariance by the isolation of islands from the Chinese continent was described and established in these papers.

Insecta consists of Apterygota and Pterygota, and we included Apterygota of Archaeognatha (jumping bristletail) and Zygentoma (silverfish) as outgroup applying sequence data from GenBank / DDBJ. Pterygota consists of Palaeoptera, ancestral group of insects that lacks/lacked the ability to fold the wings back over the abdomen, and Neoptera in contrast to Palaeoptera (Ishiwata et al. 2011). Odonata as a main target and Ephemeroptera (mayfly) belong to Palaeoptera, and we included Ephemeroptera in our analyses applying sequence data in GenBank / DDBJ. Odonata consists of Anisoptera (dragonfly) and Zygoptera (damselfly), and includes Anisozygoptera with an intermediate form between them. *Epiophlebia superstes* (Epiophlebiidae) is a representative species of Anisozygoptera in the Japanese islands, and historically considered to be extant, relict, and primitive (Asahina, 1954; Ishida et al., 1988; Hasegawa and Kasuya, 2006; Ozano et al., 2012; Busse et al., 2012, 2015; Wang et al., 2014). We ourselves collected and analyzed East Asian Odonata covering every family as possible (Figs. 1 and 2), and attempted to construct a credible Odonata timetree including *E. superstes* with the initial goal of resolving the apparent chronologic discordance problem noted above. The expectation was that *E. superstes* lineage should be very old up to 200 Ma, rather than several million years. Apterygota and Ephemeroptera lineage should be very old up to the Paleozoic.

Divergence time estimation is possible employing conventional MCMCTree (Yang, 2007). As for the fossil calibration, minimum and maximum age constraints or bounds for a specific node are defined (Benton and Donoghue, 2007). The minimum age is the oldest fossil age known, and younger than the branch node. The maximum age is such as a stem age defined by additional fossil age older than that branch node. The latter is usually difficult to determine and unclear, and overestimated in many cases. The overestimate affects on large error bar at node and shifts the divergence estimate older date found in Kohli et al. (2020). Note that two age data set specifying a node is needed for MCMCTree analysis. Another complex treatment is required; building concatenated gene and re-partitioning using PartitionFinder. We are unfamiliar with the command line user interface.

BEAST v1.X released on 10th June, 2018 (Bayesian Evolutionary Analysis Sampling Trees; Suchard et al., 2018) has a function of calibration in the associated software of BEAUti (Bayesian Evolutionary Analysis Utility). The calibration protocol of BEAUti is to input a time of the most recent common ancestor (tMRCA = minimum age) of the ingroup species (e.g., calibration point H for Libellulidae species; Fig. 1), either by fossil calibration or by the geological event calibration noted above. Using BEAST v1.X, without fixing maximum age constraint, we can simply build a reliably-dated phylogeny of Apterygota and Palaeoptera including the target Odonata (Fig. 1). The combined gene analysis of the mitochondrial COI (795 bp), COII (548 bp), and 16S rRNA (517 bp), and the nuclear 28S rRNA (825 bp) can be performed by simply setting partitions of these genes in BEAUti, and concatenation and re-partitioning using PartitionFinder is not required. Note that our employed mitochondrial genes have high resolution compared to the nuclear genes, applicable for very old age without saturation of mutation (Osozawa et al., 2012, 2017c), but unrelated to the above chronological problem.

The topology of the resulting timetree was controlled by these calibration points and date sets in BEAUti, and prior age (input in BEAUti) and posterior age (output in FigTree) shown in Fig. 2 must be coincident.

This means that the BEAST v1.X calibration is an “active” one. If the timetree has unreasonable shape and very distinct input and output age, we reset the calibration and repeatedly run BEAST until to get reasonable one.

In our previous analyses such as for *Coelicerca* damselfly in Osozawa et al. (2017c), we calibrated by geological event of 1.55 Ma and applied strict clock model in BEAST v1.8.2 analyses with the same calibration function to v1.X, and represented the base substitution rates of rapid mitochondrial and slower nuclear genes respectively. The rate was comparable to the previously reported rates such as by Brower (1994), although the rate was represented as relatively high (solution of the above problem is addressed below). Our present BEAST v1.X analyses applied relaxed clock model alternative to the strict clock model in Osozawa et al. (2017c), and the rate median at each node is not constant but valuable.

We made rate vs age diagram (Figs. 1 and 2 inset) to verify the variability, and as a main result we indicate an order of magnitude increase of base substitution rate in Odonata evolutionary history found in the Quaternary and also in the Carboniferous. We propose here that the consequent increased biodiversity of Odonata was driven by severe environmental change that began in the Quaternary, and by global glaciation during the Carboniferous. We also discussed the ultimate factor of these glacier events, which was connected to the plant evolution.

Results

Timetree (Figs. 1 and 2)

Insecta basal node was estimated at 413 Ma, Archaeognatha was represented as the oldest lineage in insecta, and Zygentoma is second. Ephemeroptera is a sister of Odonata differentiated at 347.56 Ma, and this Palaeoptera is a sister of Apterygota (Archaeognatha + Zygentoma).

Anisoptera is a sister of Zygoptera, and each forms distinct major clade. Although *Epiophlebia superstes* is included in the Anisoptera clade, it can be considered to represent a distinct clade as an intermediate Anisozygoptera, concordant to Hasegawa and Kasuya (2006) and Misof et al. (2014). *E. superstes* persists as an old lineage with a long terminal branch, and the species age is estimated at 180.17 m.y. expressing the deep evolutionary history.

