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MOLECULAR PHYLOGENY OF MAIDENHAIR FERN GENUS ADIANTUM (pteridaceae) FROM LESSER SUNDA ISLANDS INDONESIA BASED ON RBCL AND TRNL-F

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ABSTRACT
LESTARI, W. S., ADJIE, B., JARUWATANAPHAN, T., WATANO, Y. & PHARMAWATI, M. 2014. Molecular phylogeny of Maidenhair fern genus Adiantum (Pteridaceae) from Lesser Sunda Islands Indonesia based on rbCL and trnL-F. Reinwardtia 14 (1): 143 – 156. — The Lesser Sunda Islands of Indonesia are composed of small islands scattered from Bali to Timor Island. We analyzed a molecular phylogeny of Adiantum collected from Lesser Sunda Islands to reveal its phylogenetic relationships. A total of 12 species of Adiantum from this region and seven species from Java Island were collected and used in this study. Two cpDNA regions (rbCL and trnL-F) were chosen as markers and phylogenetic analyses were conducted using Neighbour-Joining (NJ) and Maximum Parsimony (MP) methods. The tree topologies reconstructed by NJ and MP from specimens used in this study and other species downloaded from GenBank are congruent in which trees are divided into five major clades. Adiantum species of Lesser Sunda Islands are not monophyletic and comprises three clades, i.e. Clade I composed of A. hispidulum group, Clade III composed of A. peruvianum group and Clade IV or A. caudatum group, each together with extra-Lesser Sunda samples. No sample from Lesser Sunda Islands examined is located in Clade II (A. tenerum group) and V (A. capillus-veneris group).

Key words: Adiantum, cpDNA, Lesser Sunda Islands, phylogenetic.

Kata kunci: Adiantum, DNA kloroplas, hubungan kekerabatan, Kepulauan Sunda Kecil.
INTRODUCTION

*Adiantum* (Pteridaceae) is a well known group of ferns and probably the most enthusiastic fern genus (Jones, 1998). The genus called Maidenhairs, is easily recognized by the polished black leaf stalks and the sori covered by specialized reflexed margins of the lamina called false indusia. The members of *Adiantum* are distributed worldwide, mainly in the tropical and subtropical regions (Korpelainen et al., 2005). *Adiantum* consist of 200 species (Hoshizaki & Moran, 2002). Many new species of *Adiantum* were published recently and it has been assumed that the number of *Adiantum* are 280 species globally (Patil et al., 2013). Six species occur in Fiji (Brownsey & Perrie, 2011). Seven species are found in New Zealand (three of them are endemic) (Large & Braggins, 1993; Bouma, 2008). About 60 species are native to Asia (Lu et al., 2012). Holttum (1968) recorded seven species in Malaya. Fifteen species were reported in Vietnam (Phan, 2010). Twenty species and two varieties occur in India (Patil et al., 2013), while eight species are recorded in Japan (one of them is endemic) (Iwatsuki et al., 1995). China is inhabited by 34 species and five varieties (16 species are endemic) (Zhang et al., 2013).

Various groupings of *Adiantum* have been proposed, primarily based on regional studies (Afriastini, 2003; Lu et al., 2012). There is no up-to-date, worldwide revision within this genus, incorporating all findings or comprehensive classification yet (Afriastini, 2003; Korpelainen et al., 2005; Bouma, 2008). Recent studies by Bouma (2008) and Lu et al. (2012) showed that the result of molecular approach analyses are in incongruence with the previous in-group classification based on morphological characters done by Ching (1957) or Tryon & Tryon (1982).

The genus *Adiantum* is monophyletic (Lu et al., 2012). Previous studies showed that it is in need of thorough taxonomic revision since the vittarioid ferns were embedded within this genus and treated as a single subfamily (Schuettelz et al., 2007; Schuettelz & Pryer, 2007; Bouma, 2008; Christenhusz et al., 2011). Recent study by Lu et al. (2012) using the combined three-marker (atpA, atpB, rbcL) and the combined five-marker (atpA, atpB, rbcL, trnL-F, rps4-trnS) showed that the *Adiantum* is monophyletic. Rothfels & Schuettelz (2013) used six-locus data set (atpA, atpB, rbcL, atp1, nad5, gapCp) to prove the very strong support for the monophyly of *Adiantum*. The relationship between *Adiantum* and the vittarioid ferns were clear as they are sisters to each other and formed the adiantoids clade (Lu et al., 2012; Rothfels & Schuettelz, 2013).

