**RESEARCH ARTICLE**

# First insights into the evolutionary history of the *Davallia repens* complex

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**Abstract**   *Davallia repens* and its close relatives have been identified as a species complex in this study because of the existence of continuously morphological variation. To decipher its evolutionary history, integrated methodologies were applied in this study including morphology, cytology, reproductive biology and molecular phylogeny. Analysis of morphological characters reveals several important discriminating characteristics, such as the shape of stipe scales, frond and indusium. Both diploid and polyploid forms are present in the complex and reproduce sexually and by apogamy, respectively. The incongruence between cpDNA and nDNA phylogeny indicates a hybrid origin for most polyploid individuals. Based on the present results, we hypothesize that there were at least two ancestral lineages distributed in the Malesian region. Through hybridization, polyploidization and apogamy, some polyploid genotypes dispersed outwards to shape the extant distribution.

## INTRODUCTION

Polyploidy has long been identified as an important evolutionary mechanism especially for plants (Stebbins 1950, Grant 1971, Rieseberg 1997). Polyploidy may involve the duplication of the genome of a single species (autopolyploidy) or originate through hybridization (allopolyploidy). Under such conditions, speciation through polyploidization and hybridization are usually depicted in the form of a reticulate network rather than a bifurcating tree. Compared with seed plants, both polyploidization and hybridization play an even more critical role in diversification of ferns and it has been estimated that 31 % of all fern speciation events is accompanied by ploidy increase (Wood et al. 2009). High incidences of hybrid species (c. 40 %) also have been recorded in the flora of Japan (Nakaike 2004). Polyploidization and hybridization events may give rise to progenies with continuous morphology and ambiguous boundaries and thus to taxonomic entities that are usually described as species complexes.

Many species complexes have been found in ferns, crossing a wide phylogenetic spectrum (e.g. Filmy ferns, Ebihara et al. 2005, Nitta et al. 2011; Pteroids, Paris & Windham 1988, Nakato & Kato 2005, Huang et al. 2007, Grusz et al. 2009, Chao et al. 2012; Cystopterioid, Haufler et al. 1990; Asplenioid, Warren & Wagner 1954, Yatabe et al. 2001, Chang et al. 2013; Thelypteroid, Ebihara & Nakato 2013; Blechnoid, Chambers & Farrant 1998; Athyrioid, Ohta & Takamiya 1999; Dryopteroid, Lee et al. 2006, Lee & Park 2013; Polygrammoid, Haufler et al. 1995, Petchsri et al. 2012). Nooteboom (1994) revised the genus

*Davallia* and reduced 53 taxa into a single species, *D. repens* (L.f.) Kuhn*,* with extremely phenotypic plasticity, and therefore *D. repens* is considered as a species complex in this study. Although a few studies have reported apogamous triploids in this species complex from Sri Lanka (Manton & Sledge 1954) and Japan (Kato 1995), the ploidy, reproduction and phylogeny of this morphologically variable species complex have to date not been integrated in an evolutionary context. In this study, with an integrated approach including morphology, cytology, reproductive biology and molecular phylogeny, we aim to decipher the evolutionary history of the *D. repens* complex.

## MATERIALS AND METHODS

### Plants sampling

Fresh materials of *D. repens* complex sensu Nooteboom (1994) and seven other species chosen as outgroup on basis of the molecular phylogenetic analysis were collected from China, Indonesia, Japan, Malaysia, Taiwan, the Philippines and Vietnam. Voucher specimens are deposited in the herbarium TAIF of the Taiwan Forestry Research Institute or TNS of the National Science Museum, Tokyo (Table 1, Appendix). To conduct a more representative morphological analysis and spore size measurement, herbarium specimens covering the global distribution of the *D. repens* complex were also loaned from the herbaria KYO, L, P and TAIF (Table 3).

### Morphological analysis

Morphological analysis was conducted both regionally (Taiwan) and globally. Thirteen morphological characters, seven of which were derived ratios, were measured from 97 specimens (Table 3). For all specimens, 13 characters were measured from three representative fertile fronds (because of the lack of sterile fronds in some specimens). These characters were chosen based on previous studies (Kato 1985, Nooteboom 1994, Shieh 1994) and on our preliminary observations, to represent dissection and shape of fronds, stipe scales and indusia (Table 2, Fig. 1).

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**Table 1** Fresh material of *Davallia repens* used in this study for molecular phylogenetic analysis, ploidy determination, spore number per sporangium (S/S) counting, spore sizes (μm) measurement and gametophyte cultivation. Vouchers are deposited in the herbarium TAIF, if not otherwised indicated. Morphological types are indicated for Taiwanese specimens.

