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Phylogenetic Study of *Metroxylon* Palms in Southeast Asia and Oceania Based on 5S nrDNA Spacer Sequence Data

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Introduction

The genus *Metroxylon* is distributed from Southeast Asia (Thailand, Malaysia, Brunei, the Philippines, and Indonesia) to Micronesia, Melanesia (Papua New Guinea, the Solomon Islands, Vanuatu, and Fiji), and Polynesia (Samoa). It is divided into two sections, *Metroxylon* (*Eumetroxylon*) and *Coelococcus* (Beccari 1918; Rauwerdink 1986), representing the western half and the eastern half of this distribution, respectively (McClatchey 1999). *Metroxylon sagu* Rottb. (called the true sago palm: hereafter "sago palm") is the only species in the section *Metroxylon*, although the monophyly of this section remains uncertain. The sago palm extends across Southeast Asia and northwestern Melanesia (Papua New Guinea and the Solomon Islands). The sago palm produces the largest amount of starch in the trunk among the Arecaceae genera, and it is used variously for food and processed food or floured for noodle making. This species has long been used as a food similar to bananas and taro (Barrau 1959) and is one of the oldest crops used by human beings since ancient times (Takamura 1990). In Southeast Asia and Melanesia, the leaves of the sago palm are used for making roof thatch, and the petioles are used as construction materials of temporary houses and huts.

Metroxylon amicarum (H. Wendl.) Becc. (Caroline ivory nut palm (Jones 1995)) of the section

Coelococcus is restricted to Micronesia. The other four species are distributed across Melanesia and Polynesia from the Solomon Islands, Vanuatu to Fiji, and Samoa. *Metroxylon salomonense* (Warb.) Becc. (Solomon sago palm) is distributed in the Solomon Islands (McClatchey 1999) and Vanuatu (Ehara et al. 2003a), and *M. vitiense* (H. Wendl.) H. Wendl. ex Benth. & Hook. f. (Fiji sago palm) (Watling 2005) is distributed in Fiji. *Metroxylon warburugii* (F. Heim) Becc. is distributed in Vanuatu, Fiji, and Samoa; and *M. paulcoxii* McClatchey (*M. upoluense* Becc.) is distributed in Samoa. *Metroxylon warburgii* is sometimes called the Vanuatu sago palm. McClatchey (1998) reported that people on Rotuma in Fiji consume sago (starch obtained from the trunk of the palm) produced from *M. warburugii*. In other areas, *Metroxylon* palms have been used occasionally. For example, *M. amicarum* was used on Moen in Micronesia until the 1940s, and *M. warburgii* was used on Gaua in Vanuatu until at least the 1950s (Ehara et al. 2003a). On Malekula Island in Vanuatu, *M. warburgii* is sometimes used as an emergency food. On the other hand, Indo-Fijian people often harvest *M. vitiense* to get the pre-emergent young leaves around the growing point (apical bud together with the very young leaf sheathes and leaves) (Ehara 2015a). This harvested part is known as "palm cabbage" and, in cooking, is used similarly to bamboo shoots. The leaves of *Coelococcus* palms are also important as building and houseware materials similar to uses of the sago palm (*M. sagu*). The hard endosperm of *M. amicarum* and *M. warburgii* seeds is called "palm ivory" and is utilized as material for craftwork (Ehara 2015c).

There have been reports on the specifications of the *Metroxylon* section *Coelococcus* based on morphological characteristics (McClatchey 1998, 1999). However, few studies of the molecular phylogenetics of the genus *Metroxylon*, including the section *Coelococcus*, exist. McClatchey (2002) suggested that genetic studies of sections *Metroxylon* and *Coelococcus* should be conducted in order to support or refute the phylogeny determined by morphology alone. Barrow (1998) showed that DNA sequences from the nuclear 5S nontranscribed spacer were useful for a phylogenetic analysis of the palm genus *Phoenix*. Baker et al. (2000) reported the molecular phylogenetics of *Calamus* and related rattan genera based on 5S nrDNA spacer sequence data. The phylogenetic analysis based on 5S nrDNA spacer sequence is, therefore, considered to be an available substitute for the genus *Metroxylon*, as it belongs to the same tribe (Calameae) as does the genus *Calamus*. Thus, we used DNA sequences from the nontranscribed spacer of 5S nrDNA in this study to investigate phylogenetic relationships among the *Metroxylon* palms distributed in Southeast Asia and Oceania.

