Spermatophyta timetree, accelerated base substitution rates at mid-Cretaceous and the Recent

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Abstract

We constructed a whole of Spermatophyta timetree by employing BEAST v1. X applying the nuclear ribosomal ITS, and chloroplastic matK and rbcL. Robust multipoint calibrations were done by applying fossil ages up to the Jurassic for 20 genera and a Quaternary geological event age of 1.55 Ma for 6 genera. The resultant topology was concordant to the APG system, and we successfully and precisely dated the phylogeny. Through the BEAST analyses, we discovered the exponential increase in base substitution rate in recent geologic time, and suggested that a potential cause was generation of C4 plants and the triggered Quaternary climatic change. The raised rate might have resulted in the increasing of Spermatophyta diversity including endemic Asarum and Viola species. Another rise of base substitution rate was found around 120 Ma, reflecting the order level radiation and diversification of Angiospermae at the middle Cretaceous time.

Introduction

A goal of botany may be the correlation of botanical evolutionary events with the timeline of Earth history (Wilf & Escapa, 2015). We prepared a Bayesian inference (BI) tree constructed using the latest and the most advanced version of BEAST (v1. X; Suchard *et al* ., 2018), because a credible timetree of Spermatophyta have not been constructed to date probably by employing the old version and by the unpractical use of functions (Smith *et al* ., 2010; Beulieu *et al* ., 2015). The protocol of calibration is simply to input the time of the most recent common ancestor (tMRCA) of ingroup species in the associate software BEAUti, and both the fossil and geological event calibrations for multiple points can be simultaneously apply to run in the main software BEAST. Combined gene analysis is recommended to build a reliable tree, and the concatenation of sequence data and the related complex treatment of partitioning, which was yet found in Magallon *et al* . (2015), are not needed for this simultaneous combined gene analysis in BEAST v1. X. We selected the nuclear ribosomal internal transcribed spacer (ITS), and the chloroplastic maturase K (matK) and the ribulose-1, 5-bisphosphate carboxylase/ oxygenase large subunit (rbcL) in our BEAST analyses. The high resolution ITS data is not included in analyses such as in Soltis *et al* . (2011) and APG IV (2016), and the ITS sequence data for *Amborella trichopoda* , considered as the oldest species in Angiospermae in every study, could not find or constrain from whole genome in DDBJ / GenBank.

For Gymnospermae, we include *Ginkgo biloba* (Ginkgoidae), and representative species of Pinales (conifers) and Cycadales. For Angiospermae, we include dicots as well as monocots. Whole order of paleodicots and representative order of eudicots with older lineages in APG system are included. If the sequence data are not available in DDBJ / GenBank, we ourselves analyzed for representative Angiospermae species including *Amborella trichopoda* , and will newly upload totally 91 specimens (Table 1).

The topology of the resulting timetree was controlled by the calibration point and date set and the selection

of ingroup species in BEAUti, and prior age (input) and posterior age (output) shown in Figure 3 must be coincident. Paleodicots, eudicots, and monocots must be monophyletic as checked in BEAUti and the crown age of the paleodicots must be older than eudicots and monocots, following the established topology of the APG system. This means that the BEAST v.1.X calibration is an "active" one, in contrast to other software that employs a "passive" calibration. If the timetree has unreasonable topology different from the APG system and mismatched input and output ages, we reset the calibration and repeatedly run BEAST until to get reasonable one. Figure 3 shows the input ages of A to Q as well as the output data.

We surveyed literatures including fossil calibration reviews such as by Smith et al. (2010), and employed chronologically reliable calibration dates as shown in Methods bellow. Some are based on radio-isotopic dating 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 (Wilf & Escapa, 2015). 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; as of this writing the most recent version available is v v2020/03, and the explanatory paper related to the generation of the time scale is Cohen*et al* . (2013).

FigTree v1.4.2 draws the consequent BI tree, and has a function to show posterior probability, posterior age, mean base substitution rate, and others at each node. This function was not fully used in any previous paper, and we found in this paper the inconsistent rates through the time as suspected by the relaxed clock model of BEAST (Drummond *et al* ., 2012).

Angiosperm was diversified in Cretaceous time, and the adaptive radiation may increase the base substitution rate (Magallon *et al* ., 2015). They also predicts that while substantial amounts of angiosperm morphological and functional diversity have deep evolutionary roots, extant species richness was probably acquired later (= Quaternary). Ho *et al* . (2005), developers of BEAST v.1.3 (Drummond *et al* ., 2006), noted higher mutation rate after 1 to 2 Ma (Quaternary) compared to the prior lower rates, although they considered the accelerated rates a systematic overestimation. To revisit the issue of accelerated mutation rates requires calibration with younger ages such as those of Quaternary age.

Genus *Asarum* , section *Heterotropa* is suitable for a Quaternary calibration. *Heterotropa* is diversified in the Japan, Ryukyu, and Taiwan islands (Figs. 1 and 2; Sugawara, 2006), and this radiation is expected to have been triggered by isolation of these islands that began at 1.55 ± 0.15 Ma (Osozawa *et al* ., 2012). Whereas Takahashi & Setoguchi (2017) suggested the diversification was due to distribution range fragmentation by dispersal from China, a suitable land bridge did not exist at that time (Osozawa *et al* ., 2012). The time of the MRCA of *Heterotropa* endemic species in these islands was input as 1.55 ± 0.15 Ma for a geological event calibration, and using this we can evaluate the base substitution rate for all of Spermatophyta in relatively recent time. Duchene & Bromham (2013) showed that higher substitution rates correlate with species-richness and diversification in the Proteaceae (our analyses include tree species of Proteales, eudicots), and our study also evaluates this in the case of *Heterotropa* . Matsuda *et al* . (2017) also pointed out that the *Asarum* radiation is in progress in the Amami islands, a part of the Ryukyu islands. We additionally included *Viola* , *Lilium* , and *Pinus* for the Quaternary calibration, because the endemic species are known also from the Japan, Ryukyu, and Taiwan islands.

This paper includes phylogenetic analyses especially of*Heterotropa* wild gingers collected from the Japan, Ryukyu, and Taiwan islands, under the newly built robust timetree of whole Spermatophyta as a main subject (Fig. 3), and also attempts to assess the details of their endemism and radiation relative to the base substitution rate (Fig. 3 inset). There are no previous botanical studies of this type in this island region of East Asia, possibly because a straightforward evolutionary model cannot assume a simple dispersal and colonization process as supposed by Takahashi & Setoguchi (2017) and Nakamura *et al* . (2010).

Materials and Methods

Taxon sampling (Table 1)

Amborella trichopoda , New Caledonia origin, was offered by the Koishikawa Botanical Garden, the University of Tokyo. Some Angiospermae specimens unavailable in DDBJ / GenBank were collected from Experimental Station for Medical Plant Studies, Graduate School of Pharmaceutical Sciences, Tohoku University (Table 1). Endemic *Viola* and others were collected (Table 1), collaborated the *Asarum* sampling below.

The genus *Asarum* consists of three sections of *Heterotropa*(evergreen, main section), *Euasarum* (deciduous, stolon; 16 species in Kelly, 1998; previous *Asarum* ; renamed by Sinn *et al* ., 2015b), and *Asiasarum* (deciduous; not known from the Ryukyu and Taiwan islands; two species). The phylogenetic relationship of these sections, focused on section *Euasarum,* was studied by Kelly (1998), Sugawara *et al* . (2005), and Sinn *et al* . (2015ab). The phylogeny of section *Asiasarum* , distributed in East Asia, including the Japan islands, was studied and subdivided into species by Yamaji *et al* . (2007). Section *Heterotropa* has not been analyzed over the entirety of the Japan, Ryukyu, and Taiwan islands (Fig. 1) except for a recently published paper of Takahashi & Setoguchi (2017). Note that we submitted our *Heterotropa* sequence data to DDBJ / GenBank in 2015, and they were released in 2019 (Table 1).