| Location | Voucher | Morphological type | Ploidy | S/S | Spore size |
|---|---|---|---|---|---|
| China | *Lu17812* | – | – | – | – |
| China | *Wade1320* | – | – | – | – |
| China | *Wu974* | – | – | – | – |
| Indonesia | *Wade1063* | – | – | – | – |
| Indonesia | *Wade1113* | – | 3× | 32 | 54.3 ± 4.54 |
| Indonesia | *Wade1125* | – | 2× | 64 | 37.4 ± 3.25 |
| Indonesia | *Wade1860* | – | – | – | – |
| Indonesia | *Wade1866* | – | – | – | – |
| Indonesia | *Wade1961* | – | – | – | – |
| Japan | *100371* (TNS) | – | – | – | – |
| Japan | *101619* (TNS) | – | – | – | – |
| Japan | *Kuo0295* | – | – | – | – |
| Malaysia | *101981* (TNS) | – | – | – | – |
| Taiwan | *Lu16409* | I | 4× | 32 | 54.44 ± 7.29 |
| Taiwan | *Lu16812* | B | 3× | 32 | 63.22 ± 5.35 |
| Taiwan | *Wade0142* | B | 3× | 32 | – |
| Taiwan | *Wade0165*[*] | I | 4× | 32 | 50.60 ± 8.26 |
| Taiwan | *Wade0398*[*] | D | 3× | 32 | – |
| Taiwan | *Wade0399*[†] | E | 3× | – | – |
| Taiwan | *Wade0475* | B | 3× | 32 | – |
| Taiwan | *Wade0548*[*] | G | 4× | 32 | – |
| Taiwan | *Wade0591*[*] | G | 4× | 32 | – |
| Taiwan | *Wade0613* | D | 3× | 32 | – |
| Taiwan | *Wade0714* | I | 4× | 32 | 52.61 ± 6.63 |
| Taiwan | *Wade0726*[*] | B | 3× | 32 | 59.16 ± 6.23 |
| Taiwan | *Wade0729*[*] | C | 3× | 32 | – |
| Taiwan | *Wade0785*[*] | E | 3× | 32 | 56.74 ± 7.36 |
| Taiwan | *Wade0788*[*] | F | 3× | 32 | 57.52 ± 5.59 |
| Taiwan | *Wade0789* | I | 4× | 32 | 57.11 ± 4.57 |
| Taiwan | *Wade0796* | D | 3× | 32 | 55.17 ± 5.13 |
| Taiwan | *Wade0841*[†] | E | 3× | 32 | 51.44 ± 5.56 |
| Taiwan | *Wade0843*[*] | A | 3× | 32 | 49.72 ± 8.54 |
| Taiwan | *Wade0844* | C | 3× | 32 | 52.73 ± 5.55 |
| Taiwan | *Wade0849* | E | 3× | 32 | 49.19 ± 5.66 |
| Taiwan | *Wade0885* | D | 3× | 32 | 46.34 ± 9.45 |
| Taiwan | *Wade0941*[†] | E | 3× | – | – |
| Taiwan | *Wade0948* | H | 4× | – | – |
| Taiwan | *Wade0951*[*] | H | 4× | 32 | 52.51 ± 8.54 |
| Taiwan | *Wade0967* | B | 3× | 32 | – |
| Taiwan | *Wade1148* | C | 3× | 32 | 47.18 ± 3.52 |
| Taiwan | *Wade1149* | A | 3× | – | – |
| Taiwan | *Wade1470* | G | 4× | 32 | – |
| Taiwan | *Wade1472* | A | 3× | – | – |
| The Philippines | *Kuo2069* | – | – | – | – |
| The Philippines | *Kuo2070* | – | – | – | – |
| The Philippines | *Kuo3424* | – | – | – | – |
| The Philippines | *Wade0870*[†] | – | 4× | – | – |
| The Philippines | *Wade0871*[†] | – | 4× | 32 | 51.49 ± 6.97 |
| The Philippines | *Wade0873* | – | 2× | 64 | 33.53 ± 3.98 |
| Vietnam | *Wade1056*[*] | – | 3× | 32 | 62.4 ± 4.32 |

[*] Specimens used for both cpDNA and nDNA phylogeny.
[†] Specimens used for reproductive mode observation.

The images of these characters were taken by a digital camera (EOS 7D, Canon) under light microscope (Wild M8, Leica) and then used for measurement with Image-Pro Plus 5.0 (IPP; Version 5.0; Media Cybernetics, Silver Spring, Mo., USA). The datasets obtained were subjected to Principal Component Analysis (PCA) using SPSS v.14.0 (IBM Inc.).

### Ploidy analysis

The ploidy level was determined by two methods. Firstly, the root tips of two plants (*Lu16409* & *Wade0785*, Table 1) were used for observations of the mitotic chromosomes by the following protocol: The root tips were pre-treated with 2 mM solution of 8-hydroxyquinoline for 8–16 hr at c. 20 °C, fixed in 45 % acetic acid for 15–30 min, hydrolyzed in 1 N HCl at 60 °C for 5 min and then squashed in 2 % aceto-orcein (Sharma 1982).

**Table 2** Loadings of the first two principal components for 13 characters of the *Davallia repens* complex.

| Characters | Regional PC1 | Regional PC2 | Globally PC1 | Globally PC2 |
|---|---|---|---|---|
| ch1: no. of pairs of petioled pinnae | -0.15 | 0.84 | 0.87 | -0.07 |
| ch2: frond division | -0.08 | 0.80 | 0.84 | -0.08 |
| ch3: scale length (mm) | -0.58 | 0.21 | -0.09 | -0.17 |
| ch4: scale width (mm) | 0.75 | 0.46 | 0.23 | 0.67 |
| ch5: indusium length (mm) | 0.14 | -0.06 | 0.02 | 0.00 |
| ch6: indusium width (mm) | 0.36 | 0.52 | 0.66 | 0.25 |
| ch7: petiole length / leaf length | -0.19 | -0.59 | -0.17 | -0.03 |
| ch8: frond length / frond width | 0.76 | 0.15 | -0.01 | 0.82 |
| ch9: frond length / basal pinna length | 0.78 | -0.22 | -0.44 | 0.72 |
| ch10: basal pinna length / basal pinna width | 0.70 | 0.36 | 0.39 | 0.61 |
| ch11: basal pinnule length / basal pinnule width | 0.49 | 0.59 | 0.64 | 0.22 |
| ch12: scale length / scale width | -0.84 | -0.17 | -0.23 | -0.71 |
| ch13: indusium length / indusium width | -0.34 | -0.78 | -0.61 | -0.28 |
| Eigen value | 5.29 | 2.55 | 3.69 | 2.37 |
| Variance explained (%) | 40.68 | 19.58 | 28.39 | 18.25 |
| Variance cumulative (%) | 40.68 | 60.26 | 28.39 | 46.64 |