No study has been made about this genus in Indonesia since Posthumus (1944) who enumerated 11 species from Lesser Sunda Islands of Indonesia. Lesser Sunda Islands (LSI) are composed of small islands scattered from Bali to Timor Island. This eastern region is quite different from other parts of Indonesia in the drier and more seasonal climate, resulting in a different flora and fauna (de Lang, 2011).

In the present study we analyzed a molecular phylogeny of *Adiantum* occurring in Lesser Sunda Islands of Indonesia by using the rbcL gene and trnL-F region of the chloroplast genome to reveal the phylogenetic relationship. rbcL is a gene (more than 1.400 bp) that encodes the large subunit of ribulose 1,5-biphosphate carboxylase/oxygenase (RUBISCO) and being the most characterized plastid coding region in GenBank with sufficient variation to discriminate among species (Soltis & Soltis, 1998; Avise, 2001; Newmaster et al., 2006). The other plastid marker, the trnL-F, is a region between trnL (UAA) 5’ exon and trnF (GAA) (Adjie et al., 2008). This region is the most variable across ferns because it contains an intron of trnL and an intergenic spacer between trnL and trnF, which displays relatively high rates of mutation, making it an ideal locus for detecting variation at the interspecific and intraspecific taxonomic levels (Taberlet et al., 1991; Bouma, 2008; Li et al., 2009). The combination between these two plastid marker rbcL and trnL-F possesses all the necessary qualities to form a powerful barcode for species identification of pteridophytes (de Groot et al., 2011).

MATERIALS AND METHODS

Taxon Sampling

A total of 12 species (22 specimens) of *Adiantum* were collected from Lesser Sunda Islands (Bali, Lombok, Sumbawa, Sumba and Timor Island), and seven species (eight specimens) were collected from Java. Three species (five specimens) of *Antrophyum* and one species (two specimens) of *Vittaria* collected from Java, Bali, Lombok, Sumbawa and Molucca Island were used as outgroup based on the previous study (Lu et al., 2012). The sequences of non-Lesser Sunda Islands taxa registered at GenBank were also added to the dataset. All taxa included in this study with voucher information, locality and sequence are listed in Table 1. The figure of some specimens are presented in Appendix 1 (Figs. 3, 4 & 5). Living specimens and vouchers were deposited as living and herbarium collection in Bali Botanic Garden, Indonesia (THBB).
Table 1. List of specimens examined

