A DNA barcode database for the woody plants of Japan

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Abstract

DNA barcode databases are increasingly available for a range of organisms facilitating the wide application of DNA barcode-based pursuits. Here we announce the development of a comprehensive DNA barcode database of the Japanese woody flora representing 43 orders, 99 families, 303 genera and 834 species and comprising 77.3% of genera and 72.2% of species of woody plants in Japan. A total of 6,216 plant specimens were collected from 223 sites (municipalities, i.e. city, town, village) across the subtropical, temperate, boreal and alpine biomes in Japan with most species represented by multiple accessions. This database utilised three chloroplast DNA regions (rbcL, trnH-psbA and matK) and consists of 14,404 barcode sequences. Individual regions varied in their identification rates with species-level and genus-level rates for rbcL, trnH-psbA and matK being 57.4%/ 96.2%, 78.5%/ 99.1 % and 67.8%/ 98%, respectively. Identification rates were higher using region combinations with total species level rates for two region combinations (rbcL & trnH, rbcL & matK, and trnH-psbA & matK) ranging between 90.6–95.8%, and for all three regions equal to 98.6%. Genus level identification rates were even higher ranging between 99.7–100% for two region combinations and being 100% for the three regions. These results indicate that this DNA barcode database is an effective resource for investigations of woody plants in Japan using DNA barcodes and provides a useful template for development of libraries for other components of the Japanese flora.

KEYWORDS

conifers, species discrimination, DNA barcoding, Japan, woody flora, vascular plants

1 | INTRODUCTION

DNA barcodes are short DNA fragments that are able to accurately and rapidly identify to the lowest taxonomic level possible (ideally to the species level) any unidentified organism including whole or fragmented specimens, wood, pollen, subfossils or environmental DNA. The ability to identify plant species via DNA barcoding has a great range of uses including for human health (such as identifying sources of pollen (Kraaijeveld *et al.*, 2015) or house dust (Craine *et al.*, 2017), in forensics (Ferri *et al.*, 2015), bio-security (Ashfaq & Hebert, 2016), nature conservation (such as environmental monitoring (Fahner *et al.*, 2016), biodiversity assessment (Burgess *et al.*, 2011) and enforcing trade laws of endangered species (Dormontt *et al.*, 2015)), agriculture (e.g. monitoring pollination and gene flow of crops (Richardson *et al.*, 2015) and various applications for scientific research (e.g. understanding past impacts of climate change (Giguet-Covex *et al.*, 2014) or for use in plant taxonomy and species discovery (Kress *et al.*, 2015)). Creating DNA barcode libraries, that is, a collection of DNA sequences associated with specimens that have verified taxonomic identifications (Kress *et al.*, 2015), is essential for use as a reference in order to identify unidentified samples. DNA barcoding libraries are now available for a wide range of organisms such as animals and fungi due to the availability of universal barcodes for these groups. However, unlike animals or fungi, there is no single universal DNA fragment for use in DNA barcoding of plants mostly due to the low level of mutation of organelle genomes in plants (Wolfe *et al.*, 1987). This has made it necessary to use multi-locus barcodes (Kress & Erickson, 2007) and in some cases to develop specific barcodes for the targeted plant species meaning that the development of DNA barcode libraries for plants is more complex and time consuming. Nonetheless, in the last decade such libraries have become available for plants of specific countries or regions (de Vere *et al.*, 2012; Kim *et al.*, 2012),

specific taxonomic groups (Liu *et al.*, 2018; Nevill *et al.*, 2013) or individual biomes (Costion *et al.*, 2016; Saarela *et al.*, 2013).

Due to the enormous effort required to completely represent the full range of genetic diversity within species in DNA barcode libraries, especially for those covering many diverse taxa, the full range of sequence diversity may not be fully captured in DNA barcode libraries. This factor, together with low sequence divergence between closely related species, which is particularly common in species rich clades, along with taxonomic uncertainty, means that reliable identification to species level can be difficult (Parmentier *et al.*, 2013; Raupach *et al.*, 2014). However, for many applications of DNA barcoding assignment to higher taxonomic levels, such as the genus-level, is of considerable value and, in many plant groups, accuracy of assignment at this level is more reliable than at the species level (Wilson *et al.*, 2011).

In Japan, DNA barcode libraries have been developed for a range of taxonomic groups (Japanese DNA Barcode Database Committee, 2014). However, these have focussed exclusively on animals such as birds (Nishiumi, 2012), ticks (Takano *et al.*, 2014), snails (Hirose *et al.*, 2015) and snapping beetles (Oba *et al.*, 2015) with plants, excluding ferns (Ebihara *et al.*, 2010), so far having been overlooked. The Japanese archipelago has a highly diverse vascular plant flora with 6,000 species (of which approximately 1,000 are woody plants in ~100 families (Satake *et al.*, 1989)) of which around 2,900 are endemic (Biodiversity Center of Japan Nature Conservation Bureau Ministry of the Environment, 2010) and is one of the 35 hotspots of plant diversity in the world (Mittermeier *et al.*, 2004).

In this paper we announce the development of a DNA barcode database for nearly the entire woody plant flora of Japan (available on the publicly available Barcode of Life Data System (BOLD: https://www.boldsystems.org (Ratnasingham & Hebert, 2007)). This database

consists of 6,216 specimens of woody plants (i.e. those plants with above ground perennial parts having lignified wood formed by secondary growth) sampled from 223 sites across the entire Japanese Archipelago representing subtropical, temperate, boreal and alpine biomes with multiple accessions collected across the range of each species where possible (Figure 1). We utilized three chloroplast regions (rbcL, matK and trnH-psbA) which have become widely used in plant DNA barcoding studies and have achieved high rates of species resolution (Burgess *et al.*, 2011; Kress *et al.*, 2009).

2 | MATERIALS AND METHODS

2.1 | Laboratory Work

Leaf samples for DNA extraction were collected from 223 sites across the whole of the Japanese Archipelago (Figure 1). These sites encompass all major vegetation types and biomes of Japan. For each sample, the latitude and longitude were recorded and identification was done to the lowest taxonomic level possible (i.e. subspecies or variety). For 92.4 % of samples, voucher herbarium specimens were prepared and stored in the herbaria in Japan. Most of them are housed in the xylarium (TWTw) and herbarium (TF) of the Forestry and Forest Products Research Institute (FFPRI), and most images of these voucher herbarium specimens are available at the database of Japanese Woods, FFPRI (https://db.ffpri.go.jp/WoodDB/index-E.html) and BOLD SYSTEMS. The rest vouchers of the samples are housed in the herbaria of Tohoku University Botanical Garden herbarium (TUS) and Herbarium of the Kyushu University Museum (FU) in Japan (Specimen details including museum ID together with GenBank ID and BOLD process ID is available from Supplementary table 1 and BOLD SYSTEMS).