Secondly, by using these materials (i.e. *Lu16409* & *Wade0785*) with known ploidy level as standard, flow cytometry was used for rapid determination of ploidy levels of other specimens (Ebihara et al. 2005, Nitta et al. 2011). The tissue (c. 4 cm$^2$) of fresh leaves (Table 1) was chopped by a razor in 1.0 mL of buffer (1.0 % Triton X-100, 140 mM 2-mercaptoethanol, 50 mM $Na_2SO_3$, 50 mM Tris-HCl, 40 mg/mL polyvinyl-pyrrolidone and 0.1 mg/mL ribonuclease) together with leaf tissue of known ploidy material as internal standard. The crushed tissue was placed on ice for 5 min and filtered through 30 μm nylon mesh. Twenty-five mg/mL PI was added to the suspension and incubated at 37 °C for 15 min, then 4 °C for 30 min. Genome sizes were analysed by BD FACSCount System (BD Biosciences, California, USA).

The correlation between ploidy level and spore size was examined by measuring the spore size of the samples of which the ploidy level was determined by flow cytometry as described above. Mature fertile fronds of 21 selected specimens (Table 1) were collected then air-dried for 1 day to release the spores. The long axis of 100 spores from each sample was measured. One-way ANOVA analysis was used to determine whether there is significant difference between the spore size of diploids, triploids and tetraploids. In addition, 44 specimens (Table 3), representing the total distribution range of the *D. repens* complex, were selected, and 100 spores from each specimen were measured. The ploidy level of the herbarium specimens was then estimated afterwards by post hoc tests based on the result of the ANOVA analysis.

### Reproductive mode and spore number per sporangium

Spores were collected from five living plants including three triploids and two tetraploids (*Wade0399*, *Wade0841*, *Wade0870*, *Wade0871*, *Wade0941*, Table 1), then sown on Kingroot medium (4 : 4 : 2 vermiculite–peat–perlite, South Sea Vermiculite & Perlite Co., Taipei, Taiwan) to observe gametophyte morphology and reproductive mode. Cultures were maintained under white fluorescent illumination at about 24 μmole m-2s-1, 12 h per day, 20–28 °C.

Many previous studies have shown that plants with sexual and apogamous reproduction produce 64 and 32 spores, respectively (e.g. Takamiya et al. 1999, Takamiya et al. 2001, Huang et al. 2006). Thus, the spore number was also counted to predict the reproductive mode in this study. For each sample (Table 1), five randomly selected sporangia were isolated from mature sori and the numbers of spores were counted under the light microscope (Wild M8, Leica).

**Table 3** Specimens of *Davallia repens* used in this study for morphological analysis and spore size (in μm) measurement used to inferred ploidy. Taiwanese materials are indicated with morphological types. Vouchers are deposited in the herbarium KYO, L, P and TAIF (*KYO, L, P* at the beginning of the voucher number represent the herbarium deposited; others are in TAIF).