As for another relict species, Petaluridae (Ware et al., 2015), age of the MRCA was estimated at 141.33 Ma as calibrated by point F of 142.4 ± 2.6 Ma, and differentiated from Gomphidae at 149.7 Ma (branching order 2 in Figs. 1 and 2). However, the differentiation into each species was relatively young around 10 Ma.

Aeshnidae occupies a relatively old position as old as 149.01 Ma (branching order 1), and a sister of Cordulegastridae + Chlorogomphoidea differentiated at 146.11 Ma (branching order 3). Excepting this sister relationship, the topology is concordant to that of Kohli et al. (2020).

Date of MRCA of monophyletic Gomphidae is estimated at 130.57 Ma, and the topology is totally concordant to the North American genera reported by Ware et al. (2017; not a dated tree). Epigomphinae is an old lineage in Gomphidae, and a sister of the other Gomphidae species.

Libellulidae is a sister of Corduliidae, and differentiated at 138.38 Ma (branching order 4). The MRCA is estimated at relatively young age of 51.25 Ma

For analyzed Zygoptera, *Sinostica ogatai* (Platystictidae) and *Sympecma paedisca* (Lestidae) constituted an old lineage and was a sister of the resting Zygoptera. Each Zygoptera family constituted a distinct clade, excepting for *Mesopodagrion tibetanum* (see discussion, timetree).

Multi-furcation into the Rykyu-Taiwan-Japan island species including Chinese species, reflecting vicariance started at 1.55 Ma, is observed for four ingroup species for Anisoptera (Q1 to Q4), and two ingroup species for Zygoptera (Q5 and Q6), as calibrated by this 1.55 Ma date. However, minor discordance between input date and output date was observed for *Asiagomphus*, *Anotogaster*, *Chlorogomphus*, and *Coeliccia*.

Base Substitution Rates

Base substitution rate vs age diagram (Figs. 1 and 2 inset) shows that the rate is not constant as applied relaxed clock model, but tends to have exponentially increased since ca. 20 Ma, and the equation of the trendline is shown on the inset. The rates less than 0.01 substitutions/site/myr (s/s/myr) between ca. 200 Ma and 20 Ma were exponentially increased up to 0.2183 s/s/myr of vicariantly speciated *Rhipidolestes* in the Recent time.

Because our timetree covered after 400 Ma of post Silurian by calibrated by up to 400 Ma by point A, the base substitution rate vs age diagram (Figs. 1 and 2 inset) recorded such ancient variation of rates. We found another peak of rate around 320 Ma of late Carboniferous. The peak rate was 0.063 at the basal node of Palaeoptera (Ephemeroptera + Odonata).

Discussion

Timetree (Fig. 3)

Insecta basal node was estimated at 413 Ma, younger than estimate of 479 Ma by Misof et al. (2014), and that of Palaeoptera (Ephemeroptera + Odonata) of Pterygota was estimated at 317.29 Ma, also younger than 406 Ma for whole Pterygota and 362 Ma for Palaeoptera by Misof et al. (2014). Misof et al. (2014) employed BEAST v.1.8 with the same calibration function to BEAST v1.X, and the discordance might be simply arisen from our robust calibrations. As we note in methodology bellow, the Kyrgyzstan damselfly fossil date applied by Misof et al. (2014) was not useful.

Note that ancient divergence times were successfully estimated by our mitochondrial gene application, with no sign of saturation of mutations, and application of nuclear gene was not affected on the

topology and divergence time. Actually the base substitution rate of combined gene (Figs. 1 and 2 inset) is comparable to that of solely mitochondrial gene (Osozawa et al., 2017c).

The Odonata topology is comparable to Kohli et al. (2020). Epiophlebiidae is the oldest lineage. In Anisoptera, as numbered in Figs. 1 and 2, branching order is Aeshnidae (1), Petaluridae (2) and its sister of Gomphidae (2), Cordulegastridae (3) and its sister of Chlorogomphoidea (3), Corduliidae (4) and its sister of Libellulidae (4), same as the order of Kohli et al. (2020). Aeshnidae is a sister of Cordulegastridae + Chlorogomphoidea, which differs from Kohli et al. (2020). In Zygoptera, Lestidae and also Platystictidae is an old lineage relative to the resting. Discordance of divergence times might reflect overestimate of maximum bounds in Kohli et al. (2020).

In introduction, we noted a problem that the basal node age was estimated much younger than expected age when calibrated solely by the Quaternary geological event at 1.55 Ma. This problem was solved by contemporaneously applying old ages calibrated by fossil calibration points from A (400.45 Ma; Devonian) to I (1.76 Ma; Quaternary) in addition to geological event at 1.55 Ma (Quaternary) from Q1 to Q6, and the consequent dated tree (Figs. 1 and 2) is reliable.