<table>
<thead>
<tr>
<th>No.</th>
<th>Voucher</th>
<th>Species</th>
<th>Locality</th>
<th>Length of Accession Number</th>
<th>Length of Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>AG 326</td>
<td><em>Adiantum sp. AG326</em></td>
<td>Sumbawa¹</td>
<td>1142 bp</td>
<td>924 bp</td>
</tr>
<tr>
<td>2.</td>
<td>WN 118</td>
<td><em>Adiantum sp. WN118</em></td>
<td>Bali¹</td>
<td>-</td>
<td>926 bp</td>
</tr>
<tr>
<td>3.</td>
<td>WN 114</td>
<td><em>A. capillus-veneris</em> L.</td>
<td>Java¹</td>
<td>1148 bp</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>WN 154</td>
<td><em>A. capillus-veneris</em> L.</td>
<td>Java¹</td>
<td>1380 bp</td>
<td>814 bp</td>
</tr>
<tr>
<td>5.</td>
<td>WN 140</td>
<td><em>A. caudatum</em> L.</td>
<td>Bali¹</td>
<td>-</td>
<td>898 bp</td>
</tr>
<tr>
<td>6.</td>
<td>WN 117</td>
<td><em>A. concinnum</em> Humb. &amp; Bonpl. ex Willd.*</td>
<td>Bali²</td>
<td>-</td>
<td>913 bp</td>
</tr>
<tr>
<td>7.</td>
<td>SH 1129</td>
<td><em>A. concinnum</em> Humb. &amp; Bonpl. ex Willd.*</td>
<td>Lombok²</td>
<td>-</td>
<td>923 bp</td>
</tr>
<tr>
<td>8.</td>
<td>WN 150</td>
<td><em>A. concinnum</em> Humb. &amp; Bonpl. ex Willd.*</td>
<td>Java²</td>
<td>1367 bp</td>
<td>966 bp</td>
</tr>
<tr>
<td>9.</td>
<td>WN 112</td>
<td><em>A. diaphanum</em> Blume</td>
<td>Bali¹</td>
<td>-</td>
<td>868 bp</td>
</tr>
<tr>
<td>10.</td>
<td>BA 754a</td>
<td><em>A. diaphanum</em> Blume</td>
<td>Bali¹</td>
<td>-</td>
<td>949 bp</td>
</tr>
<tr>
<td>11.</td>
<td>BA 742</td>
<td><em>A. edgeworthii</em> Hook.</td>
<td>Timor¹</td>
<td>1182 bp</td>
<td>843 bp</td>
</tr>
<tr>
<td>12.</td>
<td>WN 157</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Bali¹</td>
<td>1062 bp</td>
<td>858 bp</td>
</tr>
<tr>
<td>13.</td>
<td>WN 158</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Timor¹</td>
<td>1376 bp</td>
<td>910 bp</td>
</tr>
<tr>
<td>14.</td>
<td>WT 797</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Lombok¹</td>
<td>-</td>
<td>852 bp</td>
</tr>
<tr>
<td>15.</td>
<td>WN 120</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Bali¹</td>
<td>1365 bp</td>
<td>934 bp</td>
</tr>
<tr>
<td>16.</td>
<td>BA 706</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Timor¹</td>
<td>1380 bp</td>
<td>900 bp</td>
</tr>
<tr>
<td>17.</td>
<td>BA 809</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Sumba¹</td>
<td>1336 bp</td>
<td>901 bp</td>
</tr>
<tr>
<td>18.</td>
<td>WN 144</td>
<td><em>A. hispidulum</em> Sw.</td>
<td>Java¹</td>
<td>1366 bp</td>
<td>906 bp</td>
</tr>
<tr>
<td>19.</td>
<td>WN 119</td>
<td><em>A. peruvianum</em> Klotzsch</td>
<td>Bali³</td>
<td>1373 bp</td>
<td>858 bp</td>
</tr>
<tr>
<td>20.</td>
<td>WN 128</td>
<td><em>A. philippense</em> L.</td>
<td>Bali¹</td>
<td>1173 bp</td>
<td>-</td>
</tr>
<tr>
<td>21.</td>
<td>WN 142</td>
<td><em>A. philippense</em> L.</td>
<td>Lombok¹</td>
<td>1221 bp</td>
<td>-</td>
</tr>
<tr>
<td>22.</td>
<td>SH 1130</td>
<td><em>A. philippense</em> L.</td>
<td>Lombok¹</td>
<td>1244 bp</td>
<td>-</td>
</tr>
<tr>
<td>23.</td>
<td>WN 153</td>
<td><em>A. philippense</em> L.</td>
<td>Java¹</td>
<td>1156 bp</td>
<td>-</td>
</tr>
<tr>
<td>24.</td>
<td>WN 116</td>
<td><em>A. polyphyllum</em> Wild.*</td>
<td>Java¹</td>
<td>-</td>
<td>909 bp</td>
</tr>
<tr>
<td>25.</td>
<td>WN 111</td>
<td><em>A. raddianum</em> C. Presl.</td>
<td>Bali²</td>
<td>1380 bp</td>
<td>910 bp</td>
</tr>
</tbody>
</table>
DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted from silica-gel-dried leaf tissue using a modification of the CTAB extraction procedure (Doyle & Doyle, 1987). The \textit{trn}L-F region was amplified and sequenced with primers "cF" and "fR" (Taberlet et al., 1991), and the \textit{rbc}L gene was amplified and sequenced using primers "aF" and "cR" (Hasebe et al., 1994). All amplification were performed in a 25 µl reaction-mixture volume, contained 17.375 µl distilled deionized water, 2.5 µl 10x Buffer, 2 µl dNTP, 1 µl primer (F) 10 µM, 1 µl primer (R) 10 µM, 0.125 µl Ex Taq™ (TaKaRa Bio) and 1 µl DNA sample. For \textit{trn}L-F, reactions were incubated at 95°C for 3 min, then cycled 35 times (94°C for 1 min; 55°C for 1 min; and 72°C for 2 min), followed by a final extension for 10 min at 72°C (Taberlet et al., 1991). For \textit{rbc}L, reactions were incubated at 95°C for 3 min, then cycled 35 times (95°C for 45 s; 55°C for 45 s; and 72°C for 90 s), followed by a final extension for 10 min at 72°C.