| Location | Voucher | Spore size | Inferred ploidy | Location | Voucher | Spore size | Inferred ploidy |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Admiralty Island | P00637357 | 32.3 ± 2.72 | diploid | Taiwan A | Wade1149 | – | – |
| Australia | L0796454 | – | – | Taiwan B | Wade0140 | – | – |
| Caroline Island | L0796460 | 43.3 ± 3.71 | polyploid | Taiwan B | Wade0141 | – | – |
| China | P00635838 | 49.1 ± 4.52 | polyploid | Taiwan E | Wade0149 | – | – |
| China, Hainan | P00635805 | 43.1 ± 5.09 | polyploid | Taiwan E | Wade0153 | – | – |
| Gabon | P00637304 | – | – | Taiwan H | Wade0160 | – | – |
| Indonesia, Celebes | L0796483 | – | – | Taiwan I | Wade0166 | – | – |
| Indonesia, Celebes | L0796488 | – | – | Taiwan D | Wade0398 | – | – |
| Indonesia, Celebes | P00637153 | – | – | Taiwan E | Wade0474 | – | – |
| Indonesia, Celebes | P00637154 | – | – | Taiwan B | Wade0475 | – | – |
| Indonesia, Celebes | P00637362 | 30.6 ± 3.82 | diploid | Taiwan E | Wade0478 | – | – |
| Indonesia, Java | P00637406 | – | – | Taiwan G | Wade0548 | – | – |
| Indonesia, Java | P00637408 | 35.1 ± 2.7 | diploid | Taiwan G | Wade0591 | – | – |
| Indonesia, Sumatra | L0796511 | – | – | Taiwan B | Wade0592 | – | – |
| Indonesia, Sumatra | L0796512 | 45.3 ± 6.1 | polyploid | Taiwan G | Wade0601 | – | – |
| Indonesia, Sumatra | P00637185 | 43.5 ± 7.57 | polyploid | Taiwan D | Wade0613 | – | – |
| Indonesia, Sumatra | P00637186 | 43.8 ± 4.93 | polyploid | Taiwan H | Wade0621 | – | – |
| Indonesia, Sumatra | P00637361 | – | – | Taiwan I | Wade0622 | – | – |
| Japan | L0796539 | 49.7 ± 8.41 | polyploid | Taiwan B | Wade0715 | – | – |
| Madagascar | P00637202 | 45.3 ± 4.98 | polyploid | Taiwan B | Wade0726 | 59.16 ± 6.23 | polyploid |
| Madagascar | P00637235 | 50.4 ± 6.94 | polyploid | Taiwan C | Wade0729 | – | – |
| Malaysia, Borneo | L0796499 | 44.8 ± 4.66 | polyploid | Taiwan E | Wade0785 | 56.74 ± 7.36 | polyploid |
| Malaysia, Borneo | P00637390 | – | – | Taiwan D | Wade0786 | – | – |
| Malaysia, Borneo | P00637458 | – | – | Taiwan F | Wade0788 | 57.52 ± 5.59 | polyploid |
| Malaysia, Malay Peninsula | P00635807 | – | – | Taiwan D | Wade0796 | 55.17 ± 5.13 | polyploid |
| Malaysia, Malay Peninsula | P00635855 | 48.2 ± 5.13 | polyploid | Taiwan E | Wade0833 | – | – |
| Malaysia, Malay Peninsula | P00637133 | 31.5 ± 4.33 | diploid | Taiwan D | Wade0834 | – | – |
| Malaysia, Sarawak | L0796498 | – | – | Taiwan E | Wade0841 | 51.44 ± 5.56 | polyploid |
| Malaysia, Sarawak | P00637385 | – | – | Taiwan A | Wade0843 | 49.72 ± 8.54 | polyploid |
| Malaysia, Sarawak | P00637461 | – | – | Taiwan C | Wade0844 | 52.73 ± 5.55 | polyploid |
| Mauritius | P00637283 | – | – | Taiwan D | Wade0942 | – | – |
| New Caledonia | L0796450 | 42.2 ± 6.29 | polyploid | Taiwan H | Wade0948 | – | – |
| New Caledonia | L0796451 | 49.9 ± 8.55 | polyploid | Taiwan I | Wade0950 | – | – |
| New Caledonia | P00636686 | 44.5 ± 6.19 | polyploid | Taiwan H | Wade0951 | 52.51 ± 8.54 | polyploid |
| Papua New Guinea | L0796453 | 30.4 ± 4.09 | diploid | Taiwan I | Wade0973 | – | – |
| Papua New Guinea | L0796463 | 33.8 ± 4.05 | diploid | The Philippines | L0796490 | – | – |
| Papua New Guinea | L0796467 | – | – | The Philippines | L0796494 | 44.1 ± 6.84 | polyploid |
| Papua New Guinea | L0796503 | 31.8 ± 2.98 | diploid | The Philippines | P00637114 | – | – |
| Society Islands | L0796449 | – | – | The Philippines | P00637140 | – | – |
| Solomon Islands | P00637324 | 35.4 ± 3.31 | diploid | The Philippines | P00637370 | 32.3 ± 3.34 | diploid |
| Sri Lanka | L0796532 | 46.8 ± 7.74 | polyploid | The Philippines | P00637413 | 30.8 ± 3.26 | diploid |
| Sri Lanka | P00635846 | – | – | The Philippines | P00637416 | 45.8 ± 4.17 | polyploid |
| Taiwan I | KYO4752 | 51.8 ± 5.34 | polyploid | Vanuatu | P00591152 | 32.2 ± 2.83 | diploid |
| Taiwan G | KYO7240 | 48.5 ± 7.01 | polyploid | Vanuatu | P00591153 | – | – |
| Taiwan I | Lu16409 | 54.44 ± 7.29 | polyploid | Vanuatu | P00591156 | – | – |
| Taiwan B | Lu16812 | 63.22 ± 5.35 | polyploid | Vietnam | P00635769 | 48.5 ± 8.04 | polyploid |
| Taiwan C | TAIF268915 | – | – | Vietnam | P00635773 | 48.6 ± 4.08 | polyploid |
| Taiwan C | TAIF288308 | – | – | Vietnam | P00635829 | 55.1 ± 7.01 | polyploid |
| Taiwan C | Wade1148 | 47.18 ± 3.52 | polyploid | | | | |

### Molecular phylogeny

Total genomic DNA was extracted from fresh fronds (Table 1) using either modified CTAB or Plant Genomic DNA Mini Kit (Geneaid, Taipei, Taiwan) following the manufacturer's protocol. PCR amplification was performed in 15 μl reaction volumes containing 10–100 ng template DNA, 7.2 μl ddH$_2$O, 1.5 μl 10× buffer, 1.2 μl of 10 μM dNTPs, 1.5 μl of 10 μM forward primer, 1.5 μl of 10 μM reverse primer and 0.5 U Taq polymerase. In total four cpDNA (i.e., *atpB-rbcL*, *rbcL-accD*, *rps16-matK* and *matK*) and one nDNA (i.e., *PgiC*) markers were used in this study, using the primers listed in Table 4. PCR conditions were completed under a standard temperature cycle procedure beginning with a 5 minute denaturation step at 94 °C; followed by 35 cycles of 94 °C for 1 min, 55–65 °C (depend on the primer set) for 1 min, 72 °C for 1 min; finishing with a 10 min elongation step at 72 °C. PCR products were checked on a 1 % agarose gel in TBE buffer. Single strand conformation polymorphism (SSCP) was used to separate different alleles for nDNA markers following Chen et al. (2012). Automated sequencing was done with the amplification primers by Genomics (Taipei, Taiwan).