On the basis of flower structure, the pollinators of *Heterotropa*are probably tiny flies and millipedes (Hiura, 1978; Sinn *et al* ., 2015b), and seeds may be carried/transported by ants (Maeda, 2013; Sinn et al., 2015b). The dispersal ability of *Heterotropa* may be small (Sugawara, 2006), and this may be a factor in generating endemic*Heterotropa* species (Takahashi & Setoguchi, 2017). Note, however, that some islands, such as Amami Oshima island, yield plural endemic species (Fig. 1; Table 1), and these are mostly sympatrically distributed within such an island including Amami Oshima (Maeda, 2013). Therefore, allopatric speciation, proposed by Takahashi & Setoguchi (2017), cannot be simply applied to explain the extraordinary diversification of *Heterotropa* in the Japan-Ryukyu-Taiwan islands.

Collected leaf samples were stored in a desiccator with silica gel filling the space under the platform. Voucher *Asarum* specimens were also prepared. Specimen numbers (= isolate), localities (= country), species, and collectors are registered in the DDBJ / GenBank. These accession numbers, and additional information on the character of calyx, leaf, and stem are shown in Table 1. Some representative calyx photos are shown in Fig. 2. Many endemic *Asarum* species are also known from China, and the Chinese sequence data have recently become available in the DDBJ / GenBank.

DNA extraction, polymerase chain reaction amplification, and sequence alignment

A problem applying molecular phylogenetic method to plants was the lack of suitable primers to amplify recognizable variation (Kanno *et al* ., 2004; Fazekas *et al* ., 2008). However, the chloroplastic matK and rbcL genes were recommended for use as markers by the CBOL Plant Working Group (2009). In addition, the nuclear ITS gene is known to be an additional suitable marker (*Asarum* : Kelly, 1996; Sugawara *et al* ., 2005; Yamaji *et al* ., 2007; China Plant BOL Group, 2011; Sinn *et al* ., 2015ab; Takahashi & Setoguchi, 2017), although the APG (1998, 2003, 2009, 2016) did not include the ITS data in their analyses. We analyzed by the combining these genes (not by the concatenated data set) in BEAST v1. X as the first trial, and found that the resolution was much improved.

DNA extraction was done using the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit by Sigma-Aldrich. Before this operation, crushed young leaf specimens were cleaned with 1% Tris and EDTA solution in 1.5 mL tube.

Primers used for amplification of the nuclear ribosomal ITS were ITS5F (5- GGA AGG AGA AGT CGT AAC AAG G -3) and ITS4R (5- TCC TCC GCT TAT TGA TAT GC -3), following the China Plant BOL Group (2011).

Primers used for amplification of chloroplastic matK were matKF (5- CGT ACA GTA CTT TTG TGT TTA CGA G-3), and matKR (5- ACC CAG TCC ATC TGG AAA TCT TGG TTC-3), following the CBOL Plant Working Group (2009). Notice that matKF was downstream primer, and matKR was upstream primer, actually.

Primers used for amplification of chloroplastic rbcL were rbcLF (5- ATG TCA CCA CAA ACA GAG ACT

AAA GC-3), and rbcLR (5- GTA AAA TCA AGT CCA CCR CG-3), following the CBOL Plant Working Group (2009).

Amplification was done by GoTaq G2 Green Master Mix, Promega, and the temperature of incubation was 94@C for 60 seconds, with denaturation at 94@C for 30 seconds, annealing at 53@C for ITS (56@C for matK; 54@C for rbcL) for 60 seconds, extension at 72@C for 60 seconds, cycled 35 times, and final extension was at 72@C for 5 minutes.

The PCR product was purified using Wizard SV Gel and PCR Clean-Up System, Promega. Sequencing was done by Macrogen Japan.

Sequence alignment was done using ClustalW incorporated in MEGA X (Kumar et al., 2016). No gap was observed in aligned sequences for*Asarum* , although gapes were found in ITS region for the resting sequences even for *Aristolochia* species relative to the*Asarum* sequences. The ITS gene (694 bp), the chloroplastic matK gene (833 bp), and the chloroplastic rbcL gene (544 bp) were readable for *Asarum* and the number of base pair were similar to the resting. Codon translation for the matK and rbcL sequences was checked using the ExPASy- Translate tool, Bioinformatics Resource Tool. These sequences were also checked by BLAST (Basic Local Alignment Search Tool) offered by DDBJ / GenBank, and such data are reflected in our registered data in the DDBJ / GenBank.

PHYLOGENETIC ANALYSES ASSOCIATED WITH FOSSIL + GEOLOGICAL EVENT CALIBRATION BY BEAST

A Bayesian inference (BI) tree (Fig. 3; *Heterotropa* tree extends to the right in this diagram) was constructed using the software BEAST v1. X released on 10th June 2018 (Bayesian Evolutionary Analysis Sampling Trees; Suchard *et al* ., 2018), running BEAUti (Bayesian Evolutionary Analysis Utility), BEAST, TreeAnnotator, and FigTree, in ascending order. Before operating the BEAST software, the BEAGLE Library must be downloaded.

For graphic explanation of the operation of this software, see the "BEAST operating manual" at:

http://kawaosombgi.livedoor.blog/

Calibration point A: *Gonkgo biloba* is often described as a living fossil and an extant genus, and *Gonkgo* fossil wood was reported from the Liaoning province, northern China (Jiang *et al* , 2016). Chang et al. (2009, 2014) reported Ar-Ar ages of 160.7 ± 0.4 Ma and 166.7 ± 1.0 Ma, respectively, and we adopted the latter as the prior input and stem age (Fig. 3).

Calibration point B: Conifer fossil woods were reported from the Eocene La Meseta Formation, Antarctica (Pujana *et al* ., 2014), and marine microplanktonic fossils indicate a Priabonian (35.95 ± 2.05 Ma; Cocozza & Clarke, 1992; Fig. 3) age for these rocks.

Calibration point C: Modern but also fossil *Ceratozamia* was reported from the European Oligocene basinal strata (Kvacek, 2014; Kovar-Eder, 2016), and corresponds to calcareous nannoplankton zone NP23 (31.8 ± 2.2 Ma) and planktonic foraminifera zone P18 (32.9 ± 0.9 Ma; Fig. 3). Fossil localities of other modern cycads such as Australia are not well constrained by geochronology. Note that extinct cycad species such as *Nilssonia* cannot be adopted for calibration dates.

Calibration point D1: *Archaefructus* from the Jehol Biota, northeast China, is an extinct but earliest known genus of Angiospermae. The lower *Archaefructus* fossil horizon within the Yixian Formation (Sun *et al* ., 2011) was dated by the Ar-Ar method applied to intercalated silicic tuff, and the date was 130.7 ± 1.4 Ma (He et al. 2006). We applied this date for the crown age of paleodicots (monophyletic), rather than the whole Angiospermae. Calibration point D2: No fossil *Amborella* has not been found, but this species is the oldest lineage of paleodicots in the APG system, and the stem age was applied also as 130.7 ± 1.4 Ma.

Calibration point E: *Leefructus mirus* also from from the Jehol Biota is included in a basal eudicot family of Ranunculaceae (Sun*et al* ., 2011). The fossil horizon within the middle Yixian Formation (Sun *et al* ., 2011)

was dated by the Ar-Ar method applied to sanidine included in intercalated silicic tuff, and the date was 124.60 ± 0.25 Ma (Yang *et al* . 2007).

Calibration point F: Palynological data of the Early Cretaceous continental sequences of western Portugal revealed that monocot radiation was in Aptian time (119 ± 6 Ma) (Hochuli *et al* ., 2006). They stated that it preceded the radiation of dicots by at least 10 m.y., but it conflicts to the above calibration of D and E and the APG system, and we do not follow this statement.