The PCR products were evaluated with 1% agarose gel electrophoresis and purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix), 1 µl ExoSAP-IT/10 µl sample, then incubated (37ºC, 1.5 h; 80ºC, 15 min). All cycle sequencings were performed in a 10 µl reaction-mixture volume, containing 6.44 µl distilled deionized water, 1.98 µl 5× Sequencing Buffer, 0.08 µl primer (F/R) 10 µM, 0.5 µl Big Dye 3.1 and 1 µl sample. Reactions were performed at 96ºC for 1 min, then cycled 30 times (96°C for 10 s; 50°C for 5 s; 60°C for 4 min), followed by a final extension for 7 min at 60°C. Each sample then added with 1 µl 125 mM EDTA and 26 µl ethanol : 3 M sodium hydroxide (50:2), and centrifuged (12.500 rpm, 25°C, 60 min). Precipitate was then added with 50 µl 70% ethanol, centrifuged (12.500 rpm, 25°C, 45 min) and air-dried, then incubated for 2 min 95°C and added with formamide then re-incubated (95°C, 3 min). After cold shock on an ice cube for 3 min, the sequencing reactions were run on an ABI 3500 Genetic Analyzer.

The sequence fragments were analyzed using Sequencing Analysis (Applied Biosystems, Foster City, California, USA), then assembled using Auto Assembler 2.1.1. A total of 50 sequences were determined as part of this study and eight sequences of it are new (Table 1). In order to complete the data, the \textit{rbc}L and/or \textit{trn}L-F sequences of 22 specimens were downloaded from GenBank (Appendix 2). Sequence aligned
Fig. 1. Neighbour-Joining tree of the rbcL sequences. Figure at the nodes indicate bootstrap values (NJ/MP; >50%). Support values under 50 are shown as hyphens (-). Bold: nucleotide sequences from LSI.
 Phylogenetic Analysis

Phylogenetic analyses were conducted separately for the two data set (rbcL and trnL-F) using Neighbour-Joining (NJ) and Maximum Parsimony (MP) on MEGA 5.05. The NJ tree was constructed with genetic distance set according to Jukes-Cantor Model (Jukes & Cantor, 1969) and bootstrapping of 1,000 replicates. MP trees was calculated with the following options: Close-Neighbour-Interchange (CNI) on Random Trees with 1,000 replicates. All characters were equally weighted where indels are coded as missing data. A 50% majority-rule consensus tree was calculated to obtain topology with average branch lengths for all resolved nodes (Adjie et al., 2008).

RESULTS

Sequence Characteristics

Among 37 specimens used in this study, 13 specimens can be sequenced for both rbcL and trnL-F regions (Table 1). Seven other specimens can be sequenced for the rbcL region. For the remaining 17 specimens, only rbcL sequences were obtained. The nucleotide sequences obtained in this study were deposited in DNA Data Bank of Japan (DDBJ) under accession number LC004106 to LC004125 for rbcL and LC004375 to LC004404 for trnL-F (Table 1).

The length of the rbcL sequence obtained in Adiantum varied from 1.062 bp in Adiantum hispidulum (WN157) to 1.380 bp in A. capillus-veneris (WN154), A. hispidulum (BA706) and A. raddianum (WN111). No insertion or deletion was observed in any sequences for this gene. The alignment of 40 rbcL sequences including 20 sequences downloaded from GenBank of the Adiantum and allied genera, included 317 (30%) variable sites and 267 (25.2%) were parsimony informative.

The length of the sequence trnL-F region obtained in Adiantum also varied between 814 bp in Adiantum capillus-veneris (WN154) to 966 bp in A. concinnum (WN150). Several indels were found in this region and alignment of 45 sequences (of which 15 sequences were downloaded from GenBank) produced 1176 characters in a matrix (available upon request), of which 748 (63.6%) were variable sites and 623 (53.0%) were parsimony informative.