Materials and Methods

Plant Materials

Two populations each of *M. amicarum* (plant materials of *M. amicarum* 1 taken from a palm planted and grown in Townsville Botanic Gardens, Australia, after germination from a seed collected on Caroline Island; *M. amicarum* 2 harvested on Pohnpei Island, Micronesia: 6° 54.063' N, 158° 17.115' E), *M. salomonense* (plant materials of *M. salomonense* 1 taken from Singapore Botanic Gardens in Singapore; *M. salomonense* 2 harvested on Gaua Island, Vanuatu: 14° 15.907'S, 167° 36.098'E) (Ehara et al. 2003a), *M. vitiense* (plant materials of *M. vitiense* 1 taken from a palm planted and grown in Townsville Botanic Garden after germination from a seed collected in Fiji; *M. vitiense* 2 harvested on Viti Levu Island, Fiji: 18° 13.241'S, 178° 5.048'E), *M. warburgii* (*M. warburgii* 1 harvested on Espiritu Santo Island, Vanuatu: 15° 29.277'S, 167° 11.621'E (2003a); *M. warburgii* 2 harvested on Upolu Island, Samoa: 13° 53.576'S, 171° 34.809'W), and one population of *M. paulcoxii* (plant materials of this palm taken on Upolu Island, Samoa: 13° 54.812'S, 171° 35.829'W) were used. In addition, two populations of *M. sagu* grown in Southeast Sulawesi (3° 58.566' S, 122° 26.668'E; the folk variety names of *M. sagu* 1

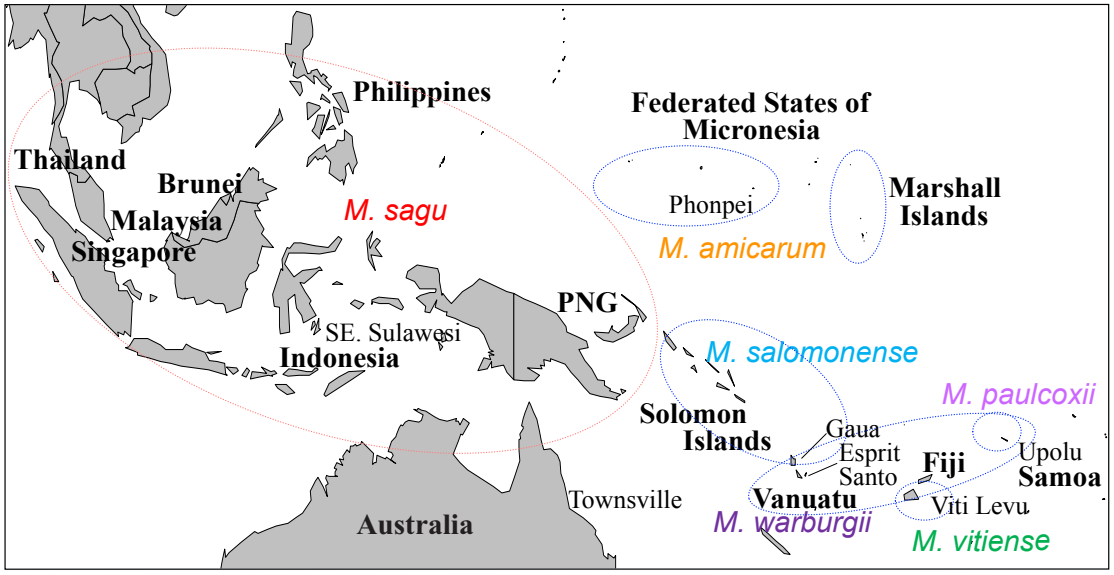

Fig 1. Distribution of sections *Metroxylon* and *Coelococcus*
Section *Metroxylon*: *M. sagu*
Section *Coelococcus*: *M. amicarum*, *M. salomonense*, *M. warburgii*, *M. vitiense*, and *M. paulcoxii*

and *M. sagu* 2 are Roe and Rui, respectively) were used. The sampling sites are shown in Fig. 1.