Epiophlebia superstes can be considered to be included in the Anisoptera major clade, and is a sister of the other Anisoptera, common to Kohli et al. (2020). The stem age was estimated to be 189.5 Ma, but younger than 200.3 ± 1.0 Ma of calibration point C. In the Anisoptera clade, *E. superstes* persists as an independent lineage, so classification to Anisozygoptera was possible, and the species age is as old as 180.17 m.y. Accordingly, *E. superstes* can be considered a living fossil. Genus *Epiophlebia* is known from isolated areas of the Japan islands, China (the same species to China was reported also from North Korea; Gunther et al., 2013), and the Himalaya, but the vicariance was indicated to be very mild, suggesting that the isolation in these areas occurred recently and is unrelated to its very old lineage (Busse et al., 2012).

Petaluridae including *Tanypteryx pryeri* and *Petalura gigantea* was dated at 149.7 Ma (early Cretaceous). Ware et al. (2014) proposed that Petaluridae is an old species dating back to the Jurassic, reflecting the breakup of the supercontinent Pangaea. As we note in methodology, the Pangaea breakup and initiation of the Atlantic Ocean was recorded in the Santana Formation, and may at 125 - 100 Ma (Martill, 2007), which is younger than the stem node age of Petaluridae. However, Petaluridae can be considered to be primitive and extant species (Ware et al., 2014).

Family level differentiations for Anisoptera were occurred during early Cretaceous time around 130 Ma, and those for Zygoptera were occurred during Paleogene to early Neogene between 72.64 to 22.19 Ma. So family age of Zygoptera is younger than that of Anisoptera. Subspecies level differentiations of Gomphidae were during Paleogene. Species level differentiation was after Paleogene, but mostly Neogene, and endemic species calibrated by 1.55 Ma as points Q1 to Q6 were differentiated during the Quaternary.

Yu and Bu (2011) cladistically analyzed damselflies, and showed that Mesopodagrion and Rhipidolestes possess in distinct clade, and Megapodagrionidae was divided into two distinct families. Two distinct clades of Megapodagrionidae in Figs. 1 and 2 reflect this subdivision.

Vicariance increased biodiversity

Q1: *Stylogomphus*, Q2: *Asiagomphus*, Q3: *Anotogaster*, Q4: *Chlorogomphus* (Osozawa and Wakabayashi, 2015), Q5: *Rhipidolestes*, Q6: *Coeliccia* (Osozawa et al., 2017c) were calibrated by the 1.55 ± 0.15 Ma geologic event, and each of the multi-furcation or polytomy indicates the generation of multi- endemic species (Fig. 2). Isolation of islands by the opening of the Okinawa trough is a physical process, but such process clearly triggered increasing biodiversity. These endemic species might have experienced a severe bottleneck by the isolation of habitat, but the bottleneck might have effect on species the low diversity and the low nucleotide substitution rate (Zhai et al., 2017), which is conflict to the present case.

Calibration point I: *Calopteryx* + *Matrona* was calibrated by fossil date of 1.76 ± 0.22 Ma, and splitting between *Calopteryx japonica* (Japan) and *Matrona basilaris* (Amami-Okinawa; failed to amplify Taiwan specimen) appears to be a consequence (or a precursor) of 1.55 Ma vicariance (Fig. 2).

In general, back arc spreading to form continental islands may trigger vicariance that increases extent of biodiversity. There are many back arc basins in the western Pacific Ocean, and some are still active, including the Okinawa trough. The high diversity of terrestrial organisms observed on continental islands separated from continental landmasses by sea-floor spreading of this sort may be a consequence of the physical isolation of these islands by the rifting process and the consequent vicariance.

In contrast, the oceanic islands are also generated back arc spreading by building of volcanic edifices in the ocean, but these had no terrestrial life prior to their formation of islands because they emerged above sea level as a result of progressive volcanism. Such islands initially acquired their terrestrial species by dispersal from other land masses, and the species diversity associated with such islands may be expected to increase by vicariance after initial colonization by dispersal (cf., Osozawa, K. et al., 2016).

We showed that lotic damselflies tended to speciate vicariantly in contrast to the lentic damselfly (Osozawa et al., 2017c), and the similar report was by Letsch et al. (2016). However, as for Octogomphinae, lotic *Davidius* and lentic *Trigomphus* show no such tendency (Fig. 2). Libellulidae is mostly lentic, and vicariance may be negligible (= same sequence between island populations), and resulting in low species diversity, but we failed to amplify enough island specimens of Libellulidae to fully evaluate this premise. This is a case also for mostly lentic Coenagrionidae damselfly by lacking corresponding sequence data.

Increase base substitution rate and biodiversity

Base substitution rates estimated by calibration with 1.55 Ma geologic event of Q1 to Q6 are relatively high (> 0.01 s/s/myr including > 0.1 s/s/myr), and comparable to those that we estimated recently for other insects using the same calibration event (Osozawa et al., 2013, 2015ab, 2016, 2017a-c; Osozawa, K.

et al., 2016; Osozawa and Wakabayashi, 2015). However, ages of older nodes were underestimated, and branch lengths were shortened, when using this Quaternary geologic calibration alone.

Base substitution rates estimated by old fossil dates of A to I for calibration are < 0.01 s/s/myr, and these resulted in older nodes and extension of branches. Both the fossil calibrations including the Quaternary of point I and the Quaternary geologic event calibrations of point Q1 to Q6 appear to be robust, so the discrepancy might be the result of changing base substitution rates that may have been relatively constant from 200 Ma at slower rate, but exponentially increased since at ca. 20 Ma, especially during the Quaternary (Figs. 1 and 2 inset).