Smith *et al* . (2010) reviewed dicot fossil data applicable to our fossil calibrations (Fig. 3). Calibration E: Nymphaeales (E1, paleodicots) fossils were from Albian of Jordan (106.75 ± 6.25 Ma; Tayler et al., 2008). Chloranthales (E2, paleodicots), Magnoliidae (Magnoliales + Laurales + Piperales; E3, paleodicots), Buxuales (E4, eudicots), and Proteales (E5, eudicots) fossils were from he Albian Potomac Group (106.75 ± 6.25 Ma; Crane et al., 1994; Crane & Herendeen, 1996).

Calibration point F: Saxifragales (F1, eudicots) and Ericales (F2, eudicots) fossils were reported from the Turonian Raritan Formation, USA (91.85 ± 2.05 Ma; Hermsen *et al* ., 2003; Nixon & Crepet, 2003).

Calibration point G: Fossil leaves of *Piper* (Piperales, paleodicots) were reported from the Maastrichtian coal seams in Colombia, South America (Martinez *et al* . 2015), so the time of MRCA of all species of Piperales including *Piper* ,*Houttuynia* , *Aristolochia* , and *Asarum* was 69.05 ± 3.05 Ma (Fig. 3).

Calibration point H: A fossil of *Aristolochia* (Piperales) was reported from the western Pannonian Basin, Austria (Meller, 2014) and estimated as Tortonian (11.62 - 7.24 Ma; Miocene) in age (Matenco and Radivojevic, 2012). In contrast, an older, Eocene *Aristolochia*fossil was reported from the Green River Formation (Grande, 1984), and an Ar-Ar age of 51.25 ± 0.31 Ma has been reported from these rocks (Smith *et al* ., 2003). Accordingly, for the time of MRCA of Aristolochiaceae including *Aristolochia* and *Asarum* , the prior age is input as 51.25 ± 0.31 Ma (Fig. 3).

Lies *et al* . (2015) reviewed monocot fossil data applicable to our fossil calibrations (Fig. 3). Calibration point I: A *Typha* fossil was reported also from the Green River Formation (Grande, 1984) with above mentioned an Ar-Ar age of 51.25 ± 0.31 Ma (Smith *et al* ., 2003). Calibration point J: A *Dioscorea* fossil was found from strata in Ethiopia with an U-Pb age of 27.23 ± 0.1 Ma (Pan *et al* ., 2014). Calibration point K1 and K2: Fossils of *Earina* and*Dendrobium* were reported from strata in New Zealand (Conran*et al* ., 2009) with an Ar-Ar age of 23.2 Ma (errors are not shown; Lindqvist & Lee, 2009). Calibration point L: *Protoyucca* was found from Nevada (Tidwell & Parker, 1990), and the silicic tuff was dated by the Ar-Ar method (15.35 ± 0.85 Ma; Perkins *et al* ., 1998)

Calibration point Q1: We considered that the *Heterotropa* endemic species of the Japan-Ryukyu-Taiwan islands (Fig. 1) simultaneously began to radiate at 1.55 ± 0.15 Ma, following the geological event of island separation presented in Osozawa *et al* . (2012).

Calibration point Q2 to Q4: Similarly, Viola endemic species are especially in the Ryukyu islands, and *Viola okinawensis* + *V. grypoceras* (A2), *Viola amamiana* + *V. tashiroi*(A3), and *Viola yedoensis* + *V. betonicifolia* (A4).

Calibration point Q5 and A6: Similarly, *Lilium alexandrae* and*Pinus luchuensis* are endemic in Ryukyu. A cone fossil of *P. luchuensis* was found from the 1.55 Ma Guga Formation, Okinawa island (Osozawa & Watababe, 2012), and the above geologic event calibration is concordant with the fossil calibration date. Fossils of*Cunninghamia lanceolata* (Pinidae), *Cerbera manghas (* Gentianales), *Schima wallichii (* Ericales), *Liquidambar formosana (* Saxifragales), *Caesalpinia crista (* Fabales), and*Quercus salicina* (Fagales) were also found from the Guga Formation (Osozawa & Watababe, 2012), and these species were included in the analyses (Table 1).

In BEAUti, the following software settings were used. Partitions: fasta files were converted into nexus files by ClustalW offered by DDBJ, and the loading was by using the Import Data or plus button. Partitions defined by the ITS, matK, and rbcL gene sequences appeared in the Partition box.

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), and the right screen: Included Taxa. As output in Fig. 3, calibration dates were set in Priors.

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 noted above as Prior Distribution: Normal, and the Mean and Standard deviation.

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

Running BEAST was done by incorporating each 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 Fig. 3 to avoid confusion. In FigTree, posterior probability ("posterior"), posterior age ("Node ages"), and "rate median" (not constant) can be output, and these are shown 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 inset).

Results

Spermatophyta time tree (Fig. 3)

Gymnospermae is a sister of Angiospermae. The tree root age was estimated to be 179 Ma.

In Gymnospermae, *Ginkgo biloba* is an old lineage, and dated back to 16.63 m.y. as calibrated. *G. biloba* is a sister of the other species of Gymnospermae. Pinales is a sister of Cycadales, and the separation in order level is estimated at 116.38 Ma. Pinales consists of two clades of Cupressaceae and Pinaceae with the basal node for family level at 37.8 Ma, and began differentiation into genus level since 26.8 Ma. Cycadales consists of two clades of Cycadaceae and Zamiaceae with the basal node at 69.13 Ma, and differentiated since 34.24 Ma.

The crown age of Angiospermae was estimated at 132.44 Ma. As we calibrated paleodicots (including *Amborella*), eudicots, and monocots at 130.7, 124.6, and 119 Ma, the crown dates were estimated at 132.44, 124.62, and 120.38 Ma, and these ingroup species were represented as monophyletic, respectively. Consequently, the Angiospermae topology of paleodicots (including *Amborella*), eudicots, and monocots was concordant to the APG system. Internal topology each of paleodicot, monocot, and eudicot was almost concordant to the APG system, and we describe the node ages below (Fig. 3).

In paleodicots, *Amborella trichopoda* is the oldest lineage. Austrobaileyales and Nymphaeales is a sister differentiated at 105.3 Ma. Chloranthales is a sister of Magnoliidae differentiated at 106.84 Ma. Magnoliales and Laurales is a sister differentiated at 66.6 Ma, and also sister to Piperales at 76.17 Ma.

In Piperales, Piperaceae is asister of Saururaceae differentiated at 16.77 Ma, and these and Aristolochiaceae were differentiated at 58.08 Ma. Genus level differentiation into *Aristolochia* and*Saruma* + *Asarum* was dated at 48.91 Ma, and *Saruma*and *Asarum* were dated at 10.54 Ma.

Saruma henryi is a sister of and an outgroup species relative to the *Asarum* species as shown by Kelly (1998) and Sinn *et al* . (2015ab). Section *Asiasarum* is a sister of section*Euasarum* + section *Heterotropa* , and section*Euasarum* is a sister of section *Heterotropa* . In section*Asiasarum* , *A. sieboldii* is not differentiated

among collection sites in Japan, but is differentiated relative to the Korean specimen. In section *Euasarum* , inter-species and intra-species differentiation is recognized, although with stolons for plant propagation (no stolon for *Heterotropa*).

The Chinese *Heterotropa* excluding *A. forbesii* (= section*Longistylis* ; Sinn *et al* ., 2015b) is a sister of *A. forbesii* + the Japan-Ryukyuan-Taiwan *Heterotropa* , and *A. forbesii* is a sister of the Japan-Ryukyu-Taiwan *Heterotropa* . Four Chinese species with rbcL data in the DDBJ / GenBank are included in our analyses, but an additional 14 Chinese species were also included in section *Longistylis* clade based on solely ITS and matK data.

Endemic species of section *Heterotropa* were severely differentiated on the Japan-Ryukyu-Taiwan islands and the divergence time was considered to be the geological event of 1.55 Ma. The differentiation tends to occur within islands, and therein constitutes distinct clades. The species of each clade tends to have common characteristics of calyx and leaf (Fig. 2; Table 1). However, sympatric occurrence of genetically distinct species within a single island without a sister relationship, differentiation into a distinct clade on the same island, and inter-island sister relationships were found.