DNA Extraction, Amplification, Cloning, and Sequencing

Two leaflets were taken from each population, cut into small pieces, and freeze-dried or silica-dried (Chase and Harold 1991). Some pieces of small dried leaflets were ground to obtain about 25 mg of comparatively fine powder that was used for DNA extraction. Total genomic DNA was extracted from the dried leaflet tissue using the DNeasy Plant Mini Kit (QIAGEN). The extracted DNA was cleaned using the QIAquick PCR Purification Kit (QIAGEN), and the purified product was eluted into 50 µl of elution buffer or water. The 5S spacer was amplified from total genomic DNA using 5S F (AGTTAAGCTTGCTTGGGCGAGAGTA) and 5S R (AGTTCTGATGGAATTCGGTGYTKTA) primers. The primers 5S R and 5S F were designed with reference to published tree sequences (Mitchell and Wen 2005) and annual herbaceous sequences (Cox et al. 1992). Fifty-microliter reactions were prepared (5 µL of 10×HybriPol Buffer provided by Bioline, 50 mM $MgCl_2$ 1.5 µL, 2 mM dNTP (4 µL) each, 10 µM each primer (0.5 µL each), 4 µL of HybriPol DNA polymerase (Bioline), and 0.5 µL of template DNA). The PCR profile used is as follows: 96°C for 2 min, 1 cycle; denaturing step of 96°C for 1 min, annealing step of 60°C for 1 min, extension step of 72°C for 1 min, 60 cycles; final extension step of 72°C for 2 min.

All PCR products were loaded in 1% agarose gel electrophoresis, and the target band was taken for further extraction using the QIAEX II Gel Extraction Kit (QIAGEN). The extracted products were used for ligation at 4°C for 16 hours with the pGEM-T Easy Vector (Promega). Ligations and transformations were prepared according to the protocol provided by the manufacturer. Ten microliters of ligation mixture was mixed with 100 µl of competent cells (*Escherichia coli* XL-1 Blue), incubated on ice for 30 min, heat shocked (42°C for 45 sec), then chilled again on ice for 3 min. Transformation *E. coli* cells were then put into 900 µl of LB liquid medium and incubated for 30 min to 1hour in a shaking incubator. Transformed *Escherichia coli* was spread onto 25 mL agar plates (LB medium, including 50 µg/mL ampicillin and 20 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 1 mM of

Isopropyl β-D-1-thiogalactopyranoside (IPTG)) and incubated at 37° C overnight. Five white colonies were selected from each plate and suspended in Milli-Q water and used for colony PCR, being spread onto an agar plate for preparing a master plate.

Fifty microliters of reaction mixture of colony PCR was prepared as follows: 15 µl of colony-suspended water, 2.5 µL of 10×HybriPol Buffer, 0.75 µL of 50 mM MgCl2, 2 mM dNTPs (each), 10 µM of M13 forward and reverse primers, and 0.25 µL of HybriPol DNA polymerase (Bioline). The colony PCR profile was as follows: 96° C for 5 min, 1 cycle; denaturing step of 96° C for 0.5 min, annealing step of 55° C for 0.5 min, extension step of 72° C for 2 min, 35 cycles; final extension step of 72° C for 2 min. The PCR products were loaded in 1% agarose gel electrophoresis to select some colonies that produced the target product. Some selected colonies were used from the master plates for additional procedures. The selected colony was suspended in LB medium including 50 µg/mL ampicillin and then incubated at 37° C overnight in the shaking incubator. Subsequently, plasmid DNA was isolated using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The isolated plasmid DNA was used as a template (2 µL) for cycle sequencing using ABI PRISM BigDye Terminator Cycle Sequencing Ver.3 with M13 forward or M13 reverse primers (1 µl of 2.5 µM primer). The products of the cycle-sequencing reaction were analyzed using an ABI PRISM 3100 Genetic Analyzer. Raw sequence data files were assembled and edited using the Clustal X 2.0.10 provided by Des Higgins, the Conway Institute, UCD Dublin.

Cladistic Analysis

Cladistic analysis was conducted using the PHYLIP (ver. 3.6) software package (Felsentein 2001), and an unrooted dendrogram was constructed using the neighbor-joining (NJ) method. The cluster dendrogram was drawn using the NJ pot program developed by Perrière and Gouy.