DNA sequences were aligned with the program MUSCLE (Edgar 2004) on default settings and edited by eye using BioEdit (Hall 1999) to correct obvious misalignments. Maximum likelihood analysis was performed using GARLI (Zwickl 2006) with a GTR+I+Γ model of sequence evolution, and the genthreshfortopoterm option was set to 20 000. Branch support was assessed with 10 000 bootstrap replicates under the same criteria.

## RESULTS

### Morphological variation of D. repens complex

The morphology of scales, fronds and indusia was observed in this study. Scales are present on rhizomes, stipes and fronds. Rhizome scales are lanceolate with marginal setae. The stipe scales can be separated into two types by their shape: ovate and lanceolate; the ovate scales usually appressed to the stipes and the lanceolate scales spreading from the stipes. The frond scales are irregular in shape and the density varies between populations. The fronds are highly variable, from slightly to
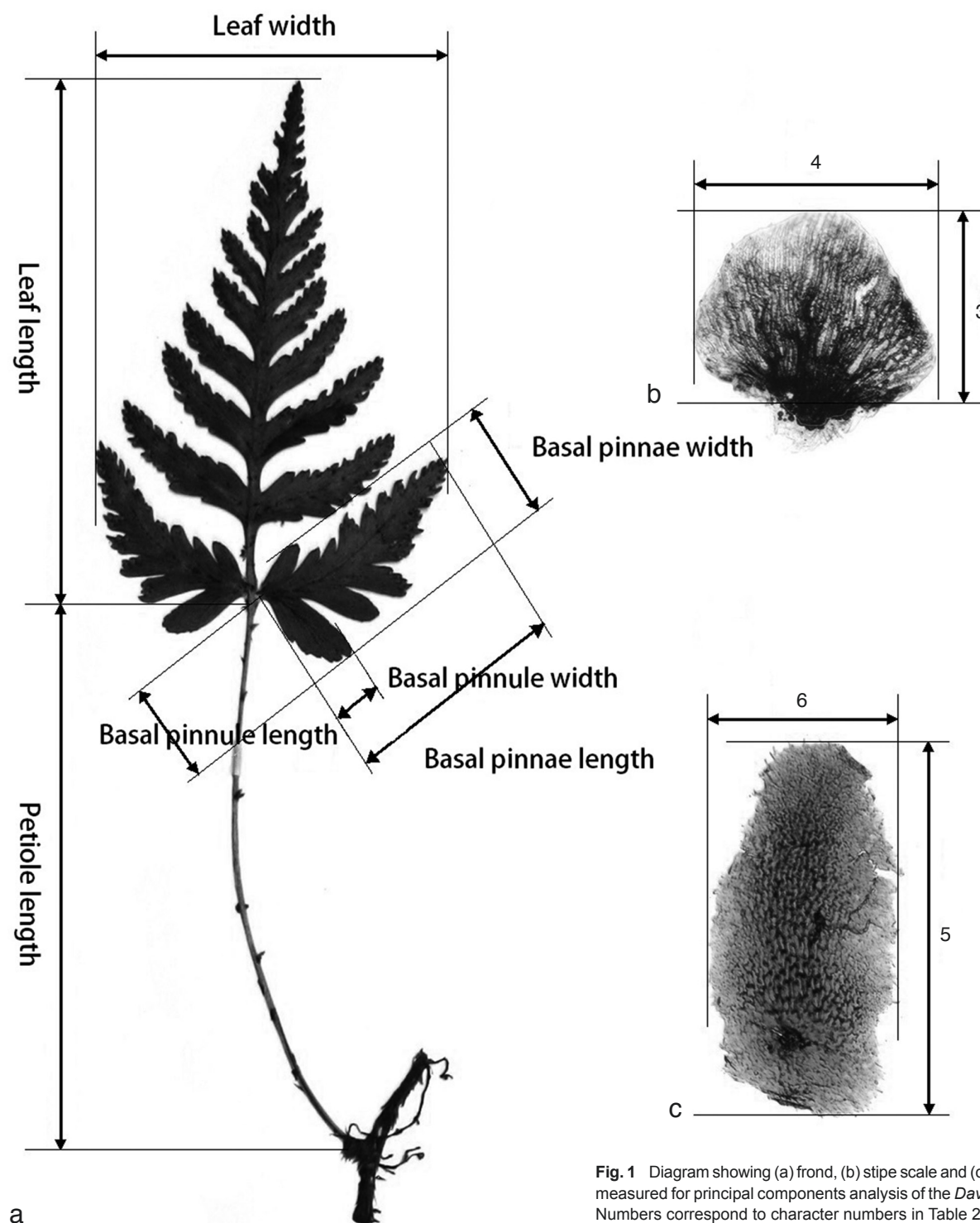
**Fig. 1** Diagram showing (a) frond, (b) stipe scale and (c) indusium characters measured for principal components analysis of the *Davallia repens* complex. Numbers correspond to character numbers in Table 2.
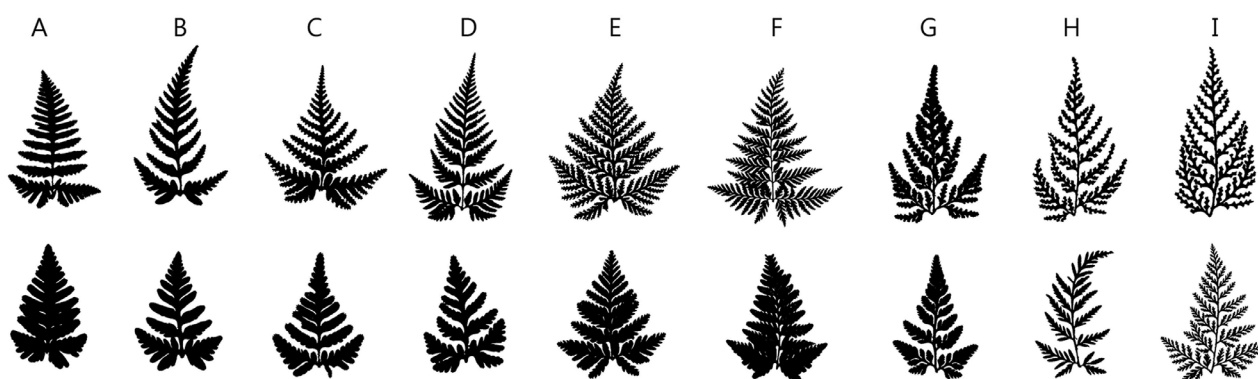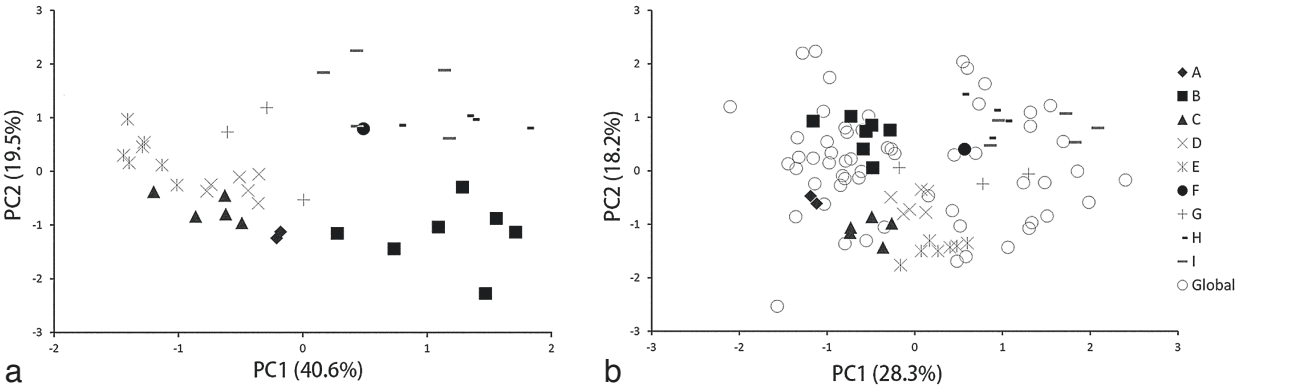