In Monocots, *Trillium smalli* (Liliales) was represented as the oldest lineage, although *Lilium* (Liliales) is a sister of Dioscoreales differentiated at 59.91 Ma. Acorales was differentiated at106.79 Ma, and Alismatales was at 94.74 Ma (Aponogetonaceae is a sister of Araceae differentiated at 63.77 Ma). The descendent consists of two clade groups of Liliales + Dioscoreales + Asparagales (Orchidaceae), and Asparagales (Asparagaeae + Iridaceae) + Poales with the basal node and start of order level differentiation at 83.22 Ma, and the youngest one is at 59.91 Ma for Dioscoreales and Liliales. Family level differentiations were at 50.62 Ma for Asparagaceae and Iridaceae of Asparagales, and at 62.33 Ma for Typhaceae and Poaceae of Poales. Poales is not absolutely the youngest lineage in monocots, started genus level differentiation at 34.97 Ma, and C4 Poales generated and started differentiation from 15.58 Ma.

In eudicots, Ceratophyllales was expressed as the oldest lineage of 120.31 Ma, Ranunculales is the next at 116.48 Ma, and Sabiales the third at 108.55 Ma, common to the APG system. Buxuales, Proteales, Trochodendrales, Vitales, Gunnerales, and Saxifragales are older lineages differentiated before 91.62 Ma, also common to the APG system.

Other than the above orders, eudicots have two major clades; Caryophyllales and asterids from Ericales to Lamiales, and rosids from Celastrales to Malpighiales, and this classification into the two clades of asterids and rosids basically followed the APG system, Soltis*et al.* (2011), and Magallon *et al* . (2015). Santalales is the basal lineage with a crown date at 98.83 Ma relative to the two major clades. Order level differentiation began at 98.26 Ma, but continued at 31.16 Ma for Apiales and Asterales. This order level Neogene date for eudicots is much younger for paleodicots and also monocots and Gymnospermae.

Totally 16 nodes of family level differentiation were found at from 63.77 to 17.25 Ma (Paleogene to Neogene). We included C4 Amaranthaceae (Caryophyllales) in our analyses, and differentiated at 25.93 Ma, although the stem age was up to 90.14 Ma.

Viola (Malpighiales; rodids) has crown node of 15.49 Ma. Endemic *Viola okinawensis* is a sister of widely distributed *V. grypoceras* . *Viola amamiana* , Amami Oshima, is a sister of *V. tashiroi* , Iriomote-jima. *Viola yedoensis* is differentiated in each island of Ryukyu, Tanwan, and HongKong. *Viola betonicifolia* is a similar species with and a sister of sympatric *V. yedoensis* .

Sister relation was found for additional calibrated species by 1.55 ± 0.15 Ma for nodes of A5 of *Lilium* (monocots) and A6 of*Pinus* (conifers).

Diversification and exponential increase in base substitution rate

As shown in Fig. 3, order level differentiation and then diversification was from the mid-Cretaceous to Paleogene, but that of eudicots extended to the late Paleogene. Family level differentiation was during the Paleogene, but that of eudicots extended to the Neogene. Genus-species level differentiation was after the

late Paleogene and mostly Neogene, and for the species calibrated 1.55 Ma of A1 to A6, the differentiation and diversification was an event of the Quaternary.

The base substitution rate has exponentially increased since 10 Ma (Miocene; Neogene), and the equation of trendline is shown on the inset in Fig. 3.

All the *Heterotropa* rates were less than 0.01 as shown in lower rates on left hand figure of *Heterotropa* (Fig. 3), and the 1.55 Ma calibration for *Heterotropa* did not affect the increasing rata in Quaternary time.

We also drew a "trendline" by smoothly connecting plots with maximum rate through the time (red curve on inset in Fig. 3). This curve shows another peak for rate around 130 Ma (mid-Cretaceous).

Discussion

Order level phylogeny, reflecting mid-cretaceous Angiospermae radiation

Gymnospermae and Angiospermae have a sister relation, and whole Spermatophyta (Gymnospermae and Angiospermae) phylogeny was presented by Rydin *et al* . (2002), by selecting chloroplastic rbcL, other chloroplastic, and two nuclear genes and using PAUP* (Swofford, 1998). Although PAUP* has no calibration function, this high level phylogeny is comparable to ours.

The crown age of *Ginkgo biloba* was estimated at 166.63 Ma, reflecting the calibration date of N: 166.7 ± 22 Ma (Fig. 3), suggesting that this species is actually a living fossil. The order level node age was 116.01 Ma between Pinales and Cycadales.

The Angiosperm phylogeny was presented by the Angiosperm Phylogeny Group (APG) (APG, 1998) and updated several times (APG II, 2003; APG III, 2009; and APG IV, 2016). APG selected the chloroplastic rbcL and two other genes, but even the most recent work appears to be hampered by connected to the concatenated data set for analyses and the use of the older software PAUP* (Swofford, 2002). The Angiosperm phylogeny of APG IV is now a base of systematic botany and much affects on it (Sokolof*et al* ., 2018). Similar phylogeny was presented by selecting both chloroplastic rbcL and matK, and other 15 genes by Soltis *et al* . (2011), but it is not possible to age calibrate in RAxML (vers. 7.1; Stamatakis, 2006). Other Angiosperm phylogeny was presented selecting four mitochondrial genes by Qiu *et al* . (2010) and selecting tree genes including rbcL by Smith et al. (2010), but this analysis was also conducted using RAxML (Stamatakis *et al* ., 2008) with the same limitations. The broadly inclusive seed plant phylogeny was by Smith & Brown (2018) using RAxML v. 8.2.11 (Stamatakis, 2014), but note that the most recent version is raxmlGUI 2.0 beta (Elder et al., 2019). Smith*et al.* (2010) also made timetree employing older version of BEAST v1.4.7 (Drummond & Rambaut, 2007).

We also estimated crown age of Amborellales at 126.23 Ma, and it represented the oldest lineage in paleodicots, also concordant to the APG system and many other papers, although any fossil was reported but we set in BEAUti to satisfy that.

If restricted to monocots, the phylogenetic tree was made by PAUP* (Swofford, 1993), and dates and gene substitution rates have been calculated by analysis of variance, selecting the rbcL gene (Bremer, 2000). Our monocot topology including the older lineages of Acorales and Alismatales, and the descendent two clades of Asparagales (Orchidaceae) + Dioscoreales + Liliales, and Asparagales (Asparagaeae) + Poales is basically common to the order level phylogeny Bremer (2000).

If restricted to eudicots, Ceratophyllales is the oldest lineage, Ranunculales is the next, Sabiales the third, and others, and the descendent two clades of asterids and rosids are common to the APG system, Soltis *et al.* (2011), and Magallon *et al* . (2015). The youngest date of order level branching for eudicots was at 31.16 Ma between Apiales and Asterales of the asterids clade. Note that order level differentiation of eudicots was extended to the younger age than the other such as paleodicots, and the diversification and evolution was the younger event for eudicots.

Our Angiospermae topology was as a whole concordant to the APG system and referenced papers above. The other word, we set BEAUti calibrations to satisfy to generate concordant topology of paleodicots, monoicots, and eudicots to the APG system. Note that, however, our calibrations were robust. Therefore, our contribution was not the Angiospermae topology, but through building a dated phylogeny we estimated dates of order level differentiation, as well as family and genus-species level differentiation below. We found base substitution rates were valuable but with some tendency (Fig. 3 inset), probably reflecting these differentiations and radiations.