Phylogenetic Analysis

Phylogenetic analysis was conducted for each dataset employing NJ and MP. These two reconstruction methods generated mostly congruent topologies for each data set. In the rbcL analysis, 40 sequences were included in dataset and the MP method recovered 15 most parsimonious trees of 653 steps (CI = 0.550000; RI = 0.862525). Thus, NJ tree with bootstrap values for the rbcL is shown in Fig 1. as representative.

The NJ and MP trees of the rbcL sequences generated five major clades. Clade I (NJ: 100/MP: 99) is composed of Adiantum hispidulum group. Adiantum hispidulum and A. raddianum from Lesser Sunda Islands formed a clade with A. hispidulum, A. cuneatum and A. concinnum from Java, A. diaphanum from Taiwan and China, A. aethiopicum from New Zealand and an unidentified specimen from Bolivia.

Clade II (100/99) is composed of Adiantum tenerum and A. princeps from Mexico, and did not include any sample from Lesser Sunda Islands. Clade III or A. peruvianum group, sister to Clade II, is composed of A. peruvianum from Lesser Sunda, A. tetraphyllum (cultivated) and an unidentified specimen from Malaysia. This clade is highly supported (100/99) and the relationships between Clade II and Clade III was also resolved in NJ (85), but was not well supported in MP (74).

Clade IV (100/99) or the Adiantum caudatum group, is strongly supported, formed by A. philippense from Lesser Sundas and Java, an unidentified specimen from Lesser Sundas (AG326), A. soboliferum, A. caudatum, A. malesianum, A. mariesii and A. sinicum from China, A. egdeworthii from Lesser Sundas, Japan and China.

Clade V (Adiantum capillus-veneris group) is also strongly supported (100/97) and perhaps sister to Clade IV with low support. This clade was composed by A. capillus-veneris from Java and China, A. jordanii from USA and A. flabellulatum from China. This clade does not include any sample from Lesser Sunda Islands examined.

For the trnL-F, 45 sequences were included in dataset and the MP method recovered 15 shortest trees of 884 steps (CI = 0.622328; RI = 0.901058). All phylogenetic trees of the trnL-F dataset recovered the same five clades with similar memberships as shown in the rbcL dataset, with some additional specimens whose rbcL sequences could not be determined. The NJ tree with bootstrap values for the trnL-F is shown in Fig 2.
Fig. 2. Neighbour-Joining tree of the trnL-F sequences. Numbers at the nodes indicate bootstrap values (NJ/MP; >50%). Support values under 50 are shown as hyphens (-). Bold: nucleotide sequences from LSI.
In this tree, Adiantum silvaticum, A. diaphanum, A. concinnum and an unidentified sample from Lesser Sundas (WN118) are included in Clade I. Again, Clades II and V do not include any sample from Lesser Sunda Islands examined. The Brittle Maidenhair from Java, A. tenerum, included in Clade II, while A. trapeziforme from Lesser Sunda and A. polyphyllum from Java included in Clade III, and A. caudatum from Lesser Sunda Islands was included in Clade IV.

**DISCUSSION**

This study examined 12 species of the Genus *Adiantum* as representative species in Lesser Sunda Islands. Six species are considered as native species, i.e. *A. caudatum*, *A. diaphanum*, *A. edge-worthii*, *A. hispidulum*, *A. philippense* and *A. silvaticum* based on their distribution record. The other species, *A. concinnum*, *A. raddianum* and *A. trapeziforme* considered as an introduced and naturalized species, while *A. peruvianum* considered as cultivated species. The two unidentified specimens, *Adiantum* sp. AG326 and WN118 are morphologically distinct from any species reported in Malay Archipelago so far and tentatively considered as native taxa in the present study.

Both phylogenetic trees of *rbcL* and *trnL-F* are comprised of five major clades. In the trees, Lesser Sunda’s samples are not monophyletic. Some species of Lesser Sunda Islands have wide distribution and were grouped with the samples of the same species from other regions. The Lesser Sunda’s species are referable to the three clades, i.e. Clade I (the *Adiantum hispidulum* group), Clade III (the *A. peruvianum* group) and Clade IV (the *A. caudatum* group).

Clade I (*Adiantum hispidulum* group) consists of seven to nine species (of which six are from Lesser Sunda). Intraspecific variation in *rbcL* and *trnL-F* sequences were observed in *Adiantum hispidulum*. The nucleotide substitution occurs in several sites and the *A. hispidulum* samples were divided into two subclades in the *rbcL* tree.