By applying both geologic and fossil calibrations and using a relaxed clock model in BEAUti, we obtained an accurate BI tree in calibration and topology (= accurate timetree; Figs. 1 and 2). But additional important result is that the molecular clock hypothesis (relatively constant rate over time; Ho 2008) is unacceptable for Odonata evolution, and the rate exponentially increased toward the Recent (Figs. 1 and 2 inset).

This phenomenon had been shown for taxa such as primates (Ho et al., 2005, 2011) and Aegean beetles (Papadopoulo et al., 2010), but generated little attention. BEAST v1.X may simultaneously apply multiple calibration points as done for constructing Figs. 1 and 2, but BEAST v.1.3 (Ho et al., 2005) and BEAST v.1.4 (Papadopoulo et al., 2010) needed to run repeatedly by applying a date at every calibration point, and a rate at every run was assumed to be constant at each node (= strict clock model). They found that a Quaternary date calibration produced a rapid rate. Note that we instantaneously calibrated by multi dates including older dates up to the Devonian as well as younger dates of the Quaternary, rather than solely calibrating by the Quaternary date, and the increasing rate is not solely an artifact of the Quaternary calibration. It should also be noted that combined gene analyses were not possible in these older versions of BEAST, and such the users needed to discuss the rate for every each gene such as COI vs 28S rRNA. Therefore, although their trendlines and the equations are similar to ours, their timetrees are actually incorrect by not reflecting drastic changing of base substitution rates through the time, but by assuming a constant rate as if governed by the strict clock.

Increased base substitution rate may be expected to increase biodiversity, as is the case for the 1.55 Ma vicariance-induced biodiversity mentioned above. Vicariance is partly explained as radiation for *Rhipidolestes amamiensis* as forming distinct clades (Fig. 2). An alternative is that greater apparent biodiversity in geologically recent time may be an artifact of the greater availability (preservation) and consequent greater number of studies of recent geological sections (Rohde and Muller, 2005). Molecular phylogenetic analyses combined with paleobiological study (Sahney et al., 2010; Neige, 2015; Buatois and Mángano, 2018) can test these alternatives.

A possible forcing factor of exponentially increasing evolution rate and probable biodiversity toward the Recent may be the start of glacial and interglacial cycle and the environment change in the Quaternary time since 2.58 Ma. The Quaternary glaciations may have been triggered by expansion of C4 land grasses and evolution of sea diatoms, because this process increased the carbon fixation that

consequently decreased atmospheric CO₂ concentration (Taira, 2007). The expansion of C₄ grasses was a global phenomena also including North America and South America beginning in the late Miocene and persisting to the present day, concerning the present glacial–interglacial period with a time lag (Cerling et al., 1997).

We found another peak of base substitution rate in the Carboniferous time (300 – 350 Ma; 0.063 s/s/myr at the node between Ephemeroptera and Odonata; Figs. 1 and 2 inset), and this may be analogous to the late Paleozoic icehouse (= the Karoo ice age in South Africa; Montañez et al., 2013). Whereas glacial episodes in Earth history appear to be influenced by landmass configuration also in this period, feedback from biologic development may have also played a role in triggering the onset on this glaciation. The late Paleozoic glaciation had been triggered by the development of terrestrial plants to form thick coal layers in the Carboniferous time (Franks et al., 2014), because this process also increased carbon fixation that effectively reduced atmospheric CO₂ (Taira, 2007).

Conclusion

Constant evolution rates that are the basis of the strict molecular clock model do not apply in the cases of Odonata and may not apply in a broader sense. The present biodiversity including Odonata may be a consequence of a geologically recent exponential increase in base substitution rate. This may reflect the onset of Quaternary glacial and inter glacial cycles associated with severe climatic change, triggered adaptive radiation of Odonata. Another peak was found in the Carboniferous time, also tied to the late Paleozoic icehouse.

Materials

East Asia Odonata sampling

Sequence data including whole mitochondrial data for several species of Archaeognatha, Archaeognatha, Ephemeroptera, and Odonata that we did not analyzed or failed to get sequence data were found in GenBank / DDBJ and included in our BEAST v1.X analysis (Fig. 2).

Location map is in Fig. 3, and representative collected specimens are shown on Fig. 4. DNA sequence data are newly registered in the DDBJ / GenBank, excluding previously uploaded data of *Chlorogomphus* by Osozawa and Wakabayashi (2015) and *Coelicerca* by Osozawa et al. (2017c). Species isolate, country, accession number, collection date, and collector data newly obtained in this paper are shown in Table 1. Species names are after Ozono et al. (2012).

Stylogomphus (Octogomphinae; Gomphidae; lotic) contains *S. suzukii* (Japan), *S. chunliuae* Chao, 1954 (China), *S. shirozui* (Yaeyama and Taiwan), *S. ryukyuanus* (southern Japan, Amami), and *S. ryukyuanus asatoi* (Okinawa).

We collected lotic *Davidius nanus*, *D. moiwanus*, and *Lanthus fujiacus* (Octogomphinae; Gomphidae; Japan; Kiyoshi and Sota, 2006), and lentic Octogomphinae species of *Trigomphus citimus tabei* and *T. interruptus* (southwest Japan), and *T. melampus* (northeast Japan). We will compare the extent of vicariance of these species with contrasting habitat, as analyzed in Osozawa et al. (2017c).