DNA was extracted using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. For each sample we aimed to sequence three chloroplast barcode regions (rbcL, matK and trnH-psbA). The rbcL gene is easily amplified across land plants and, although it does not have sufficient variability to be used alone as a species discriminator, is considered a reliable 'benchmark' locus for placing taxa into family and genera (Kress & Erickson, 2007). matK is one of the most variable coding regions found in chloroplast DNA (Shaw *et al.*, 2005), while the non-coding trnH-psbA region has highly conserved priming sites across land plants and high sequence divergence (Kress & Erickson, 2007). The first two of these fragments, rbcL and matK, have been adopted by the Consortium for the Barcode of Life (CBOL) (CBOL Plant Working Group, 2009) as the core 2-locus barcode for plants while trnH-psbA has been widely used as a single locus or in combination with other loci (Pang *et al.*, 2012; Yao *et al.*, 2009). To amplify the barcode regions (rbcL, matK and trnH-psbA) we first tested primers recommended by the CBOL Plant Working Group (CBOL Plant Working Group, 2009; Hamilton, 1999). However, due to poor amplification and sequence quality, new primers to amplify both rbcL (reverse only) trnH-psbA (both forward and reverse) regions were designed by consulting the whole chloroplast genomes of tobacco, rice and Japanese black pine (Table 1). For the matK region, we also designed a new reverse primer by consulting the matK sequence of *Hydrangea macrophylla*, and trialled a two-step PCR approach following Forrest *et al*. (2011) with separate primer pairs for each step (Table 1). However, due to poor amplification success of matK in some orders or, in some cases, families, we developed new targeted primers. For those orders and/or families, where amplification of matK was poor we downloaded available chloroplast sequences from Genbank and developed new targeted primers (Table 1).

The PCR reaction mixture contained 0.05 µl Ex Taq polymerase (5 U/µl, TAKARA), 1µl 10X Ex Taq Buffer (20 mM, Mg^{2+} plus), 0.8 µl dNTP Mixture (2.5 mM each), 0.5 µl forward and reverse primer (each 2µM), and 2 µl template DNA (approximately 10 ng) in 10 µl total volume. PCRs were carried out using the following thermocycle: 94 ºC for 3 min, then 35 cycles of 94 ºC for 30s, each annealing temperature for 60s, 72 ºC for 90s, followed by final extension at 72 ºC for 10 min. Amplicon products were sent to TAKARA Bio (Mie, Japan) and Hokkaido System Science (Sapporo, Japan) for DNA sequencing or, alternatively, sequenced using an ABI3100 Genetic Analyzer (Applied Biosystems) at the FFPRI, Tsukuba, Ibaraki Prefecture. We used not only KB Basecaller (Applied Biosystems) but also PeakTrace software (Nucleics) for accurate base calling in some sequences. Sequences for rbcL and trnH were checked by eye using Sequencher (Hitachi) and aligned in Bioedit (Hall 1994). Those for matK were checked and aligned by CodonCode Aligner (CodonCode Corporation, www.codoncode.com).

2.2 | Data analysis

Firstly, in order to grasp how well the database represents the total native woody plants of Japan we calculated the percentage of genera and species native to Japan included in the database per family using the most comprehensive reference available (The wild woody plants of Japan volumes I and II; Satake *et al.* 1989). In addition, we calculated the proportion of the flora included in the database for each of six regions of Japan (Hokkaido, Honshu, Shikoku, Kyushu, Nansei and Ogasawara Islands) with the distribution of each species based on Satake *et al.* (1989). Where new additions or changes to the woody plants have been made that are not listed in Satake *et al.* (1989) these were not taken into account in the calculations. For woody plants not listed in Satake *et al*. (1989), we included as many as possible from the 223 sites and

included them in the database. For taxonomic classifications we followed the most recent available (Green List (Ito *et al.*, 2016) or, for those not listed on Green List, we followed YList (Yonekura & Kajita, 2003)). The taxonomic classification used for BOLD is shown in Supplementary table 1. Any disagreement in classification at the order or family level between Green List and/or YList is also indicated.

The success rate of sequencing was calculated as the proportion of the number of high-quality sequences obtained to the total number of samples. The species identification ability of each barcode was evaluated using the BLAST method (Altschul *et al.*, 1990). BLAST databases were constructed not only for each region (rbcL, matK and trnH-psbA), but also combined regions (rbcL & matK, matK & trnH-psbA, trnH-psbA & rbcL, and all three regions). Sequences were concatenated and used in the BLAST databases if sequences were available. Nucleotide BLAST (blastn) search was carried out for each sequence in each database against its own database (i.e. a self-blast) with default parameters. If the top hit sequence species name was the same as that of the query sequence and was the highest BLAST hit score, we considered the query sequence was successfully identified at the species level. However, if multiple top BLAST hits had the same score, the query was considered to not be identifiable to the species level. In the case that the multiple top BLAST hits contained only sequences of the same genus or family as that of query sequence, it was considered to be identified at genus or family level, respectively.

2.3 | Phylogenetic analyses

To confirm the consistency of the data we constructed, a phylogenetic tree based on the three region barcode sequences of angiosperms included in the Japanese woody plant database and compared the result to published angiosperm phylogenies (The Angiosperm Phylogeny Group, 2003, 2009, 2016). To do so, for each angiosperm family one representative individual sample with the longest concatenated sequence of the three regions was selected. Sequence alignment was undertaken in Geneious version 2019.0.4 using Muscle alignment (Edgar, 2004) with default parameters. Phylogenetic analysis was undertaken using a Bayesian MCMC approach implemented in MrBayes v 3.2 (Ronquist *et al.*, 2012) and run with 400,000 MCMC generations, 4 chains and sample frequency of 100 and implementing the most parameter-rich substitution model, GTR+I+G, which has been shown to perform equally well as specifically selected models (Abadi *et al.*, 2019). A sequence of the basal angiosperm family Schisandraceae (Austrobaileyales) was selected as an outgroup (The Angiosperm Phylogeny Group, 2003, 2009, 2016). A consensus tree was produced using the sumt burnin=0.25 command and was then edited in FigTree v1.4.4 (Rambaut, 2020).