*Adiantum hispidulum* has been considered to be a polymorphic species, due to the variation in several morphological characters. Large and Braggins (1993) described two polymorphic members of the New Zealand *A. hispidulum* complex, *A. hispidulum* s.s. and *A. pubescens* Schkuhr., which distinguished based on their pinnule hairs. One subgroup with short (63-815 µm), stiff, often pigmented hairs with enlarged basal cells was treated as *A. hispidulum* var. *hispidulum*, while the other subgroup with long (251-1003 µm), soft, pale hairs with narrow basal cells is given varietal status as *A. hispidulum* var. *pubescens*. Bouma (2008) found another type of New Zealand’s *A. hispidulum* pinnule’s hair called type 3, but did not describe it clearly. Morphological examination of the above two subclades in the *rbcL* tree do not accord with the subdivision based on pinnule hair character.

*Adiantum hispidulum* is distributed in southern India, eastern Africa and the Pacific Islands (Hoshizaki & Moran, 2002) and also found in New Zealand (Large & Braggins, 1993), Australia (Bostock et al., 1998), Thailand (Boonkerd & Pollawat, 2013), Indonesia (Posthumus, 1944; Lu et al., 2012) and China (Lu et al., 2012). The cpDNA variation observed in *Adiantum hispidulum* s.l. would be applicable for future studies on phylogeography or on gene flow patterns among continents and islands.

In the *trnL-F* tree (Fig. 2), *Adiantum silvaticum* from Lesser Sunda is included in Clade I. *Adiantum silvaticum* was reported native in eastern Australia. This species is closely related in *A. cunninghamii* Hook., *A. fulva* Raoul. and *A. viridescens* Col., endemic to New Zealand (Parris & Croxall, 1974). In our result, *A. silvaticum* was also related to *A. diaphanum*. This family Maiden- hair is native to Asia, Australia, New Zealand and the Pacific Islands (Hoshizaki & Moran, 2002).

As shown in a recent study by Bouma (2008) and Lu et al. (2012), the result of molecular analysis incongruent with the previous ingroup classification based on morphological characters. *Adiantum concinnum* and *A. raddianum* native to tropical America (Hoshizaki & Moran, 2002) were similar to *A. capillus-veneris* morphologically. However, the chloroplast sequence data shows that *A. capillus-veneris* is distantly related to the two. In both *rbcL* and *trnL-F* trees (Figs. 1 & 2), *A. raddianum* was grouped together with *A. cuneatum* which was recorded as a synonym of *A. raddianum* (Scamman, 1960; Hoshizaki & Moran, 2002).

Among the samples examined, there was no sample from Lesser Sunda Islands in Clade II. In Clade III or the *Adiantum peruvianum* group, *A. peruvianum* and *A. trapeziforme* from Lesser Sunda were grouped together with *A. polyphyllum* from Java. *Adiantum peruvianum* is recorded native to Ecuador, Peru and Bolivia, *A. trapeziforme* is native to Central America and the West Indies, and *A. polyphyllum* occurs from Venezuela to Peru (Hoshizaki & Moran, 2002). Members of this group can be identified by having clathrate rhizome scales with minutely denticulate margins (McCarthy, 2012).
Clades IV (Adiantum caudatum group) is highly supported, although the relationships among its members are not yet well understood. This clade is characterized by the pinnate fronds with the rachis prolonged into a proliferous whip. Adiantum edgeworthii is apparently polyphyletic, where Chinese A. edgeworthii form a sub clade with A. soboliferum and A. philippense, while Japanese and Lesser Sunda’s form a sub clade with A. sinicum. But the supports are very low. Besides China, Japan and Indonesia, A. edgeworthii are also recorded from Bhutan, India (north), Malaysia, Myanmar, Nepal, Philippines, Thailand (north) and Vietnam (Zhang et al., 2013). Extensive analysis is necessary to settle the relationships of the species.

Finally, Clade V corresponds to Lu et al.’s Adiantum capillus-veneris clade (Lu et al., 2012). A. capillus-veneris is a worldwide distributed species, from warm-temperate to subtropical area (Hoshizaki & Moran, 2002). Even the morphological plasticity in frond is very high, all samples from their distribution showed no sequence variation for rbcL. Lu et al. (2012) suggested morphological heterogenity of this species may by due to its wide-spread distribution and broad ecological range, suggesting a recent geographic migration.