**Fig. 2** Nine morphological forms of the *Davallia repens* complex in Taiwan, upper and lower fronds indicated fertile and sterile frond, respectively.

**Table 4**  Primers used for molecular phylogenetic analysis in this study.

| Region | Primer | 5'-3' sequence | Reference |
|---|---|---|---|
| atpB-rbcL | CT-atpBR2 | TTTCCAACTCCAGCCCCACCRA | Tsutsumi & Kato 2005 |
|  | CT-rbcLR1 | CACCAGCTTTGAATCCAAMACCTG | Tsutsumi & Kato 2005 |
| rbcL-accD | CT-rbcLF3 | TGGCACATGCCYGCTCTAACCGA | Tsutsumi & Kato 2005 |
|  | CT-accDR1 | CCTATACCTGTTTGAACAGCRTC | Tsutsumi & Kato 2005 |
| rps16-matK | rps16F1 | GGCAAATGTATTCAGGAGGTTGG | this study |
|  | matKR1 | CAATACTTCTCTGTTCTTTCTGTTTCCAAG | this study |
| matK | matKF1 | GAATACCTCTTCTGCGTCAGC | this study |
|  | matKR2 | TARCGTACTATCACAYGAGAGTCGTG | this study |
| PgiC | 14F | GTGCTTCTGGGTCTTTTGCGTG | Ishikawa et al. 2002 |
|  | 16R | GTTGTCCATTAGTTCCAGGTTCCCC | Ishikawa et al. 2002 |

**Table 5**  Diagnostic characters of 9 morphological forms in Taiwan.

| Characters | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| Frond shape | lanceolate | lanceolate | pentagonal | lanceolate | pentagonal | pentagonal | lanceolate | lanceolate | lanceolate |
| Dissection | 2-pinnate | 2-pinnate | 2-pinnate | 3-pinnate | 3-pinnate | 3-pinnate | 3-pinnate | 3-pinnate | 3-pinnate |
| Dimorphism | moderate | moderate | moderate | moderate | moderate | moderate | strong | strong | moderate |
| Stipe scale shape | lanceolate | ovate | lanceolate | lanceolate | lanceolate | ovate | ovate | ovate | ovate |
| Indusia shape | shell-shaped | shell-shaped | shell-shaped | shell-shaped | shell-shaped | shell-shaped | shell-shaped | oblate | oblate |