3 | RESULTS

In total, 14,404 barcode sequences from 6,216 woody plant specimens were included in the database (Table 2). The average number of accessions per species was 7.5 and ranged from a single accession to a maximum of 73. Our database included 43 orders, 99 families, 303 genera, 834 species with 953 taxa which represented 77.3% of woody plant genera and 72.2% of woody plant species recognised by Satake *et al*. (1989). The missing species included those that are rare or have restricted ranges on islands, high mountain tops or serpentine regions or were otherwise

not encountered at the 223 collection sites. The sampling rate for each geographic region ranged between 84.3–88.9% except for the Nansei Islands (63.3%, Table 3).

The total sequence success for rbcL, trnH-psbA and matK were 96.2, 76.4 and 59.1%, respectively (Figure 2a). We obtained rbcL for all orders sampled while trnH-psbA did not amplify in one order (Araucariales, Figure 3a). The amplification of matK had been lower (45.0%) with no amplification in 14 orders consisting of 17 families including all gymnosperms when we used a two-step PCR approach (data not shown). However, the use of newly developed targeted primers (Table 1) resulted in the number of orders where matK was not amplifiable decreasing from 14 to 4 consisting of 10 families, and showed high sequence success rate in gymnosperms (Figure 3a, Supplementary table 2, 3).

Total identification rates for rbcL were as follows: 57.4% of samples returned a species level match, 96.2% a genus level match and 100% a family level match (Figure 2b). For trnH-psbA, there was greater identification rate with 78.5% returning a species level match, 99.1 % at genus level and 100% at the family level. For matK the identification rates were middle range with 67.8% returning a species level match, 98.1% at the genus level and 100% at the family level. Total identification rate at species level for two region combinations (rbcL & trnH, rbcL & matK, and trnH-psbA & matK) ranged between 90.6–95.8%, and for all three regions was 98.6%. Total identification rate at genus level for two region combinations (rbcL & trnH-psbA, rbcL & matK, and trnH-psbA & matK) ranged between 99.7–100%, and for three regions was 100 %.

There were some similar taxonomic patterns across the three regions for identification rate (Figure 3b). Some species rich orders had a tendency for low species level identification rate based on the individual regions such as Pinales, Pandanales, Rosales and Fagales. The lowest

order-based identification rate for each region was 33.3% for rbcL, 50.0% for trnH (both Pandanales) and 40.5% for matK (Pinales) (Supplementary table 3). For 18 families that were well sampled and sequenced (i.e. over 70% of species represented and over 50% of sequence success for all three regions) and have relatively high species diversity (over 10), we found that identification rate at the species level was high using all three regions combined (Figure 4). Only two families had identification rate below 95% (Pinaceae=91.8% and Rosaceae=93.5%). Interestingly, despite low species-level identification rates based on each individual region (25.7–66.0%), the combined data resulted in a 99.2% successful identification rate for Fagaceae (Figure 4). High identification rates at each individual region of 85.4–97.8% were observed in Rutaceae. The identification rates at the family, genus and species levels for all families is provided in Supplementary table 3.

The phylogenetic tree of Japanese native angiosperm woody plants was well resolved with most nodes having branch-support values over 95%. In addition, the phylogenetic tree has similar overall relationships to published angiosperm phylogenies (Figure 5) (The Angiosperm Phylogeny Group, 2003, 2009, 2016).

4 | DISCUSSION

This barcode database of Japanese woody plants provides a valuable resource for a range of applications in scientific, government and commercial pursuits. The high representation of woody plant species and multiple accessions per species make it one of the most comprehensive barcode libraries of any taxonomic group in the country to date. This database is significant advancement in terms of the barcode resources available for the Japanese flora with the only

previous barcode database being for ferns (Ebihara *et al.*, 2010). For well sampled families the species identification rate achieved using all three barcodes was high (over 90%). The database can be accessed and samples analysed directly at the Barcode of Life Data System website (BOLD: Ratnasingham & Hebert (2007)), GenBank and ForestGEN (https://forestgen.ffpri.go.jp/jp/index.html) or alternatively, data exported and analysed in other programs (e.g. Sonet *et al*., 2013; Steinke *et al*., 2005; Vences *et al*., 2021). In the case that an unknown samples sequence is not included in the database identification to the nearest species and/or genus is likely to be accurate. Identification accuracy of unknown samples may be improved by utilising specific programs that take into account identification uncertainty due to incomplete sampling of sequence diversity (Sonet *et al.*, 2013).

Based on this database, the utility of each individual region differed substantially. rbcL had the highest sequence success rate but the lowest species-level identification rate. matK had the lowest sequence success rate and moderate species-level identification rate. In contrast, trnH-psbA had moderate sequencing success but the highest species-level identification rate. The CBOL Plant Working Group recommend the rbcL and matK combination as a standard barcode for land plants with trnH-psbA not included due to alignment issues caused by high variability including at mononucleotide repeats. However, if the application of barcodes is focussed at the family or genus level trnH-psbA is useful for species level identification given its high variability. On the hand, the rbcL and matK pair is more appropriate than trnH-psbA for revealing phylogenetic relationships across a diverse range of taxonomic groups. Lastly, we show that for orders where PCR amplification using universal primers was poor the development of targeted primers can significantly improve results. This was especially evident in gymnosperms and Sapindales where improvements of up to 100% were observed for matK

(Supplementary table 2). This finding suggests that for reliable use of matK in DNA barcode libraries the development of targeted primers may be necessary.

The lowest species level identification rates based on individual regions were observed in Pinales, Pandanales, Rosales and Fagales. Rosales and Fagales have high species diversity but are also characterised by families where recurrent past or ongoing gene flow via hybridisation and/or hybrid species is widespread in Japan (Iwatsuki *et al.*, 1999, 2001, 2006). In contrast, Pinales have lower number of species but undergo extensive chloroplast sharing resulting in a lower identification rate in some genera (Aizawa & Iwaizumi, 2020; Watano *et al.*, 2004). Similar identification rates have been observed in European *Pinus* (Celiński *et al.*, 2017). Hybridisation between congeneric species can lead to chloroplast haplotype sharing (McKinnon *et al.*, 1999; Petit *et al.*, 2002) which in some cases makes species level identification difficult or even impossible, especially using a single chloroplast-based barcode. However, despite this even for orders with low species level identification rates based on individual regions such as Pinales, Rosales and Fagales, identification rates were over 90% using all three regions. Another reason for low species level identification rates observed in this study is exemplified by the genus *Pandanus* whose two Japanese species occur on separate islands but due to very recent speciation have not diverged at the chloroplast.