Angiosperm timetree was drawn by more recent but two generation older version of BEAST v1.7.5 (Drummond *et al* ., 2006) selecting rbcL, matK, and other three genes (Magallon *et al* ., 2015), and every family was contemporaneously differentiated between 140 and 120 Ma due to their calibrations comparable to ours. Magallon *et al* . (2015) found that crown eudicots are approximately contemporaneous with crown paleodicots and monocots, which was a noteworthy difference from the previous studies estimated to the younger crown date of eudicots. According to the statements: "Eudicots are morphologically characterized by tricolpate pollen (or derived from this condition; Walker & Walker, 1984; Donoghue & Doyle, 1989), which probably evolved on the stem lineage of this clade. Tricolpate pollen grains are morphologically distinctive, can easily become preserved as fossils, and unequivocally indicate membership to a single clade, thus providing an exceptionally good calibration. The eudicots were estimated as being nearly contemporaneous with paleodicots and monocots; therefore, the major components of angiosperm extant diversity began to diversify in the middle Cretaceous." Note that Ceratophyllales expressed as the basal eudicots (Fig. 3 and the APG system) is tricolpate.

In our timetree of Fig.3 as noted above, crown age of Angiospermae was estimated at 132.44 Ma, paleodicots; 131.53 Ma, monocots at 116.68 Ma, and eudicots: 120.31 Ma, and almost contemporaneous, concordant to timetree by Magallon *et al* . (2015) but also the APG system. Rather than the order of the branching into paleodicots, monocots, and eudicots, such the contemporaneous differentiation event should be notable.

Family to species level phylogeny, reflecting the relatively recent angiospermae radiation

In Gymnospermae, Pinales consists of two clades of Cupressaceae and Pinidae, common to Ran *et al* . (2018), who employed RAxML v.8.2.4 (Stamatakis, 2014) and MCMCTree (Yang, 2007). As for Cycadales, the timetree was successfully drawn by BEAST v.1.3 (Drummond *et al* ., 2006) selecting rbcL and matK genes and by fossil calibrations (Nagalingum *et al* ., 2011), and their Cycad timetree consisting of the Cycadaceae and Zamiaceae clades was comparable to ours. We estimated the family level node age at 37.99 Ma for Pinales differentiation and at 69.36 Ma for Cycadales differentiation.

Nagalingum *et al* . (2011) pointed out that Cycadales is a living fossil, but each speciation is a relatively young event in the Neogene. We estimated genus level differentiation of Cycadaceae was after 39.56 Ma, and Zamiaceae was after 34.35 Ma. In a case of Pinales, Cupressaceae was after 26.32 Ma, and Pinaceae was after 21.79 Ma.

As for paleodicots, family level differentiations were found in Piperales, and at 51.26 Ma for Piperaceae + Saururaceae and Aristolochiaceae, and at 16.77 Ma for Piperaceae and Saururaceae. It was also found out between Nymphaeaceae and Cabombaceae (Nymphaeales). Genus level differentiation was after 51.26 Ma for *Aristolochia* and*Asarum* and the restings, and succeeded until recent. See*Asarum* radiation, next section.

As for monocots, family level differentiation was at 63.77 Ma for Araceae and Aponogetonaceae, at 50.62 Ma for Asparagaceae and Iridaceae, and at 62.33 Ma for Typhaceae and Poaceae. The oldest genus level differentiation or speciation for Typhaceae at 51.2 Ma was succeeded by many speciation events until recent.

As for eudicots, totally 15 nodes of family level differentiation were found at from 61.66 to 17.25 Ma, and it succeeded to much younger dates than paleodicots and monocots. It was succeeded by genus-species level differentiation for eudicots since at 34.03 Ma for Nelumbonaceae, and *Viola* speciation was such radiation event as noted in Results.

Adaptive radiation of Japan- Ryukyu- Taiwan *Heterotropa*

Six species groups calibrated by A1 to A6 at 1.55 ± 0.15 Ma (Fig. 3) were typical examples reflecting extensive radiation, and we discuss here *Heterotropa* (*Asarum* ; Aristolochiaceae; Piperales; paleodicots) radiation.

Regarding solely *Heterotropa* , our timetree is comparable to Takahashi & Setoguchi (2017). They employed BEAST v.1.7.5, analyzed by solely ITS (or matK) data, and applied a suspected and constant base substitution rate of ITS (0.0413 /m.y.) for calibration (strict clock model).

Allopatric speciation is expected to have acted on allopatric island populations of *Heterotropa* , but these endemic species are not always allopatric but partly sympatric within an island, such as on the Amami Oshima islands (Maeda, 2013). Amami Oshima yields many*Heterotropa* species, and we analyzed six endemic species. All these differentiated species splitting into three distinct clades (Fig. 3 right bottom; red colored) may not have been generated solely by the vicariant speciation (cf., Osozawa *et al* ., 2014, 2015ab, 2016, 2017abc; Osozawa & Wakabayashi, 2015).

The geological event that formed isolated islands of Ryukyu at 1.55 Ma is also tied to climatic and other environmental changes. The isolation of Ryukyu islands from continental Asia also prevented detrital sediment from the Yangtze and Yellow rivers from reaching these locations, so coral reefs began to form around each island in the absence of this detrital input. The separation of the Ryukyu islands from the mainland also resulted in the inflow of the Kuroshio current through the trough (Fig. 1), and this current, thus rerouted, flowed into and warmed the present East China Sea (Osozawa *et al* ., 2012). The rifting event resulted in a sea or seaways where the mainland once was, and this affected storm patterns. Typhoon tracks once passed over land and lost energy, but after the rifting remained over the sea and maintained their strength further along their course (Osozawa & Wakabayashi, 2015). In the case of the Honshu of the Japan islands, the Tsushima warm current, a branch of the Kuroshio current, began to flow into the Japan Sea as a result of the rifting event (Fig. 1). This current warmed the sea water during the winter, and the consequent wet, northwestern seasonal wind caused heavy snowfall along the Japan Sea coast (Fig. 1; Osozawa*et al* ., 2012).

As a consequence, the *Heterotropa* endemic speciation and severe diversity in the Japan- Ryukyu- Taiwan islands (=the simultaneous genetic and morphological expansion; Fig. 1 right; Figs. 2 and 3) might have rather resulted from the mechanism of adaptive radiation from a single ancestral *Asarum* inhabiting the eastern margin of original Asia continent (Fig. 1 left), similar to that observed for the Hawaiian silversword alliance (Baldwin and Sanderson, 1998; Blonder *et al* ., 2015; cf., Alpine plants in Boucher *et al* ., 2015).

On Amami Oshima, adaptive radiation may be in progress (Matsuda *et al* ., 2017). *A. fudsinoi* has many morphotypes of calyx and leaf as if distinct species, and *A. fudsinoi* is genetically similar to other *A. gusk* , *A. celsum* , and *A. pellucidum* with distinct morphology (Fig. 3 right center).

Similar to *Fagus crenata* (Fig. 3; although the MRCA age is estimated at 20.39 Ma), *A. megacalyx* might have adapted to a heavy snowy environment and radiated in northern Honshu (Fig. 1). Hiura (1978) originally proposed the idea of adaptation to a snowy climate, and this included the premise that the increased size of calyx during the several month growth period indicated adaptation to the heavy snow accumulation.

Asarum forbesii is a sister of the island *Heterotropa,* and it differentiated at 1.91 Ma; these have a sister relation to the other Chinese *Heterotropa* (section *Longistylis*) differentiated at 3.72 Ma (Fig. 3; *Longistylis* species were also radiated since 2.63 Ma). At 3.72 Ma, a single common ancestor of *A. forbesii*inhabited along the Yangze river and the *Heterotropa* colonized the eastern area of the Asian continent partly overlapped in habitat, and started to differentiate at 1.91 Ma (Fig. 1). This date may be related to start of glacier and inter-glacier climatic change of the Quaternary time since 2.58 Ma.

Triggers for the recent and mid-Cretaceous increases in base substitution rate

A strict clock model assumes that every branch evolves according to the same evolutionary rate (Drummond *et al* ., 2006), but the rate and branch length is clearly not proportional, and thus we should apply relaxed clock model. We can now discuss the variable rate, through building a credible Spermatophyta timetree by fully applying multiple point calibrations and partition functions of BEAST v1. X.