Results and Discussion

All of the PCR products using 5S F and 5S R primers were at approximately 500 bp. From the result of sequencing, the insert was confirmed at 499–500 bp in *M. amicarum*, at 508–509 bp in *M. salomonense*, at 489–512 bp in *M. vitiense*, at 503–509 in *M. warburgii*, at 493–510 in *M. paulcoxii*, and at 501–514 in *M. sagu*. The ends of the 5S spacer were readily identified using published data (e.g., Udovicic et al., 1995). All of the sequence data used in this study were deposited in public DNA databases under Accession Numbers LC312133 to LC312143. The average length of the 5S spacer of the genus *Metroxylon* was 301 bp (SD # 12.8, range # 288–326).

Figure 2 shows the results of the phylogenetic analysis using DNA sequence data from the nontranscribed spacer of 5S nrDNA to investigate phylogenetic relationships among the species of sections *Metroxylon* and *Coelococcus* in the *Metroxylon* genus. In the NJ dendrogram from the cladistic analysis, the clade of section *Metroxylon* was apparently distant from the species of the section *Coelococcus*. Palms belonging to the Calamoideae subfamily of the Arecaceae, including the genus *Metroxylon*, produce fruits that are covered with scales. The *Metroxylon sagu* produces fruits with 18 rows of longitudinally arranged scales with approximately 5 cm at the longest diameter (Ehara et al. 1998). The other species in the section *Coelococcus* bear large fruits covered with 24 to 28 rows of scales 7 to 11 cm in diameter (Ehara 2015a). The *Metroxylon sagu* can be propagated from both seeds and suckers; however, the germination percentage of seeds is very low (Ehara et al. 1998). The *Metroxylon sagu* produces the largest amount of starch (310 kg dry starch per plant), depending on its higher dry matter percentage in pith (41%) and starch concentration on the basis of pith dry matter (77%) among *Metroxylon* palms (Ehara 2015a). The starch yield, dry matter percentage in pith, and starch concentration in the *Coelococcus* palms are 60–160

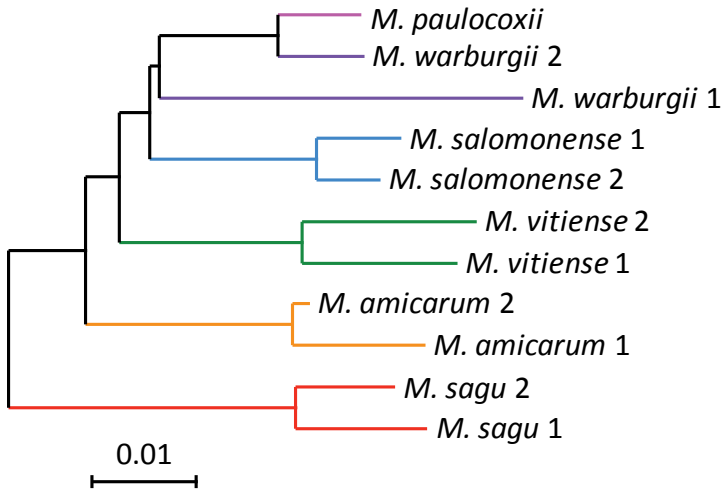

Fig 2. NJ dendrogram based on sequence data of 5S nrDNA in the *Metroxylon*

kg/plant, 16–33%, and 27–49%, respectively, on average in each species. *Coelococcus* palms are apparently different from the *M. sagu* because they do not produce suckers. Instead, the germination percentage of seeds is very high in the section *Coelococcus*, and those palms depend for their propagation only on seeds (Ehara 2015a). Such a high starch concentration in the pith of the *M. sagu* may be related to its propagation style; this species will mainly depend on vegetative propagation as compared with the propagation of other species of *Coelococcus* palms that depend entirely on seed germination. As described above, there are distinct differences in morphological and physiological characteristics between *M. sagu* and *Coelococcus* palms.

In the NJ dendrogram constructed by the current study, *M. sagu* folk varieties were located in distant positions, such as outgroups of *Coelococcus* palms. Besides, the folk varieties of *M. sagu* used in this study, namely 'Roe' and 'Rui' in Southeast Sulawesi, are a spineless and spiny type, respectively. It is believed that Southeast Sulawesi is comparable to the folk variety 'Molat' of the spineless type and 'Tuni' of the spiny type in the Maluku Islands, Indonesia, respectively.