The availability of the Japanese woody plant barcode database is likely to open new avenues for scientific research and environmental monitoring in Japan. DNA Barcodes combined with NGS technology have already formed the basis of new, powerful and less invasive approaches to environmental monitoring in other organisms. For example, DNA barcodes for fish species used in metabarcoding of environmental DNA from seawater samples has the potential to revolutionize management of fish resources in Japan (Miya *et al.*, 2015) by

improving the accuracy of assessments of fish species diversity (Yamamoto *et al.*, 2017) and providing a new tool to assess fish species biomass (Yamamoto *et al.*, 2016). Some research fields where DNA barcodes for Japanese woody plants may have significant impact, either as the sole investigative tool or together with existing methods, includes assessing present diversity of plants in biodiversity surveys (Taberlet *et al.*, 2012; Yoccoz *et al.*, 2012), diet analysis of endangered or invasive animals (Ando *et al.*, 2013; Nakahama *et al.*, 2021), or monitoring of pollen sources (Nakazawa *et al.*, 2013). The database could also have applications aimed at surveying past plant diversity from the decade to potentially thousands of years scale. For example, DNA metabarcoding could be a useful tool in deciphering the plant species composition of natural vegetation before conversion to plantations in the mid-late 20th century. Reconversion to native forest and grasslands of some areas of under-exploited plantations in Japan has been an important issue in the last decade (Yamaura *et al.*, 2012) but given that plantation forests can cover large areas, with up to 66% of totals forest area in parts of southwestern Japan being planted forests (Forestry Agency, 2017), understanding past natural vegetation can be difficult. Chloroplast DNA barcodes are highly suited to investigations using degraded samples, including ancient DNA, because of the high copy number of chloroplast in plant cells (Wagner, 1992). However, given that shorter DNA fragments survive for longer (Deiner *et al.*, 2017), optimization of shorter targeted fragments of the three barcodes would most likely be required.

The barcode database for Japanese woody plants announced in this paper provides a valuable resource for both research and non-research-based pursuits investigating the countries flora. Due to the high species diversity and high number of geographically restricted species constructing a barcode database for the entire Japanese flora was not considered feasible in one

study. However, we hope that this study provides a useful template from which further country- or region-based databases can be developed including for herbaceous plants and rare species that were not targeted in this study. For this database the use of nuclear loci such as the internal transcribed spacer (ITS) was not considered because of poor amplification success across land plants (Hollingsworth, 2011) and paralogous copies (Poczai & Hyvönen, 2010). However, future studies, especially those focussed on specific taxonomic groups, could have success using the shorter ITS2 region (China Plant B. O. L. Group *et al.*, 2011), or potentially other low copy nuclear loci (Kurian *et al.*, 2020).

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AUTHOR CONTRIBUTIONS

Hiroshi Yoshimaru, Kensuke Yoshimura and Suzuki Setsuko conceived and designed the research. Toshio Katsuki, Shuichi Noshiro, Tomoyuki Fujii and Takahisa Arai conducted the field work. Hiroshi Yoshimaru, Kensuke Yoshimura, Takahisa Arai and Suzuki Setsuko conducted the laboratory work. Hiroshi Yoshimaru, Kensuke Yoshimura, Saneyoshi Ueno, James Raymond Peter Worth, Tokuko Ujino-Ihara and Suzuki Setsuko analyzed the data. James Raymond Peter Worth, Saneyoshi Ueno and Suzuki Setsuko wrote the manuscript. All authors contributed to the final submitted manuscript.

DATA AVAILABILITY STATEMENT

Specimen data and DNA barcodes: BOLD and Genbank accessions are listed with specimen metadata in supporting information.

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359

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532 Table 1 Details of the primer DNA sequences for each of the three chloroplast barcode regions.

Region	Primer type	Primer name	Genbank ID	Direction	Sequence	TA(℃)	Reference
rbcL	Universal	rbcLa-F	-	Forward	ATG TCA CCA CAA ACA GAG ACT AAA GC	55	CBOL Plant Working Group, 2009
trnH-psbA	Universal	NTrbcL-626L24	Z00044, S54304, X15901, D17510	Reverse	GAT CTC TCC AAC GCA TAA ATG GTT	55	[c]
		NT55U		Forward	CCT TGA TCC ACT TGG CTA C		[c]
		PT119680L21		Reverse	GGA AGT TAT GCA CGA ACG TAA		[c]
matK	Universal	matK_Kim_f	-	Forward	CGTACAGTACTTTTGTGTTTACGAG	55	CBOL Plant Working Group, 2009
		HydmatK1041L	AB038178	Reverse	CCCATCCATCTGGAAATCTTGGTTC		[c]
	Universal, 1st PCR[a]	matK-Xf	-	Forward	TAA TTT ACG ATC AAT TCA TTC	46	Ragupathy et al., 2009
		MALPR1	-	Reverse	ACA AGA AAG TCG AAG TAT		Dunning & Savolainen, 2010
	Universal, 2nd PCR[a]	matK472F1	-	Forward	CCC RTY CAT CTG GAA ATC TTG GTT C	50	Yu et al., 2011
		matK1248R1	-	Reverse	GCT RTR ATA ATG AGA AAG ATT TCT GC		Yu et al., 2011
	Pinales, Cupressales[b]	CJmatK175U	AP009377	Forward	CCG AAC TAC ACG TAT CGT ACT T	50	[c]
		CJmatK1058L		Reverse	CGA GTA CCC TAC TCT ATT CAT CC		[c]
	Podocarpaceae[b]	Thujopsis_matKF	AB030134.1	Forward	ACT GTA GTA ATG AAA AAG ATT TAT CC	50	[c]
		Thujopsis_matKR		Reverse	TCA ATT CAT CCG GAA ATT TTG GTT		[c]
	Ranunculales[b]	Anemone_matKF	AB110530.1	Forward	GCT GTA ATA ATG CGA AAG ATT TCG GC	50	[c]
		Anemone_matKR		Reverse	CCT ATC CAT CTG GAA CTA TTG GTT		[c]
	Rosales[b]	Sorbaria_matKF	GU363761.1	Forward	ACT GTA ATA ATG AGA AAG ATT TCT GC	50	[c]
		Sorbaria_matKR		Reverse	CCC ATT CAT CTG GAA ATC TTG GTT		[c]
	Malpighiales[b]	Croton_matKF	AB233773.1	Forward	ACT ATA ATA ATG AGA AAG CTT TCT GC	50	[c]
		Croton_matKR		Reverse	CCC ATC CAT ATA GAA AAA TTA GTC		[c]

Anacardiaceae[b]	Choerospondias_matKF	HQ427341.1	Forward	GCT GTG ATA ATG AGA AAG ATT TCT GC	50	[c]
	Choerospondias_matKR		Reverse	CCC ATT CGC CCG GAA ATC TTG GTT		[c]
Sapindales[b]	Toddalia_matKF	FJ716738.1	Forward	GCT GTG ATA ATG AGA AAG ATT TCT GC	50	[c]
	Toddalia_matKR		Reverse	CCC ATT TGT CCC GAA ATC TTG GTT		[c]
Ericaceae[b]	Andromeda_matKF	JF801293.1	Forward	GCT ATA ATA ATG AGA AAG ATT TCT AT	50	[c]
	Andromeda_matKR		Reverse	CCC GTC CAT CTG GAA ATC TTG GTT		[c]

[a] Two step PCR was carried out in matK according to Forrest *et al*. (2011)

[b] Tageted primers in matK

[c] Primers designed for use in this study.