**Fig. 3**  Ordination of regional (a) and global (b) specimens of the *Davallia repens* complex along PC1 and PC2 from the principal component analysis using 13 morphological characters. The 9 morphological forms indicated correspond to those in Fig. 2.
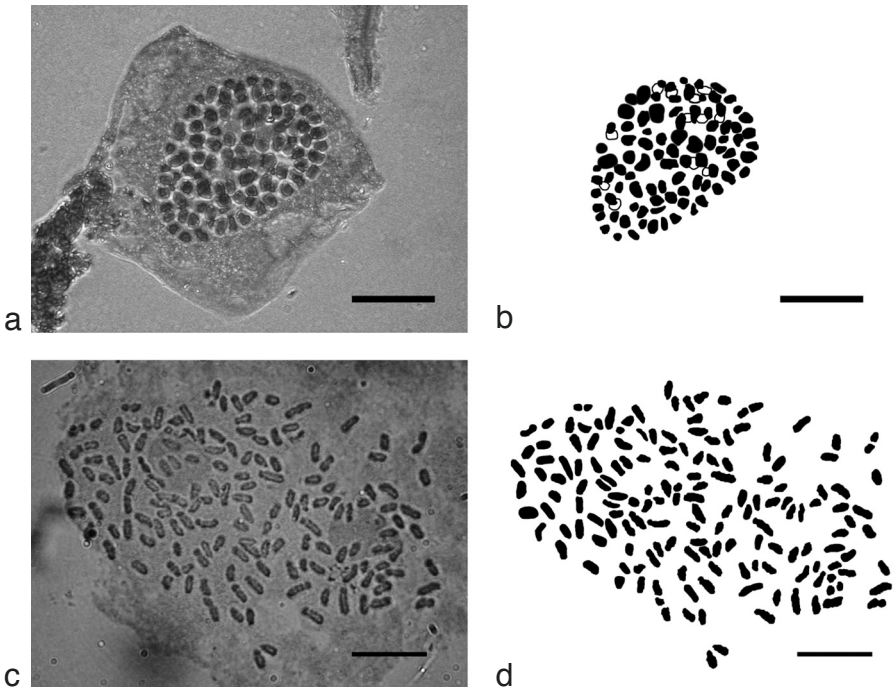


**Fig. 4**  Chromosome number of the *Davallia repens* complex. a. Chromosomes at mitosis metaphase, 2n = 120 (*Wade0785*); b. explanatory illustration of a; c. chromosomes at mitosis metaphase, 2n = 160 (*Lu16409*); d. explanatory illustration of c. The difference of chromosome length resulted from different pre-treatment time. — Scale bars = 20 μm.

**Fig. 5** Spores size distribution of *Davallia repens* complex by ploidy level. The thick horizontal line is the median, the box indicates the variation observed between the 25th and 75th percentiles and the whiskers show the variance range.

**Table 6** One-way ANOVA analysis of spore sizes including diploid, triploid and tetraploid plants of *Davallia repens* complex.

|  | diploid (n = 2) | triploid (n = 13) | tetraploid (n = 6) |
| --- | --- | --- | --- |
| Spore size | 35.47 ± 4.12a | 53.56 ± 7.91b | 53.13 ± 7.81b |

Note: values are mean ± SD.

analysis, although the individuals of each morphological form grouped together, an overlap between some forms was found (Fig. 3a). When the specimens from the global distribution were included in the analysis, there is no obvious grouping in the plot (Fig. 3b).

### *Ploidy analysis*

The chromosome counts reveal two cytotypes (Fig. 4), triploid (2n = 120) and tetraploid (2n = 160). Using these as reference, the ploidy levels of 33 plants as determined with flow cytometry, shows 2 diploids, 21 triploids and 10 tetraploids (Table 1). One way ANOVA analysis for 21 out of these 33 plants shows that the spore size of diploid plants was significantly smaller than of polyploid plants, whereas the difference between the spore sizes of triploid and tetraploid plants was not significant (Table 6, Fig. 5). By using this standard, ploidy levels (i.e., diploid or polyploid) of 44 specimens covering the global distribution of the *D. repens* complex could be estimated (Table 3). The main distribution of diploids is in Malesia (i.e., Indonesia, Malaysia, Papua New Guinea, the Philippines), whereas the distribution of polyploids was much broader but most were found in the regions surrounding the diploid distribution (Map 1).

### *Reproductive mode and spore number/sporangium*

Gametophytes of 5 individuals (3 triploids and 2 tetraploids) were cultivated and observed. The spores began to germinate 7–12 days after they were sown. Germination of the spore is of Aspidium-type following the definition of Nayar & Kaur (1971). The mature gametophytes were heart-shaped with unicellular papillate hairs on both surface and margin. Gametangia (antheridia and archegonia) began to appear after 3 months cultivation and were of the Leptosporangiate-type. Juvenile sporophytes first appeared 5 months after spores were sown and we suggest they were all reproduced by apogamy since archegonia were very rare and the channel of the archegonial neck was not open during the whole culture period.

strongly dimorphic, pinnatifid to 4-pinnate and lanceolate or pentagonal in shape. Two shapes of indusia were recognized: shell-shaped, in which the length is similar to the width vs oblate in which the width is much longer than the length. Applying these morphological characters to Taiwanese representatives of the *D. repens* complex, nine morphological forms can be recognized (Table 5, Fig. 2).