The section *Coelococcus* was divided into four clades in the current dendrogram. *Metroxylon amicarum* grown in Micronesia was genetically distant from other species in the section *Coelococcus*. Regarding inflorescence characteristics, only *M. amicarum* grows lateral inflorescences from leaf axils, while the other four species in the section *Coelococcus* and *M. sagu* produce a terminal racemose inflorescence as well. The lateral inflorescence of *M. amicarum* is pleonanthic (polycarpic); however, the terminal inflorescence of the other species is hapaxanthic (monocarpic). In this matter, *M. amicarum* is markedly different from the other species in inflorescence development. *Metroxylon salomonense* and *M. vitiense* clearly are at or near the purely hapaxanthic definition, and they are intermediate, tending to be closer to the hapaxanthic than to the pleonanthic condition (McClatchey 1999).

Metroxylon salomonense and *M. vitiense* were located next to each other in the dendrogram of this study. McClatchey (2002) pointed out that *M. salomonense* and *M. vitiense* share the characteristics of having: a) a flattened leaf sheath or petiole spines on juvenile and sometimes on adult leaves; b) secondary branches (of inflorescence) that are held horizontally or pendulously to the ground; and c) rachillae that are held horizontally or, more often, pendulously to the ground. It was, therefore, considered that there was a comparatively close

relationship between *M. salomonense* and *M. vitiense*.

Metroxylon warburgii formed a clade together with *M. paulcoxii* in this analysis. The other species of the section *Coelococcus* formed their own clade. Metroxylon paulcoxii was reported by McClatchey (1998) as a new species. However, it has been observed that the branching pattern of *M. paulcoxii* is mostly to the second order and sometimes to the third order (Ehara 2015a). The clade consisting of *M. warburgii* and *M. paulcoxii* was located next to *M. salomonense* in this analysis. *Metroxylon salomonense* also has variation in that its inflorescence branches to the third order or the second order even in a single inflorescence (Ehara et al. 2003b). Dowe (1989) reported that the inflorescence of *M. salomonense* branches to the second and third orders. Considering these reports, *M. salomonense* and *M. paulcoxii* are considered to share characteristics of variation in their branching patterns.

McClatchey et al. (2006) stated that *M. paulcoxii* is possibly an aboriginal introduction from the Santa Cruz Islands (Solomon Islands) via Rotuma (Fiji) as a cultivar of *M. warburgii*. *Metroxylon warburgii* is called '*niu o lotuma*' in the local language of Samoa, a name that means "palm from Rotuma." According to Whistler (2000), *M. warburgii* was originally named *M. upoluense*, which implies that it is native to Samoa, which it is not; it probably was introduced to Samoa in modern times, almost certainly from Rotuma. Furthermore, Whistler (2009) described in his recent publication that *M. paulcoxii* and *M. upoluense* are synonyms for *M. warburgii*. The genetic relationship between *M. paulcoxii* and *M. warburgii* should be studied in detail, employing further analysis from the molecular phylogenetic point of view. Besides, the distribution area of *M. warburgii* is very wide, from Vanuatu close to the Solomon Islands, Rotuma, and Vanua Levu of Fiji and Samoa.

From this analysis, it can be considered that *Coelococcus* palms were dispersed by two routes: one to the north and Micronesia, and the other route to the east to Polynesia through Melanesia. This is generally in agreement with a former study based on a phylogenetic analysis using morphological characteristics (McClatchey 1999). It is supposed that *M. amicarum* is possibly an aboriginal introduction to Pohnpei from the Santa Cruz Islands (McClatchey 1998, 2002). A supposition about the dispersal route of the aboriginal introduction of *M. warburgii* (and *M. paulcoxii*) to Samoa from or via Rotuma is also considered, as above. Moreover, Tucker (1998) noted that intact *M. sagu* trunks are not uncommonly washed ashore in north Queensland, Australia. Dowe (1989) wrote that for the genus *Metroxylon*, distribution through whole plants being dislodged and carried on the ocean's currents should be considered. In the case of *M. amicarum*, the seeds are disseminated by gravity or water, and fruits are often found on the shores of the Caroline Islands, having been carried by ocean waves and currents from one island to another or one part of an island to another (McClatchey et al. 2006). As McClatchey (2002) stated, since people extensively use these palms throughout their current distribution, it is difficult to estimate which parts of their ranges are natural and which have resulted from human introductions. The results of this study show some understanding toward the genetic relationship among the *Metroxylon* palms, including sections *Metroxylon* and *Coelococcus*. Genetic studies of the range of variation within populations of each species will be a further subject that will deepen our comprehension about the phylogeny of the genus *Metroxylon*.

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