537 Table 2 A summary of the representation of genera and species of each plant order native to
538 Japan included in the Japanese woody plants DNA barcoding database.

Order	No. of genera in Japan (% sampled)[a]		No. of species in Japan (% sampled)[a]		No. taxa sampled	No. samples
Cycadales	1	(100)	1	(100)	1	2
Pinales	6	(83)	22	(81.8)	20	86
Araucariales	2	(100)	2	(100)	2	11
Cupressales	9	(100)	14	(100)	19	101
Pandanales	2	(50)	4	(50)	3	6
Liliales[b]	-	-	-	-	4	11
Arecales	6	(50)	6	(50)	4	10
Poales	6	(0)	15	(0)	0	0
Ranunculales	6	(100)	10	(100)	20	111
Proteales	3	(66.7)	7	(71.4)	5	42
Trochodendrales	1	(100)	1	(100)	1	10
Buxales	1	(100)	2	(100)	3	4
Saxifragales	9	(88.9)	24	(66.7)	20	115
Vitales	4	(75)	10	(60)	9	77
Austrobaileyales	3	(100)	4	(100)	5	43
Fabales	27	(40.7)	43	(37.2)	25	144
Rosales	42	(88.1)	172	(73.8)	155	1014
Fagales	14	(100)	56	(94)	60	555
Cucurbitales	1	(100)	1	(100)	1	6
Celastrales	5	(100)	27	(74.1)	23	169
Oxalidales	1	(100)	4	(75)	3	20
Malpighiales	28	(60.7)	68	(60.3)	43	215
Myrtales	11	(63.6)	16	(56.25)	10	23
Crossosomatales	4	(100)	4	(100)	8	93
Chloranthales[b]	-	-	-	-	1	3
Sapindales	20	(90)	61	(86.9)	68	629
Malvales	14	(42.9)	31	(51.6)	17	67
Brassicales	1	(100)	1	(100)	1	2

Santalales	7	(85.7)	9	(77.8)	7	37
Caryophyllales	1	(100)	3	(33.3)	1	2
Cornales	8	(100)	28	(89.3)	28	363
Ericales	31	(90.3)	159	(74.2)	146	844
Icacinales	2	(0)	2	(0)	0	0
Garryales	1	(100)	1	(100)	3	25
Piperales[b]	-	-	-	-	2	8
Gentianales	25	(88)	47	(74.5)	45	161
Boraginales	3	(66.7)	7	(28.6)	2	6
Solanales	3	(33.3)	6	(16.7)	1	2
Lamiales	15	(80)	49	(75.5)	45	265
Aquifoliales	2	(100)	24	(91.7)	28	171
Asterales	5	(80)	6	(66.7)	7	21
Dipsacales	7	(100)	51	(68.6)	45	274
Apiales	11	(100)	20	(85)	24	161
Magnoliales	2	(50)	7	(85.7)	8	54
Laurales	10	(70)	28	(82.1)	30	253
Alismatales	1	(0)	1	(0)	0	0
Total	361	(77.3)	1054	(72.2)	953	6216

[a] The percentage of genera and species native to Japan included in the database were calculated based on Satake *et al.* (1989).

[b] denotes families that are not included in Satake *et al.* (1989) and, therefore, the percentage of genera and species represented was not calculated. However, for these orders the number of taxa and species sampled are provided.

546 Table 3 Sampling rate for each region in Japan

547

Region	No. of species	No. of species sampled	Sampling rate (%)
Hokkaido	217	193	88.9
Honshu	664	560	84.3
Shikoku	495	441	89.1
Kyushu	527	454	86.1
Nansei Islands	420	266	63.3
Ogsawara Islands	88	76	86.4