The molecular clock hypothesis states that DNA sequences evolve at a relatively constant rate over time even in a case of relaxed molecular clock, but the absolute clock needs to be calibrated (Ho, 2008). We showed that the base substitution rate of Spermatophyta has increased exponentially toward the Recent as shown by the trendline (Fig. 3 inset). This phenomenon had been shown for taxa such as primates (Ho*et al* ., 2005, 2011) and insects (Papadopoulo *et al* ., 2010), but generated little attention. BEAST v1. X is possible to run simultaneously applying multiple calibration points, 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 is assumed to be constant at each node (like as strict clock model, although relaxed clock model was applied). They found that a Quaternary date calibration produced a rapid rate. Note that we instantaneously calibrated by multi dates including older dates such as Jurassic as well as younger dates of Quaternary of 1.55 Ma, not solely calibrated by the Quaternary date, and the reason of increasing rate is unrelated only to the Quaternary calibration (*Asarum*rates were estimated less than 0.01). Also note that combined gene analyses were not possible in old versions of BEAST, and such the users needed to discuss the rate for every each gene or concatenated gene. 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 strict clock.

We justified that the recently increasing base substitution rate is an actual event. A possible trigger of increasing rate may have been the start of glacial and interglacial cycle and the severe environment change in the Quaternary time. As noted above, this may be a factor in*Heterotropa* radiation, although physical isolation of islands was probably more important. Whereas such glacial episodes in Earth history appear to be influenced by landmass configuration such as the end Permian (Rohde & Muller, 2005), feedback from biologic development may have also played a role in triggering the onset on glaciation. The Quaternary glaciations may have been triggered by expansion of land grasses prevailing Poales, and the Permian glaciations may have been triggered by the development of terrestrial tree ferns to form thick coal layers, because these processes increased carbon fixation that consequently decreased atmospheric CO2 concentration (Taira, 2007). C4 plants are efficient for such CO2 fixation (Saga, 2004), and C4 Poales plants appeared and started diversification from 15.58 Ma (Fig. 3; C4 eudicots from 25.93 Ma). Carbon isotope ratios from mammalian fossil tooth enamel show that diet change into C4 plants started at 9.9 Ma in eastern Africa (Uno *et al* ., 2011). Also the mammalian fossil tooth from the sub-Himalayan Siwalik Group, Pakistan, C4 savannah replaced C3 forest and woodland between 8.5 and 6.0 Ma (Badgley et al., 2008). 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 (Cerling et al., 1997). Note that increasing rate started at 10 Ma, not at 2.58 Ma of the Quaternary (Fig. 3 inset).

Nagalingum *et al* . (2011) showed that cycads underwent a near synchronous global diversification beginning in the Miocene. Our result revises the estimate for the initiation of this genus level diversification to 39.56 Ma (Oligocene; Fig. 3). Such a date coincides with a large and rapid drop in atmospheric CO2 and the onset of cooling also at the Eocene to Oligocene transition (33.9 Ma; c.f., Pound & Salzmann, 2017). Note that conifer radiation is also estimated at 37.99 Ma (Eocene; Fig. 3).

Fossil record indicates a dramatic increase in phylogenetic diversity and ecological abundance of angiosperms around the mid-Cretaceous (Friis*et al* ., 2006), and this event may be tied to another rate peak around 130 Ma as shown by the red trendline (Fig. 3 inset), which may express a start of order level radiation of Angiospermae (Fig. 3). Thereafter, both the paleodicots and monocots were extensively differentiated in order level, and then family level, especially for eudicots until the Neogene (Fig. 3). Insect beetles have been clarified to have a rate peak also around middle Cretaceous time (Hunt *et al* ., 2007; McKenna *et al* ., 2015; Gunter *et al* ., 2016; Toussaint *et al* ., 2016; Zhang *et al* ., 2018), and the increasing rate was explained to tie to the co-radiation of Angiospermae as the food plant. Also broad-leaved angiosperm plant species, especially dicots, initiated a global ecological transformation towards the Cretaceous biodiversity (Jan de Boer *et al* ., 2012).

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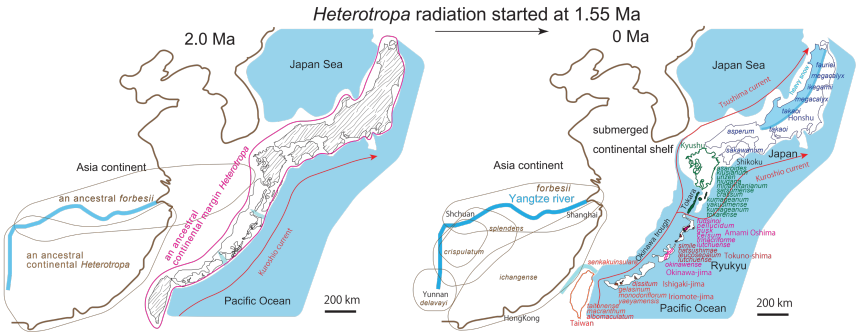
Fig. 1 Right figure: Present (0 Ma) habitats of*Heterotropa* in the Japan-Ryukyu-Taiwan islands and China. Colors reflect endemic species from an island or island unit. Note that the same island species are not always allopatric. Left figure: Original (2 Ma) habitats of a single ancestral species in the proto- Japan-Ryukyu-Taiwan islands (= marginal area of the Asia continent), that of *Asarum forbesii* along the Yangtze River, and

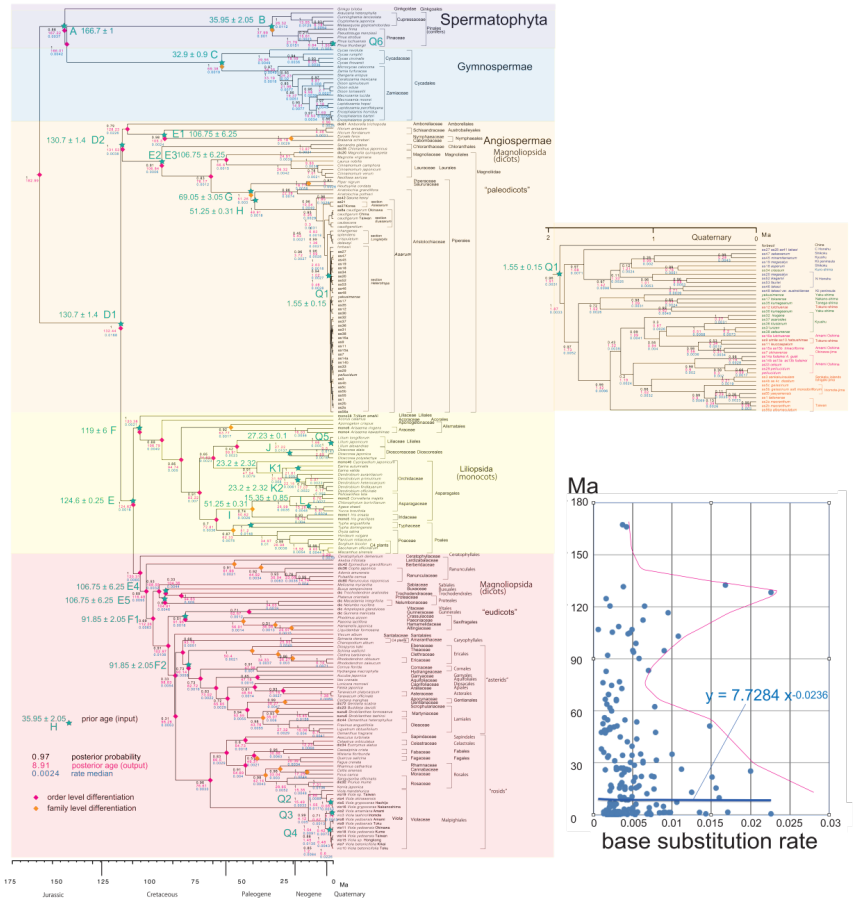
those of other Chinese *Heterotropa* . Note that *A. forbesii* is a sister of *Heterotropa* in the Japan-Ryukyu-Taiwan islands, and originally a single ancestral species before 2.54 Ma (Fig. 3), and these two habitats partly overlapped.