Sieboldius albardae (Lindeniinae; Gomphidae; Japan and Korea; lotic) is characterized by small head relative to large body.

Asiagomphus (Gomphinae; Gomphidae; lotic) contains endemic *A. melaenops* (Japan), *A. amamiensis* (Amami), *A. amamiensis okinawanus* (Okinawa), *A. yayeyamensis* (Yaeyama), and *A. hainanensis* (Taiwan and southern China, but China specimen was not obtained), expressing a typical vicariant speciation from a common ancestor. We also collected *A. pryeri* (Japan) and closely related *A. coreanus* (Korea) with sympatric lotic habitat to the above *Asiagomphus* species.

We collected *Leptogomphus* (Epigomphinae; Gomphidae) of *L. yayeyamensis* (Yaeyama) and *L. sauteri* (Taiwan), but did not find *L. elegans* (southern China). *Leptogomphus* is absent on Amami island and Okinawa.

Chlorogomphus (Chlorogomphoidea; lotic) contains *C. brunneus brunneus* (southern Japan and Amami), *C. brunneus costalis* (Okinawa), *C. iriomotensis* (Yaeyama), and *C. risi* (Taiwan). The Japanese population was probably relatively recently dispersed from Amami by typhoon (Osozawa and Wakabayashi, 2015). We failed to obtain 16S rRNA data from *C. risi*, and the species was not included in the *Chlorogomphus* ingroup in Fig. 2. We collected *C. okinawensis* (Okinawa) sympatric with *C. brunneus costalis*, but did not find *C. suzukii* (Taiwan) sympatric with *C. risi*. We collected *Chlorogomphus* sp. in China.

Anotogaster (Cordulegastridae; lotic) consists of *A. sieboldii* (Japan-Korea, Amami, and Okinawa), and *A. klossi* (Yaeyama, Taiwan, and China) (Kiyoshi, 2008; Osozawa et al., 2013), but our analyses were limited by partial mis-amplification.

Tanypteryx pryeri (Petaluridae; endemic in Japan) was considered to be morphologically and phylogenetically an extant, relict, primitive species with the other Petaluridae originated from the Pangean super continent, and the larvae live in fens and boggy seeps taking several years to mature (Ware et al. 2014). We analyzed other three Petaluridae species including *P. gigantea*, the world's largest dragonfly found in Australia, using corresponding sequence data in Ware et al. (2014), although their data were not complete.

We collected many Aeshnidae species, but we only obtained the COI- COII sequence data from *Polycanthagyna melanictera* (Okinawa; lentic), due to mis- or no amplification. *P. melanictera* is mostly known from Japan, as well as such as Tobi-shima off shore of Sakata, northern Honshu, and also Hachijo-jima of the Izu-Bonin oceanic islands, indicating its strong dispersal ability by flying. *Anaxparthenope* is a very common species in the coastal area near Sendai (northeast Honshu), persisting

after the 2011 Tsunami, but only *A. imperator* and *A. junius* data were available in GenBank / DDJB. We also failed to obtain reliable sequence data of *Oligoaeschna pryeri* (Japan), considered to be a primitive species in Aeshnidae (Ozano et al., 2012).

Although we collected Corduliidae and some are endemic species in the Ryukyu islands, we failed to amplify the sequences. However, corresponding sequence data for three Corduliidae species were available in GenBank / DDJB.

Owing to mis-amplification of COI- COII regions, our analyses of Libellulidae were limited to sg38 *Orthetrum japonicum* (Fig. 2; Table 1). To facilitate calibration using the age of fossil Libellulidae, we also used GenBank / DDJB sequence data of whole mitochondrial. Libellulidae species are mostly lentic and commonly found on even small islands of Ryukyu, but an exception is lotic *Sympetrum pedemontanum elatum* (Japan; no COI- COII sequence data). However, we included *S. eroticum* (lentic) using GenBank / DDBJ data.

We collected *Epiophlebia superstes* (Anisoptera; lotic) from the Sendai hill (newly found habitat by the senior author) of northern Honshu. The larva spends 5 to 8 years in small streams, and leaves the water just before the emergence (Ozono et al., 2012).

We included lotic Calopterygidae of *Atrocalopteryx atrata* (Japan, Korea, China), *Calopteryx japonica* (Japan and Korea), and *Matrona japonica* (Amami and Okinawa). Fossil *Calopteryx* and *Oligoaeschna pryeri* (Aeshnidae) have been identified in Quaternary strata in Kyushu, Japan (Esaki and Asahina, 1957) enabling a Quaternary fossil calibration other than geological event calibration of 1.55 Ma. Other *Matrona* species collected in Taiwan and China were mis-amplified and no data in GenBank / DDBJ.

We collected *Rhipidolestes* (Megapodagrionidae; lotic; cascade) of *R. hiraoui* (Shikoku), *R. yakusimensis* (Kyushu and Yaku-shima), *R. asatoi* (Koshiki-jima), *R. amamiensis* (Amami), *R. shozoi* (northern Okinawa), *R. okinawanus* (southern Okinawa; allopatric to *R. shozoi*), and *R. aculeatus* (Yaeyama and Taiwan). The isolated cascade habitat may affect vicariance on this species. We also included in our analyses *Agriomorpha fusca* May, 1933 (Argiolestinae; China).