548

549

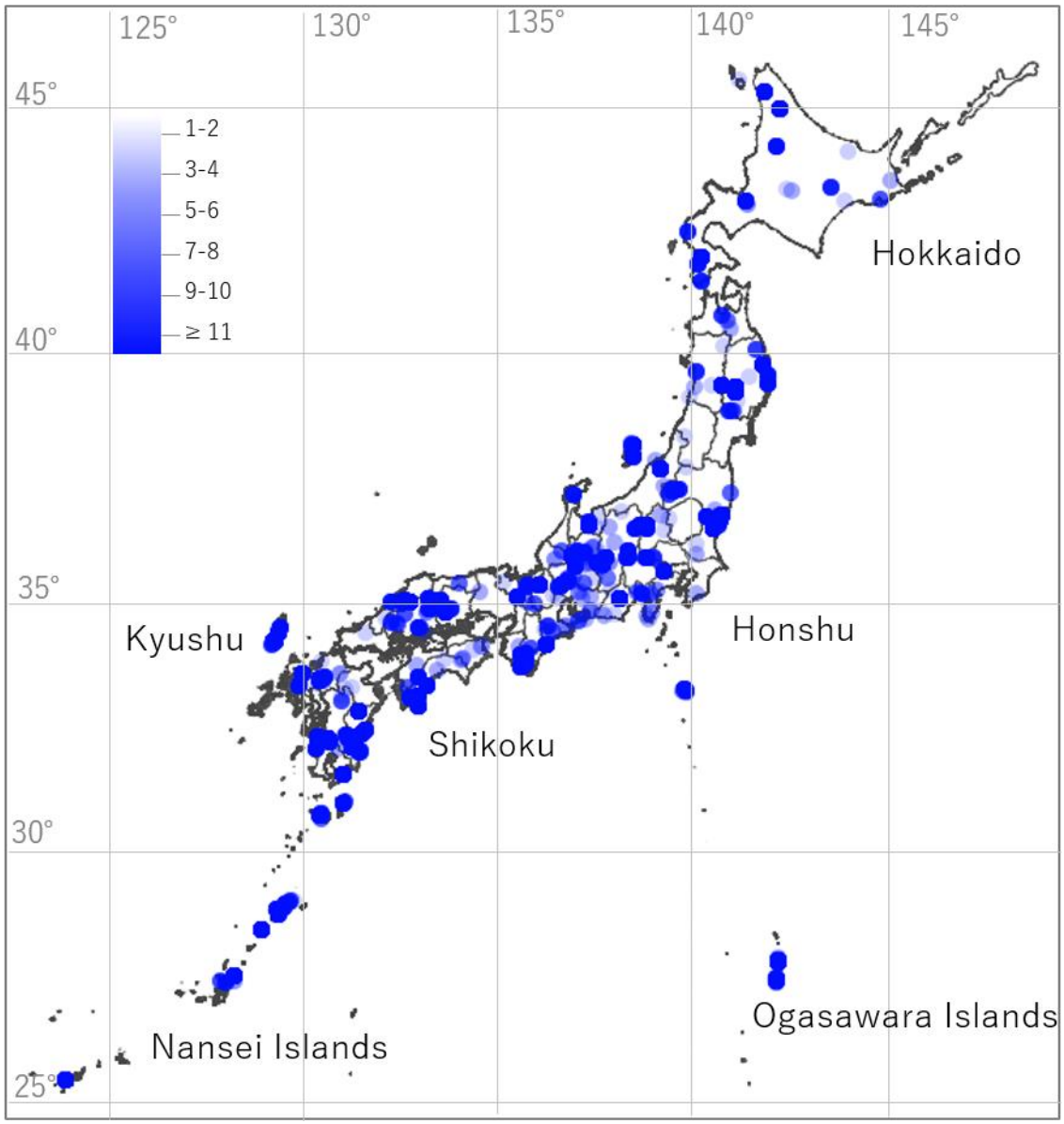
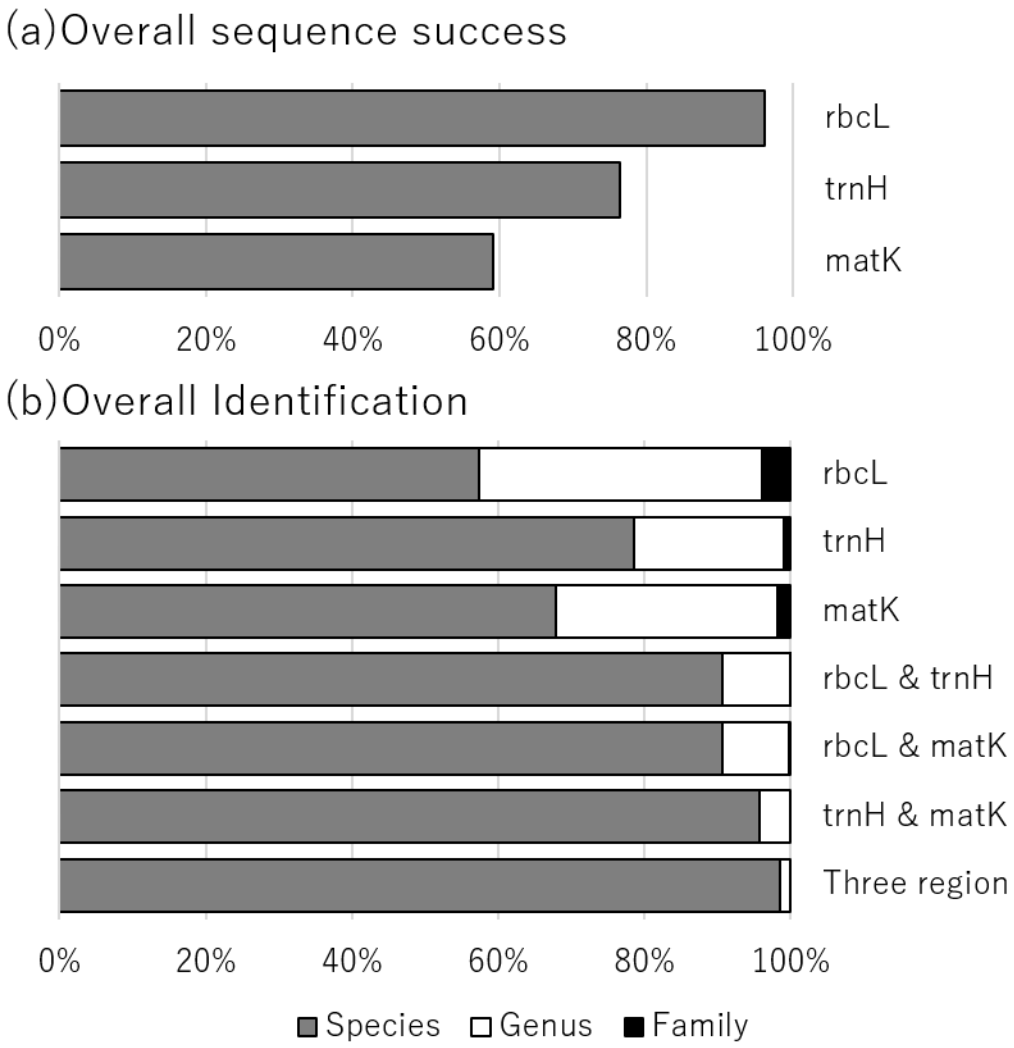