Fig. 2 Calyx photos. Calyx from the same island tends to similar, but many exceptions and variable as shown in Table 1.

Fig. 3 Bayesian inference tree of Spermatophyta using the combined sequence (not concatenation) of ITS (694 bp), matK (833 bp), and rbcL (544 bp) constructed using BEASTv.1.8.2 and applying Relaxed clock model. Red diamond: Order level branching node, Orange diamond: Family level branching node, and no diamond: Genus and species level branching node. Green star at node: Prior (input) age for calibration (with multiple calibration points). Posterior probability (black), posterior age (output; red), and rate median (blue) are shown at each node. For Gymnospermae, family and order names according to after Christenhusz *et al* . (2011) and Yonekura and Murata (2013). For Angiospermae, family and order names according to the APG III is shown aside of each species (Yonekura and Murata, 2013). Right bottom: Laterally enlarged figure for *Asarum* . Inset at right top: Base substitution rate (= rate median shown at each node) vs age (= posterior age shown at each node) diagram. Black curve with equation: Exponential trendline drawn by Excel function, Red curve: Free handed trendline drawn by connecting plots with maximum rate through the time.

Table 1 *Asarum* endemic species, mostly*Heterotropa* , collected and analyzed in this study. Morphological characteristics are also shown. Other than *Asarum* , we will load the data in DDBJ / GenBank for the blanks after the acceptance.





isolate	country	*Asarum* species	section	calyx color	calyx shape	calyx size	leaf	stem	accession number ITS	accession number matK	accession number rbcL	collected by
as53	Japan:Honshu, Yamagata, Sakekawa	*A. fauriei*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Soichi Osozawa
as19	Japan:Honshu, Yamagata, Nishikawa	*A. megacalyx*	Heterotropa	purplish	cylindrical	giant	evergreen	erect	LC068456	LC068502	LC068548	Soichi Osozawa
as20	Japan:Honshu, Yamagata, Tsuruoka	*A. megacalyx*	Heterotropa	purplish	cylindrical	giant	evergreen	erect	LC068457	LC068503	LC068549	Soichi Osozawa
as52	Japan:Honshu, Fukushima, Mishima	*A. ikegamii*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Ichi Tsunoda
as27	Japan:Honshu, Toyama, Okawaro	*A. fauriei*	Heterotropa	light purplish	cylindrical	small	evergreen	erect	LC068464	LC068510	LC068556	Kyoji Osozawa
as28	Japan:Honshu, Ishikawa, Tsurugi	*A. fauriei*	Heterotropa	light purplish	cylindrical	small	evergreen	erect	LC068465	LC068511	LC068557	Kyoji Osozawa
as41	Japan:Honshu, Gifu, Yamagata	*A. fauriei*	Heterotropa	light purplish	cylindrical	small	evergreen	erect	LC068473	LC068519	LC068565	Akio Komano
as46	Japan:Honshu, Ki peninsula	*A. takaoi*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Kinichi Watanabe
as48	Japan:Honshu, Ki peninsula	*A. takaoi var. austrokiiense*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Kinichi Watanabe
as49	Japan:Honshu, Ki peninsula	*A. takaoi*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Kinichi Watanabe
as18	Japan:Shikoku, Ehime, ShikokuChuo	*A. asperum*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068455	LC068501	LC068547	Soichi Osozawa
as47	Japan:Shikoku,Kochi	*A. sakawanum*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Kinichi Watanabe
as45	Japan:Kyushu, Miyazaki	*A. minamitanianum*	Heterotropa	light purplish	stringy	moderate	evergreen	erect				Kinichi Watanabe
as34	Japan:Kyushu, Miyazaki	*A. crassum*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect				Kinichi Watanabe
as36	Japan: Kyushu,Fukuoka	*A. kiusianum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068470	LC068516	LC068562	Kinichi Watanabe
as37	Japan: Kyushu,Fukuoka	*A. asaroides*	Heterotropa	purplish	vase	giant	evergreen	erect	LC068471	LC068517	LC068563	Kinichi Watanabe
as31	Japan: Kyushu, Nagasaki	*A. unzen*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068467	LC068513	LC068559	Kinichi Watanabe
as32	Japan: Kyushu,Miyazaki	*A. hugana*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068468	LC068514	LC068560	Kinichi Watanabe
as38	Japan: Kyushu,Kagoshima	*A. satsumense*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068472	LC068518	LC068564	Kinichi Watanabe
as30	Japan: Kyushu, Yaku-shima	*A. kumageanum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068466	LC068512	LC068558	Kinichi Watanabe
as35	Japan: Kyushu, Tanega-shima	*A. kumageanum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068469	LC068515	LC068561	Kinichi Watanabe
as17	Japan:Ryukyu, Tokara, Nakano-shijima,Mt.O-take:979 m	*A. tokarense*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068454	LC068500	LC068546	Soichi Osozawa
as16a	Japan:Ryukyu, Amami Oshima:Tatsugo,at 200 m altitude	*A. lutchuense*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068452	LC068498	LC068544	Soichi Osozawa
as16b	Japan:Ryukyu, Amami Oshima:Tatsugo,at 200 m altitude	*A. lutchuense*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068453	LC068499	LC068545	Soichi Osozawa
as13a	Japan:Ryukyu, Amami Oshima:Mt.Yuwan-dake:894 m	*A. fudsino/*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068446	LC068492	LC068538	Soichi Osozawa
as13b	Japan:Ryukyu, Amami Oshima:Mt.Yuwan-dake:894 m	*A. fudsino/*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068447	LC068493	LC068539	Soichi Osozawa
as14a	Japan:Ryukyu, Amami Oshima:Mt.Yuwan-dake:894 m	*A. fudsino/*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068448	LC068494	LC068540	Soichi Osozawa
as14b	Japan:Ryukyu, Amami Oshima:Mt.Yuwan-dake:894 m	*A. fudsino/*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068449	LC068495	LC068541	Soichi Osozawa
as33	Japan:Ryukyu, Amami Oshima:Mt.Yuwan-dake:894 m	*A. celsum*	Heterotropa	light purplish	cylindrical	small	evergreen	erect				Kinichi Watanabe
as15a	Japan:Ryukyu, Amami Oshima, Uke-jima:Mt.O-dake	*A. trinaciriforme*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068450	LC068496	LC068542	Cunio Nackejima
as15b	Japan:Ryukyu, Amami Oshima, Uke-jima:Mt.O-dake	*A. trinaciriforme*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068451	LC068497	LC068543	Cunio Nackejima
as9	Japan:Ryukyu, Tokuno-shima:Mt.Inokawa-dake:845 m	*A. simile*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068442	LC068488	LC068534	Soichi Osozawa
as10	Japan:Ryukyu, Tokuno-shima:Mt.Amagi-dake:533 m	*A. hatsushimae*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068443	LC068489	LC068535	Soichi Osozawa
as11	Japan:Ryukyu,Tokuno-shima:Mt.Sasoritsuj-dake:496 m	*A. ikeucosepalum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068444	LC068490	LC068536	Soichi Osozawa
as12	Japan:Ryukyu,Tokuno-shima:Mt.Sasoritsuj-dake:at 400 m altitude	*A. lutchuense*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068445	LC068491	LC068537	Soichi Osozawa
as7	Japan:Ryukyu,Okinawa-jima,Motobu:Mt.Katsu-dake:452 m	*A. okinawense*	Heterotropa	light purplish	cylindrical	small	evergreen	erect	LC068438	LC068484	LC068530	Cunio Nackejima
as3	Japan:Ryukyu,Senkaku,Uotsuri-jima	*A. senkakumsulare*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068432	LC068478	LC068524	Kinichi Watanabe
as4b	Japan:Ryukyu,Ishigaki-jima:Mt.Omoto-dake:526 m	*A. dissitum*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068433	LC068479	LC068525	Soichi Osozawa
as4c	Japan:Ryukyu,Ishigaki-jima:Mt.Omoto-dake:526 m	*A. dissitum*	Heterotropa	light purplish	cylindrical	moderate	evergreen	erect	LC068434	LC068480	LC068526	Soichi Osozawa
as5b	Japan:Iriomote-jima:Mt.Koza-dake:420 m	*A. gelasinum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068435	LC068481	LC068527	Soichi Osozawa
as5c	Japan:Iriomote-jima:Mt.Koza-dake:420 m	*A. gelasinum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068436	LC068482	LC068528	Soichi Osozawa
as6	Japan:Iriomote-jima:Kanpira waterfall:100 m	*A. monodoniflorum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068437	LC068483	LC068529	Soichi Osozawa
as55	Japan:Iriomote-jima	*A. yaeyamensis*	Heterotropa	purplish	vase	moderate	evergreen	erect				Kinichi Watanabe
as1	Taiwan:Yangmingshan:Mt.Datun-shan:1087 m	*A. taitoense*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068429	LC068475	LC068521	Kyoji Osozawa
as2a	Taiwan:Wusubi:at 500 m altitude	*A. macranthum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068430	LC068476	LC068522	Soichi Osozawa
as2b	Taiwan:Wusubi:at 500 m altitude	*A. macranthum*	Heterotropa	purplish	vase	moderate	evergreen	erect	LC068431	LC068477	LC068523	Soichi Osozawa
as59a	Taiwan:Alishan:,at 900 m altitude	*A. elbomaculatum*	Heterotropa	purplish	vase	moderate	evergreen	erect				Soichi Osozawa
as8a	Japan:Ryukyu,Okinawa-jima,Motobu:Mt.Yae-dake:453 m	*A. caudigerum*	Eusasarum	purplish	stringy	moderate	deciduous	stolon	LC068439	LC068485	LC068531	Kunio Nakajima
as8b	Japan:Ryukyu,Okinawa-jima,Motobu:Mt.Yae-dake:453 m	*A. caudigerum*	Eusasarum	purplish	stringy	moderate	deciduous	stolon	LC068440	LC068486	LC068532	Kunio Nakajima
as8c	Japan:Ryukyu,Okinawa-jima,Motobu:Mt.Yae-dake:453 m	*A. caudigerum*	Eusasarum	purplish	stringy	moderate	deciduous	stolon	LC068441	LC068487	LC068533	Kunio Nakajima
as21	Japan:Honshu, Yamagata, Tsuruoka	*A. sieboldii*	Asiasarum	purplish	bell	moderate	deciduous	erect	LC068458	LC068504	LC068550	Soichi Osozawa
as22	Japan:Honshu, Aomori, Hirosaki	*A. sieboldii*	Asiasarum	purplish	bell	moderate	deciduous	erect	LC068459	LC068505	LC068551	Soichi Osozawa
as23	Japan:Honshu, Miyagi, Sendai	*A. sieboldii*	Asiasarum	purplish	bell	moderate	deciduous	erect	LC068460	LC068506	LC068552	Soichi Osozawa
as25	Japan:Honshu, Yamagata, Tozawa	*A. sieboldii*	Asiasarum	purplish	bell	moderate	deciduous	erect	LC068461	LC068507	LC068553	Soichi Osozawa
as26	Japan:Honshu, Nagano, Sakae	*A. sieboldii*	Asiasarum	purplish	bell	moderate	deciduous	erect	LC068462	LC068508	LC068554	Kanjiro Ogura
as27Korea	Korea: Busan	*A. sieboldii*	Asiasarum	purplish	bell	moderate	deciduous	erect	LC068463	LC068509	LC068555	Soichi Osozawa
as42	China	*Saruma henryi*		yellow	petal	moderate	deciduous	erect	LC068474	LC068520	LC068566	Akio Komano