We had previously analyzed vicariantly speciated lotic *Coelicerca* (Platycnemididae) of *C. ryukyuensis amamii* (Amami), *C. ryukyuensis ryukyuensis* (Okinawa), *C. flavicauda masakii* (Yaeyama), *C. flavicauda flavicauda* (southern Taiwan; The Yilan basin and Lanyang valley is the barrier), and *C. cyanomelas* (northern Taiwan and China), with comparison to lentic *Copera* (Platycnemididae) of *C. marginipes* (Taiwan and China) and *C. annulata* (Japan), and lotic *Platycnemis* (Platycnemididae) of *P. foliacea sasakii* (Sendai, northern Honshu; northern limit) and *P. phyllopoda* (Korea) (Osozawa et al., 2017c).

We included two Zygoptera species of lotic *Sinostica ogatai* (Platystictidae; China; Osozawa et al. 2017c), and lentic *Sympecma paedisca* (Lestidae; Japan; GenBank / DDJB; primitive in Zygoptera; Ozono et al., 2012), other than several Euphaeidae and Calopterygidae species found in GenBank / DDJB.

Methods

DNA Extraction and Polymerase Chain Reaction Amplification, Sequence Alignment

We used the sequence data of the mitochondrial COI gene (3P; 795 bp), COII gene (5P; 548 bp), 16S rRNA (517 bp), and the nuclear 28S rRNA gene (825 bp). See our newly obtained data with accession numbers shown in Table 1. See Osozawa et al. (2017c) for analytical details, including the primer set used.

Note that these COI and COII gene data are intersecting the nonprotein coding tRNA-Leu region (Osozawa et al., 2017c), and the corresponding region data are seldom found in the DDBJ / GenBank, but we included sequence data in our analyses from DDBJ / GenBank as noted above (no isolate and only species name in Fig. 2).

Quaternary geologic event: Ryukyu continental islands formed at 1.55 Ma for BEAST v1.X analyses

Osozawa et al. (2012) showed that the Ryukyu islands formed by back-arc spreading of the Okinawa trough that separated them from the Chinese mainland. This sea-floor spreading resulted in separation of the islands by 1.55 ± 0.15 Ma and this separation has increased progressively since then. Subsidence to form each island progressed rapidly, and these islands were simultaneously separated from the China mainland and isolated from one another by the development of the Okinawa trough, as well as other major seaways between the islands, including the Tsushima and Taiwan straits (Fig. 3). These straits, including minor ones in some cases, are expected to have acted as migration barriers to trigger vicariance, so 1.55 Ma is a geologically robust calibration date applied to our phylogenetic analyses in BEAST v1.X for six Odonata groups of *Stylogomphus* (Gomphidae; calibration point Q1), *Asiagomphus* (Gomphidae; Q2; *A. hainanensis* in China was not collected; Sympatric *A. pryeri* and *A. coreanus* were not included in the *Asiagomphus* ingroup), *Chlorogomphus* (Chlorogomphoidea; Osozawa and Wakabayashi, 2015; Q3), *Anotogaster* (Cordulegastridae; Kiyoshi, 2008; Osozawa et al., 2013; Q4), *Rhipidolestes* (Megapodagrionidae; Q5; Chinese species was not collected), and *Coelliccia* (Platycnemididae; Osozawa et al., 2017c; Q6) in Fig. 2 (Locality map: Fig. 3).

Geologic assessment of Odonata fossil dates for BEAST analyses

Misof et al. (2014) constructed a whole insect timetree including three representative suborder species of Odonata (including *E. superstes*) by applying voluminous data of 1478 protein-coding genes, and estimated the basal node at 479 Ma (= basal node age of Archaeognatha) by employing BEAST v.1.8. They stated that their calibration date strictly follows the protocol proposed by Parham et al. (2012), that is geologically robust in the opinion of the present authors. We agree with their statements that “Review the published geological age and the stratigraphic range of the fossil and synchronize the data with the most updated standard global geochronology”. “Review the quality of the stratigraphic information for each fossil. In particular, amber deposits such as those from the Baltic or Dominican Republic could not be adequately correlated with the geological time scale”. Therefore, they have largely excluded amber fossils from their analysis, with an exception of Burmese amber, which was dated at 99 Ma using U-Pb

method (Shi et al., 2012). “Biostratigraphic information has been updated and adapted to the current geological time scale” (the most recent International Chronostratigraphic Chart by the International Commission on Stratigraphy).

Misof et al. (2014) used the calibration date for fossil egg insertion scars into host plant by damselfly (Moisan et al., 2012) and considered the basal node of Odonata to be late Triassic (235-221 Ma). However, the Madygen Formation in Kyrgyzstan (Voigt et al. 2006) that yielded this fossil has associated no constraining radioisotopic age data and no reliable biostratigraphic information (no index fossils that can be related to robust absolute age determination), so we consider this late Triassic date to be poorly supported, and we used alternatively reliable early Jurassic date for damselfly (see below).

Kohli et al. (2016) examined many crown dates available for Odonata fossil calibration. Family level classification of Odonata is by wing venation patterns also as for fossil wings, as illustrated in Ozono et al. (2015). Kohli et al. (2016) reviewed such fossil data, and proposed the crown age for each family. However, there is some uncertainty of the fossil ages (NOT fossil identifications), so we reevaluated the geologic constraints associated with the fossil locations. We adopted several pre-Quaternary calibration dates, after evaluating their geological reliability, including, but not limited to, whether the fossil ages were constrained by modern and more accurate radioisotopic dating or by stratigraphical correlation. We applied 11 fossil calibration points from A to I (Figs. 1 and 2), and resulting in robust time calibration as old as 400 Ma. Note that the Devonian calibration point A was for Archaeognatha, and the Carboniferous calibration point B was for Ephemeroptera (our own bibliographic survey and reevaluation; Figs. 1 and 2).