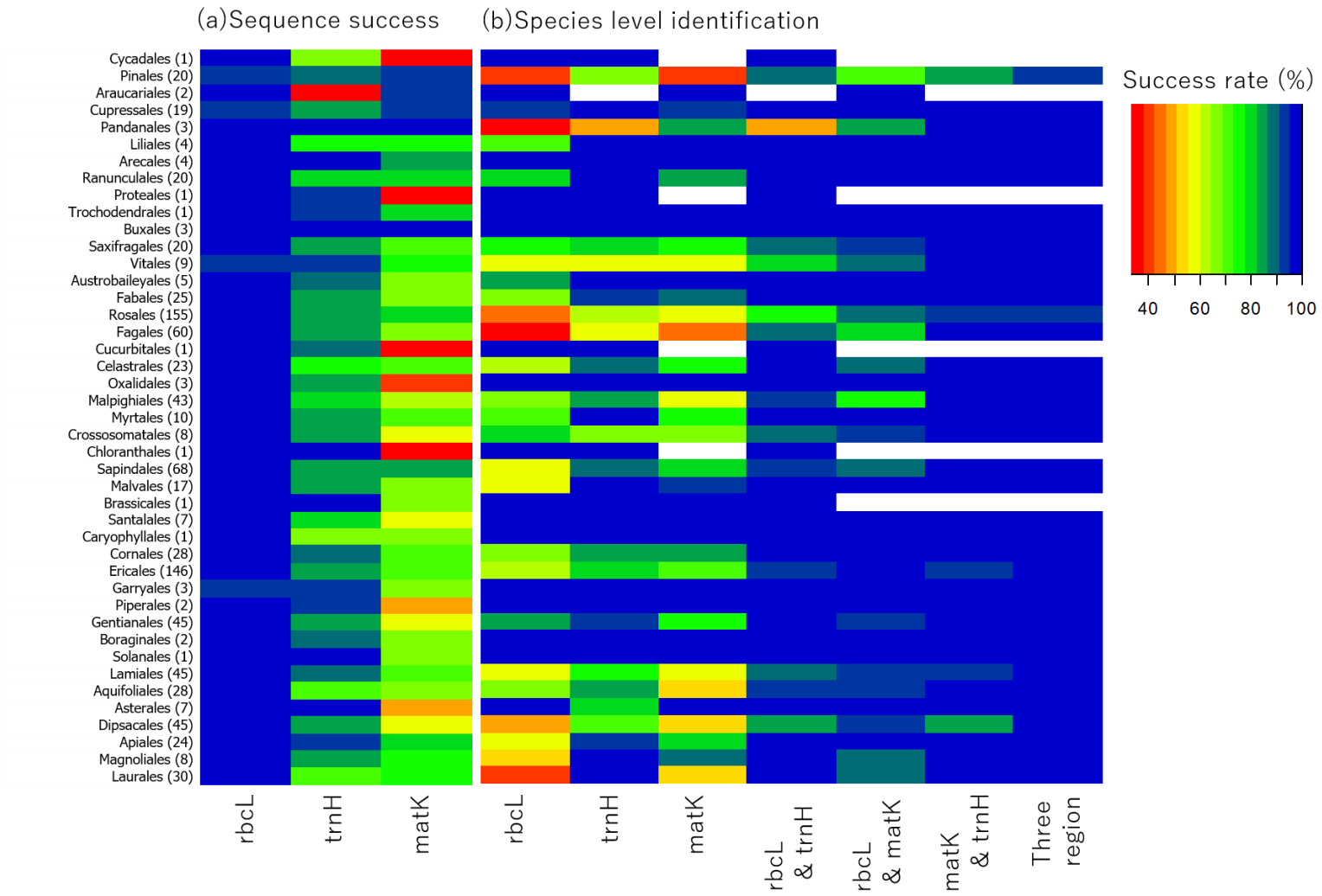
Figure 1 Location of all 223 sampling sites used to collect woody plants for the barcoding database. Opacity of the circles represent the number of samples per site ranging from 1 to 209 (average=24.9).



555

556 Figure 2 The overall level of sequencing success rate for each barcode region (a) and taxonomic
557 identification rate (at the species, genus or family level) for each individual barcode region,
558 barcode pair and all three regions (b).

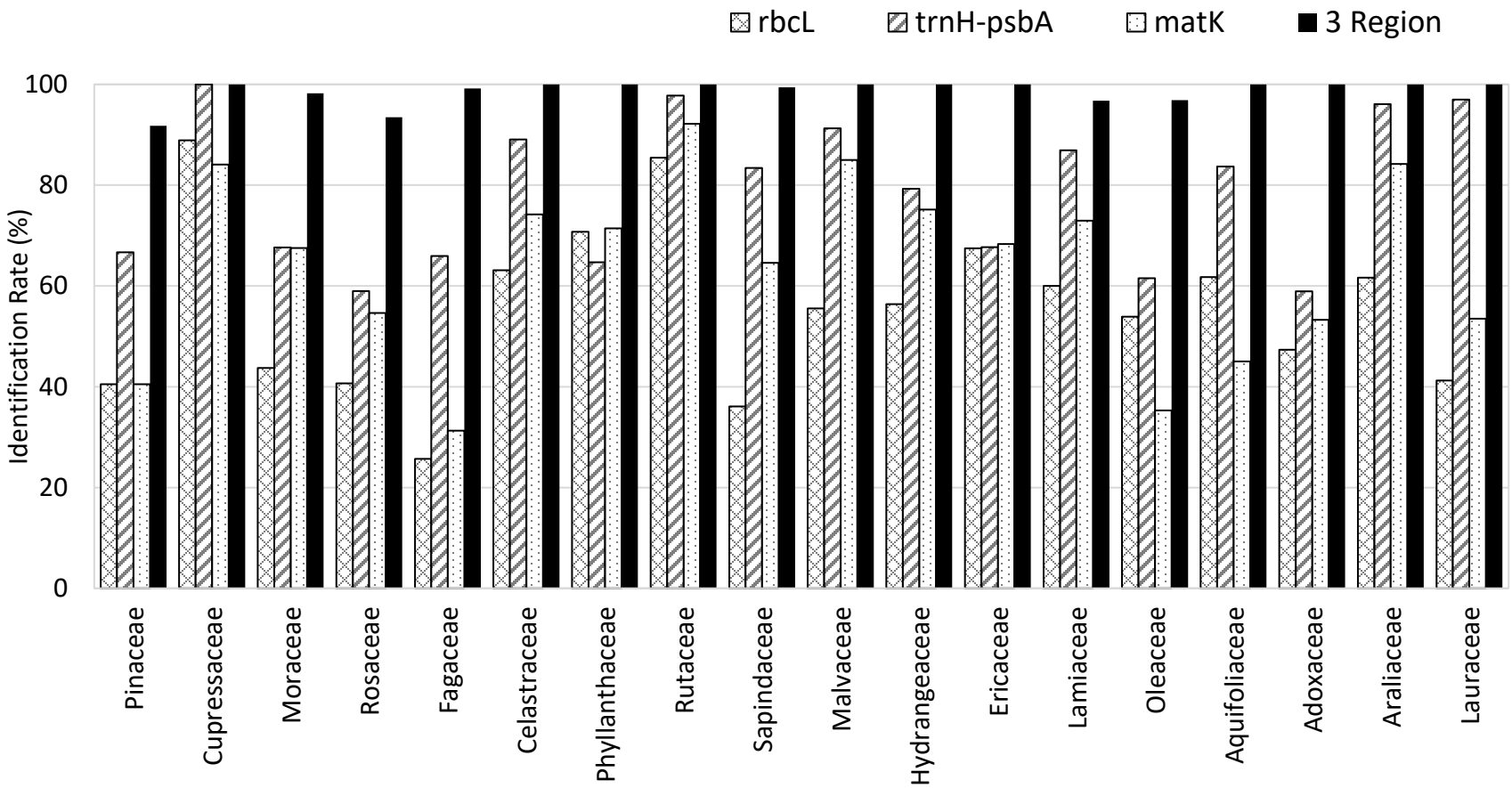
559



560

561 Figure 3 Sequencing success rate for each barcode region and species level identification rate for each barcode region, barcode pair
562 and all three regions for each order. Number of taxa represented by an order is shown in brackets. White blanks show no sequence
563 region available at the order.