**CONCLUSION**

Adiantum species of Lesser Sunda Islands are not monophyletic and the relationships are in incongruent with previous morphological in-group classification either. Lesser Sunda’s Adiantum divided into three main clades based on two plastid markers (rbcL, trnL-F). Clade I of Lesser Sunda’s Adiantum is composed of Adiantum hispidulum, A. silvaticum, A. diaphanum, A. radianum and A. concinnum, Clade III is composed of A. peruvianum and A. trapeziforme, and Clade IV is composed of A. caudatum, A. philippense and A. edgeworthii. No sample from Lesser Sunda Islands examined is placed in Clades II (A. tenerum group) and Clades V (A. capillus-veneris group).

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Appendix 1.

Fig. 3. A. *Adiantum* sp. WN118 (Bali). B. *Adiantum* sp. AG326 (Sumbawa). C. *A. capillus-veneris* WN114 (Java). D. *A. caudatum* WN140 (Bali). E. *A. concinnum* WN117 (Bali). F. *A. diaphanum* WN112 (Bali). G. *A. edgeworthii* BA742 (Timor). H. *A. hispidulum* WN144 (Java).
Appendix 1. Continued

Appendix 1. Continued.

Appendix 2.

List of nucleotide sequences used for phylogenetic analyses

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Origin</th>
<th>Accessions No.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rbcl</td>
<td>trnL-F</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td><em>Adiantum</em> sp.</td>
<td>Bolivia</td>
<td>JF935335</td>
<td>JF980679</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td><em>Adiantum</em> sp.</td>
<td>Malaysia</td>
<td>JF935344</td>
<td>JF980689</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td><em>A. aethiopicum</em></td>
<td>New Zealand</td>
<td>JF935350</td>
<td>JF980695</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td><em>A. capillus-veneris</em></td>
<td>China</td>
<td>JF935322</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td><em>A. caudatum</em></td>
<td>China</td>
<td>JF935296</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td><em>A. cuneatum</em></td>
<td>Indonesia</td>
<td>JF935339</td>
<td>JF980684</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td><em>A. diaphanum</em></td>
<td>Taiwan</td>
<td>AB574797</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td><em>A. diaphanum</em></td>
<td>China</td>
<td>JF935301</td>
<td>JF980647</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td><em>A. edgeworthii</em></td>
<td>Japan</td>
<td>AB574798</td>
<td>-</td>
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<td>10.</td>
<td><em>A. edgeworthii</em></td>
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<td>JF935311</td>
<td>JF980660</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td><em>A. flabellulatum</em></td>
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<td>JF935315</td>
<td>JF980663</td>
<td></td>
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<tr>
<td>12.</td>
<td><em>A. jordani</em></td>
<td>USA</td>
<td>JF935348</td>
<td>JF980693</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td><em>A. malesianum</em></td>
<td>China</td>
<td>JF935297</td>
<td>JF980642</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td><em>A. mariesii</em></td>
<td>China</td>
<td>JF935302</td>
<td>JF980648</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td><em>A. philippense</em></td>
<td>Phillipines</td>
<td>-</td>
<td>JF980675</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td><em>A. princeps</em></td>
<td>Mexico</td>
<td>JF935356</td>
<td>JF980701</td>
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</tr>
<tr>
<td>17.</td>
<td><em>A. sinicum</em></td>
<td>China</td>
<td>JF935300</td>
<td>JF980646</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td><em>A. soboliferum</em></td>
<td>China</td>
<td>JF935299</td>
<td>JF980644</td>
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</tr>
<tr>
<td>19.</td>
<td><em>A. tenerum</em></td>
<td>Mexico</td>
<td>JF935355</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td><em>A. tetraphyllum</em></td>
<td>Cultivated</td>
<td>EF452135</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td><em>Antrophyum callifolium</em></td>
<td>-</td>
<td>EU024556</td>
<td>-</td>
<td></td>
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<tr>
<td>22.</td>
<td><em>Vittaria</em> sp.</td>
<td>China</td>
<td>-</td>
<td>JF980705</td>
<td></td>
</tr>
</tbody>
</table>

(Schuettpelz et al., 2007; Ruhfel et al., 2008; Ebihara et al., 2010; Lu et al., 2012).
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