The labels preceding each text section below correspond to that given in the following section as calibration points on actual BEAST v1.X analysis (Figs. 1 and 2). These fossil dates are evaluated in order of decreasing age from A to I. As will be seen in the subsequent section, the ages with larger uncertainties were not used in BEAST analyses for generating Figs. 1 and 2.

A: *Gigamachilis triassicus* gen. et sp. nov. (Archaeognatha) was collected from the Kalkschieferzone (239.51 ± 0.15 Ma; Stockar et al., 2012) of Monte San Giorgio (UNESCO World Heritage Site, Switzerland-Italy) (Montagna et al., 2017). They noted that *G. triassicus*, possessing morphological features that prompt its assignment to the extant archaeognathan ingroup Machilidae, places the origin of modern lineages to Middle Triassic. However, the oldest fossil considered as Archaeognatha was from the Devonian (Emsian; 407.6 - 393.3 Ma; 400.45 ± 7.15 Ma) from Gaspé, Québec (Labandeira et al., 1988), and we adopted this date for the calibration point A of Archaeognatha.

B: Knecht et al. (2011) reported an evidence of a full body impression of a flying insect from the Late Carboniferous Wamsutta Formation of Massachusetts, representing the oldest trace fossil of Pterygota, i.e., Ephemeroptera (mayfly). We considered the date the Gzhelian between 298.9 - 303.7 Ma (following Cohen et al., 2013; 301.3 ± 2.4 Ma).

C: Wing pattern of fossil *Liassophlebia* resembles to that of *Epiophlebia superstes* included in the former Anisozygoptera (Nel et al., 1993), and the basal node represents crown Epiprocta (Anisozygoptera +

Anisoptera) (Kohli et al., 2016). *Liassophlebia* fossil was found from the Bayreuth Formation between the Keuper (one of the three Triassic strata in Germany) and the Gryphaeensandstein Formation (Kohli et al., 2016). The stratigraphy is well established, and the correlated age of the Hettangian (Lowermost Jurassic) is 201.3- 199.3 Ma (200.3 ± 1.0 Ma).

D: *Sinacymatophlebia mongolica*, included in Anisoptera (Nel and Huang 2009), was from the Daohugou Biota, Inner Mongolia. The U-Pb age of the strata, that also constrains the age of feathered dinosaurs, was determined as 160.54 ± 0.99 Ma (Liu et al., 2012). U-Pb ages of ca.160 Ma from the Daohugou beds were also obtained by Liu et al. (2006).

E1: The Solnhofen limestone famous for *Archaeopteryx* yielded *Prohemeroscopus jurassicus*, of which wing pattern is similar to the present Chlorogomphoidea (Beckly et al., 1998). The biostratigraphic age using ammonite fossils is Tithonian (152.1- 145.0 Ma; 148.5 ± 3.6 Ma; Stuttgart 2007). E2: The Solnhofen limestone also yielded Zygoptera fossils (Beckly, 2019), and the calibration point E2 is at 148.5 ± 3.6 Ma.

F: The upper Purbeck Limestone in England yielded insect fossils including Petalurida dragonfly, *Anglopetalura magnifica* (Nel, 2009). The age is stratigraphically correlated to the Berriasian (the lowermost Cretaceous; 145- 139.8 Ma; 142.4 ± 2.6 Ma). Petaluridae fossils are also known from Argentina, and the Ar-Ar age for these strata is 119.65 ± 0.45 Ma (Petruleviciusa and Nel, 2003; but not adopted because the age is younger than the above fossil locality).

G: *Liogomphus yixianensis* (Gomphidae; G1), *Rudiaeschna limnobia* (Aeshnidae; G2), and *Mesocordulia boreala* (Corduliidae; G3) were described from the Jehol Biota, northern China (Ren and Guo, 1996). The Jehol Biota horizon was dated by the Ar-Ar method applied to intercalated silicic tuff, and the date is 130.7 ± 1.4 Ma (He et al., 2006).

The Santana Formation at northeast Brazil (near the Atlantic margin) is famous for well-preserved fish fossils but also includes fossils of *Cordulagomphus*, Proterogomphidae dragonfly (Petrulevicius et al., 2012). Although no reliable index fossil are present and no volcanic rock is interbedded for radiometric dating, the age is considered to be coeval with the breakup of the supercontinent Gondwana and initiation of the Atlantic Ocean, and may be Aptian, Albian or possibly Cenomanian in Cretaceous, a rather loosely-constrained date (ca.125- 100 Ma; Martill, 2007); so this calibration point was not adopted.

The Burmese amber yielded Aeshnoptera (ancestral Aeshnidae; Huang et al., 2017) and other well preserved damselfly fossils (Euphaeidae, Coenagrionidae included in BEAST v1.X analyses; Fig. 2), and the U-Pb age is 98.79 ± 0.62 Ma (Shi et al., 2012; not adopted because of the younger age).