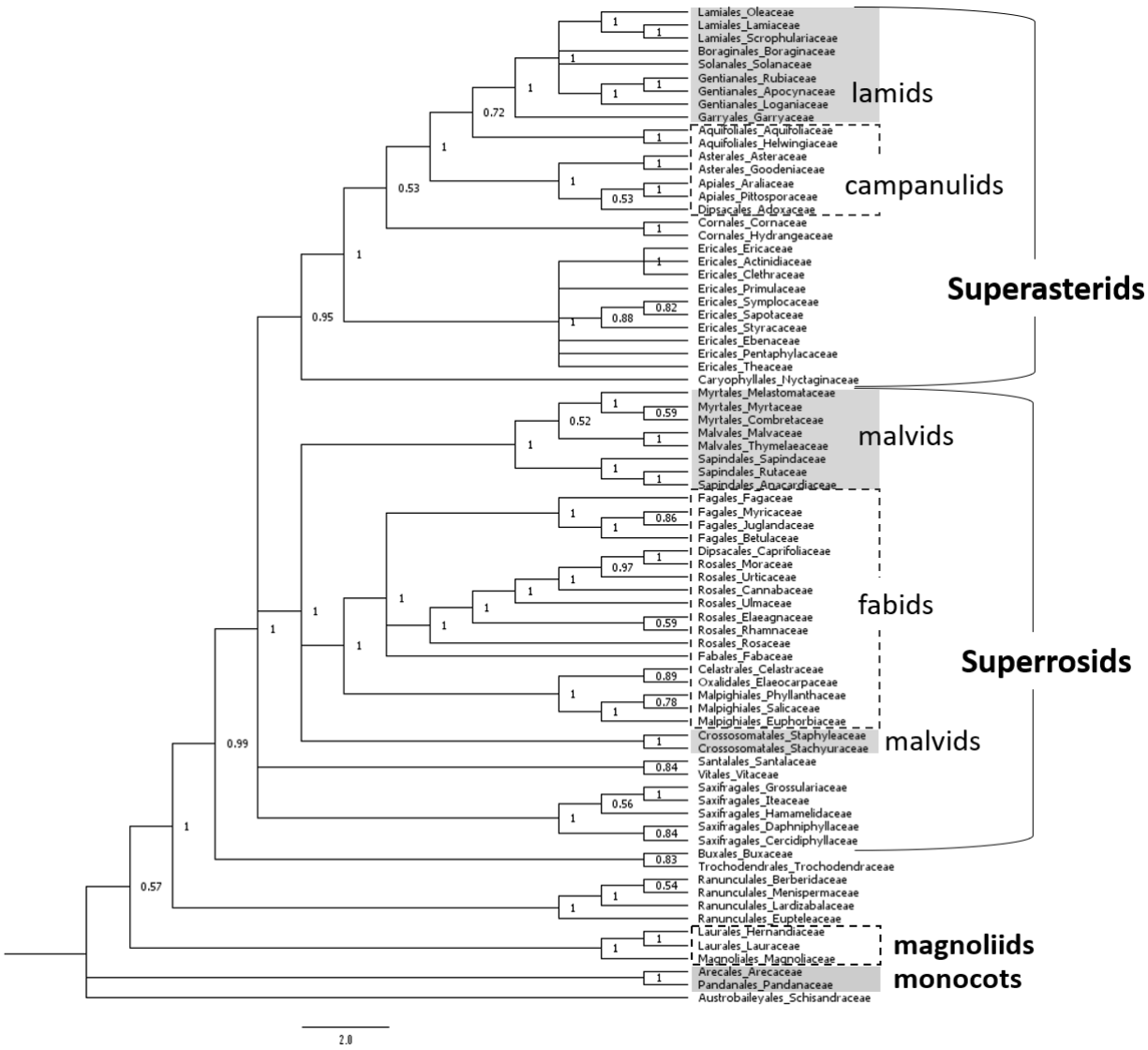
564



565
566 Figure 4 The species level identification rate for 18 selected plant families for which there are over 10 species in Japan and over 70%
567 of species were sampled and over 50% sequence success for each three regions.

568

569



570

571 Figure 5 The phylogenetic tree of Japanese woody plants using all three barcode fragments (rbcL
572 + trnH-psbA + matK).

573

574

575 Supplementary tables

576

577 Supplementary table 1 List of samples used in this study

578 Can be downloaded from https://doi.org/10.6084/m9.figshare.16947391

579

580 Supplementary table 2 Sequencing success rates of matK using two-step PCR and order specific
581 primers

582

Order	Family	Primer name	Sequence success rate for two-step PCR (%)	Sequence success rate for order specific primers (%)
Pinales	Pinaceae	CJmatK	0.00	89.6
Araucariales	Podocarpaceae	Thujopsis_matK	0.00	93.3
Cupressales	Cupressaceae	CJmatK	0.00	94.9
	Sciadopityaceae	CJmatK	0.00	100.0
	Taxaceae	CJmatK	0.00	92.9
Ranunculales	Berberidaceae	Anemone_matK	44.44	52.6
	Lardizabalaceae	Anemone_matK	64.71	100.0
	Menispermaceae	Anemone_matK	69.57	19.0
	Ranunculaceae	Anemone_matK	53.33	96.6
Rosales	Rosaceae	Sorbaria_matK	34.33	93.1
Malpighiales	Euphorbiaceae	Croton_matK	14.63	40.8
Sapindales	Anacardiaceae	Choerospondias_matK	42.86	93.0
	Meliaceae	Toddalia_matK	0.00	100.0
	Rutaceae	Toddalia_matK	17.48	94.8
	Simaroubaceae	Toddalia_matK	0.00	100.0
Ericales	Ericaceae	Andromeda_matK	37.54	87.7

583

584

585 Supplementary table 3 A summary of the representation of genera and species, sequence success
586 of each barcode region, and identification rate at the speceis, genus and family level for each
587 plant family native to Japan included in the Japanese Woody Plant DNA Barcoding Database.

588 Can be downloaded from https://doi.org/10.6084/m9.figshare.16947391

589