isolate	country	species	accession number ITS	accession number matK	accession number rbcL	collected by
mono1	Japan:Honshu, Fukushima, Mishima	*Iris ensata*				Soichi Osozawa
mono3	Japan:Honshu,Miyagi, Sendai (unknown origin)	*Convallaria majalis*				Soichi Osozawa
mono6	Japan:Ryukyu,Okinawa-jima,Motobu:Mt.Katsu-dake:452 m	*Arisaema ringens*				Soichi Osozawa
dic20	Japan:Honshu,Miyagi, Sendai (unknown origin)	*Magnolia cuinquepeta*				Soichi Osozawa
dic23	Japan:Honshu,Miyagi, Sendai (unknown origin)	*Buddleja devidii*				Soichi Osozawa
dic32	Japan:Honshu,Miyagi, Sendai (unknown origin)	*Prunus mume*				Soichi Osozawa
dic34	Japan:Honshu,Miyagi, Sendai (unknown origin)	*Euonymus alatus*				Soichi Osozawa
dic35	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Chloranthus japonicus*				Soichi Osozawa
dic36	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Coptis japonica*				Soichi Osozawa
mono38	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Trillium smallii*				Soichi Osozawa
mono40	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Cypripedium japonicum*				Soichi Osozawa
dic42	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Epimedium grandiflorum*				Soichi Osozawa
dic44	Japan:Honshu,Miyagi, Sendai	*Cornus florida*				Soichi Osozawa
dic72	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Gentiana scabra*				Soichi Osozawa
dic80	Japan:Honshu,Miyagi, Kurihara	*Ranunculus nipponicus*				Soichi Osozawa
dic81	Japan:Honshu:Tokyo, Tokyo University (New Caledonia origin)	*Amborella trichopoda*				Soichi Osozawa
mono4	Japan:Ryukyu,Tokuno-shima:Mt.Inokawa-dake:845 m	*Arisaema kawashimae*				Soichi Osozawa
mono5	Japan:Honshu,Miyagi, Sendai, Tohoku University	*Iris gracilipes*				Soichi Osozawa
suzu6	Japan:Ryukyu,Okinawa-jima,Kunigami:Mt.Yonaha-dake	*Strobilanthes tashiroi*				Soichi Osozawa
suzu8	Japan:Ryukyu,Ishigaki-jima, Yonehara	*Strobilanthes formosanus*				Soichi Osozawa
vio2	Japan:Ryukyu, Amami Oshima	*Viola amamiana*				Kinichi Watanabe
vio3	Japan:Iriomote-jima:Kanpira waterfall:100 m	*Viola tashiroi*				Soichi Osozawa
vio4	Japan:Ryukyu,Okinawa-jima,Onna, Manzamo	*Viola okinawensis*				Tokumasa Shimabukuro
vio5	Japan:Izu,Hachijo-jima	*Viola grypoceras*				Soichi Osozawa
vio7	Japan:Ryukyu, Kikai-jima	*Viola betonicifolia*				Soichi Osozawa
vio8	Japan:Ryukyu, Amami Oshima,Tatsugo	*Viola yedoensis*				Soichi Osozawa
vio9	Japan:Ryukyu,Tokuno-shima,Isen	*Viola yedoensis*				Soichi Osozawa
vio10	Japan:Ryukyu,Tokuno-shima,Isen	*Viola betonicifolia*				Soichi Osozawa
vio11	Japan:Ryukyu,Okinawa-jima,Motobu:Mt.Katsu-dake	*Viola yedoensis*				Soichi Osozawa
vio14	Taiwan:Yangmingshan	*Viola yedoensis*				Soichi Osozawa
vio15	China:HongKong	*Viola sp.*				Soichi Osozawa
vio16	Japan:Ryukyu,Tokara Nakano-shijima:Mt.O-take	*Viola grypoceras*				Soichi Osozawa
vio18	Japan:Ryukyu,Kume	*Viola yedoensis*				Soichi Osozawa
vio19	Taiwan,Mt.Hehuan:3422 m	*Viola sp.*				Soichi Osozawa