H: The Green River Formation, USA, yielded well preserved insect fossils including Libellulidae and Zygoptera (Grande, 1980). Ar-Ar dating applied to the 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).

Libellulidae is also found from the famous fossil locality of Cereste, southern France (Nel and Paicheler, 1993), including *Lethe* butterfly (Nel et al., 1993; Pfretzschner, 1998) mentioned in Osozawa et al. (2017a). The assigned age is loosely constrained as middle Oligocene (33.9- 23.03 Ma; Ducreux et al., 1985), which represents a large relative uncertainty, so this age is not adopted for calibration, also considering the younger age than the Green River Formation.

I: Wing fossils of *Calopteryx japonica* (= *Matrona basilaris japonica*; analyzed and shown in Fig. 2) and *Oligoaeschna pryori* (no reliable COI-COII sequence data were obtained) were found from the fluvial to deltatic strata of the Beppu-Shimabara graben (Esaki and Asahina, 1957), northeastern extension and branch of the Okinawa trough (Fig. 1; cf., Osozawa K. et al., 2016). The fission track age of the dacitic tuff breccia within these strata is 1.76 ± 0.22 Ma, and the strata are magnetically reversed and considered within the Matsuyama Chron (2.58- 0.77 Ma; Okaguchi and Otsuka, 1980) consistent with the above fission track age.

Phylogenetic analyses by BEAST v1.X; Age calibration

Bayesian inference (BI) trees were constructed using the software BEAST v1.X. The BEAGLE Library must be downloaded beforehand to operate the platform software of BEAST. The details are described in Osozawa et al. (2017a-c) (see the “BEAST manual” at

<http://kawaosombgi.livedoor.blog/> for a graphic explanation).

In BEAUti, “Taxa” and “Priors” (date setting is in “Prior”; date should be set in box of “tMRCA”) were set as follows for ingroup species relative to four partitions of COI, COII, 16S rRNA, and 28S rRNA. These partitions automatically appear in “Partitions” if these sequence data are uploaded by using the plus button or by “Import Data”. The protocol is that a MRCA at each calibration of point of A to I and Q1 to Q6 initiated to differentiate into the ingroup species.

Q1: *Stylogomphus*, Q2: *Asiagomphus*, Q3: *Anotogaster*, Q4: *Chlorogomphus*, Q5: *Rhipidolestes*, Q6: *Coeliccia*: 1.55 ± 0.15 (Ma) as tMRCA.

A: Archaeognatha: 400.45 ± 7.15 (Ma).

B: Ephemeroptera : 301.3 ± 2.4 (Ma).

C: *Epiophlebia superstes* (checked “Stem” box): 200.3 ± 1.0 (Ma).

D: Anisoptera excepting *E. superstes*: 160.5 ± 1.0 (Ma).

E1: Chlorogomphoidea: 148.5 ± 3.6 (Ma), E2: Zygoptera: 148.5 ± 3.6 (Ma).

F: Petaluridae: 148.5 ± 3.6 (Ma).

G1: Gomphidae, G2: Aeshnidae, G3: Corduliidae: 130.7 ± 1.4 (Ma).

H: Libellulidae: 51.2 ± 0.3 (Ma).

I: *Calopteryx* + *Matrona*: 1.76 ± 0.22 (Ma).

In FigTree, posterior probability (“posterior”), posterior age (“Node ages”), and “rate median” (not constant because we applied relaxed clock model) can be output, and these are shown on each node in Fig. 3. We made base substitution rate (“rate median” shown at each node in FigTree) vs age (“Node age” shown at each node in FigTree) diagram (Fig.3 left bottom inset).

Declarations

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Author Contributions

Soichi Osozawa (Japanese geologist) coordinated the research, carried out the BEAST analyses, and wrote this paper, and John Wakabayashi (native English-speaking American geologist) edited the writing.

Additional Information

Supplementary information accompanies this paper at

<http://kawaosombgi.livedoor.blog/archives/11386037.html>

Competing Interests

The authors declare no competing interests.

Ethical approval

Sequence data in Table 1 are available in GenBank / DDBJ.

Data Availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Simplified BI tree for combined genes of Odonata species, calibrated by both the 1.55 Ma event and older fossil dates, using BEAST v1.X. Calibration point H, for example, represents a crown node of a common ancestor of Libellulidae ingroup species, but the stem node can be specified if it is needed. In BEAUti,

monophyletic can be specified for Libellulidae ingroup species. Note that the ingroup of calibration point Q6, for example, contains Chinese Platycnemididae species as well as Ryukyu-Taiwan species. Inserted Figure: Base substitution rate vs age diagram. Red thick curve with equation: Exponential trendline drawn by Excel function, Red thin curve: Free handed trendline drawn by connecting plots with maximum rate through the time around 320 Ma. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

BI tree for combined genes of Odonata species, calibrated by both the 1.55 Ma event and older fossil dates, using BEAST v1.X. Inserted Figure: Base substitution rate vs age diagram. Red thick curve with equation: Exponential trendline drawn by Excel function, Red thin curve: Free handed trendline drawn by connecting plots with maximum rate through the time around 320 Ma.

Figure 3

Geographical map of the Ryukyu and Japan Islands, Taiwan, Korea, and China. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 4

Anisoptera species analyzed in this paper.

Supplementary Files

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- [Table1.pdf](#)