The results of the PCA based on 13 morphological characters are given in Table 2. At regional scale, the first component had relatively high loadings for the ratio of length and width of stipe scale (-0.84), leaf length and basal pinnae length (0.78) and leaf length and leaf width (0.76). At global scale, the first component loaded heavily for the number of pairs of petioled pinnae (0.87), leaf division (0.84) and indusium width (0.66). The plot of individuals projected on the first two principal components at regional and global scale is shown in Fig. 3. For the regional



**Map 1** Cytogeographical distribution of *Davallia repens* complex inferred from spore size. Open circle and close star marks represent diploid and polyploids, respectively. The global distribution of *D. repens* complex is indicated by the dotted line.

**Fig. 6** Plastid DNA phylogeny of the *Davallia repens* complex based on combined analysis of *atpB-rbaL*, *rbcL-accD*, *rps16-matK* spacer and *matK*; the best maximum likelihood topology is shown. Bootstrap value under 80 % is not shown. The morphological form and ploidy level are shown, if known, followed by collection number, species name and location.



For the spore number, a total of 30 individuals were counted. The result shows that all triploids and tetraploids contained 32 spores per sporangium. However, two diploid plants collected from Indonesia and the Philippines contained 64 spores per sporangium (Table 1).

### Molecular phylogeny of D. repens complex

The aligned sequence matrix including four cpDNA regions had a total of 3 568 characters and 535 variable sites. The maximum likelihood phylogeny (Fig. 6) shows that the *D. repens* complex can be separated into two major clades (X and Y). The X clade included part of *D. repens* complex and *D. angustata* Wall. and was sister to another species, *D. pectinata* Sm. The Y clade, including the other specimens of *D. repens* complex, was sister to the X clade plus *D. pectinata*.

The aligned nDNA data matrix had a total of 699 characters and 146 variable sites. Similar to the cpDNA phylogeny, the maximum likelihood phylogeny generated from nDNA shows that the *D. repens* complex is not a monophyletic group and can be separated into two major clades (I and II). Interestingly, the alleles of five individuals (i.e. *Wade0165*, *Wade0548*, *Wade0591*, *Wade0843*, *Wade0951*) were found in both clades, indicating that they are hybrids between members of these two clades (Fig. 7).
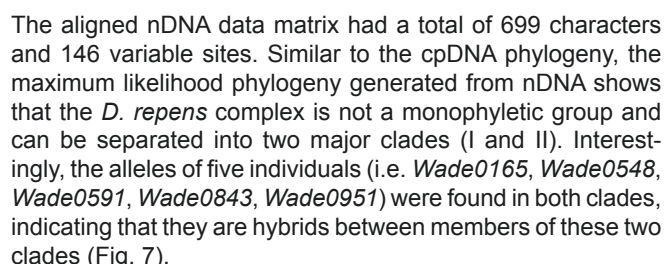
### DISCUSSION

#### Chloroplast phylogeny of D. repens complex

The chloroplast phylogeny shows that the *D. repens* complex is not a monophyletic group but includes two other species,



**Fig. 7** Nuclear DNA phylogeny of the *Davallia repens* complex based on *PgiC*; the best maximum likelihood topology is shown. Bootstrap value under 80 % is not shown. The morphological form and ploidy level are shown, if known, followed by collection number, species name, and location. The allele number is shown at the end for each individual.
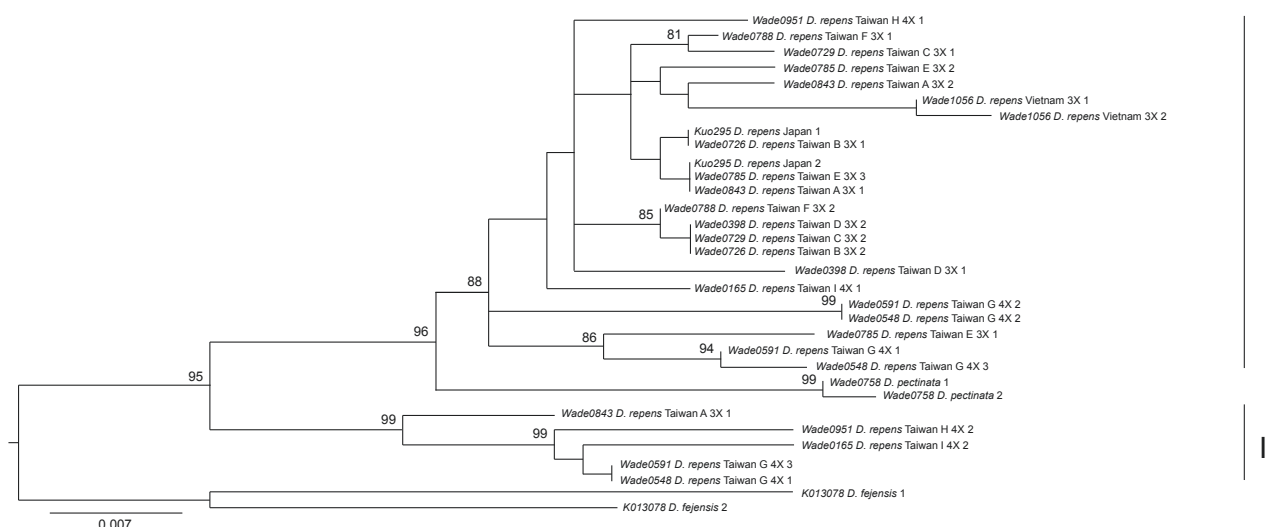
*D. angustata* and *D. pectinata*. Both species have a unique morphology (*D. angustata* has simple fronds, *D. pectinata* has pectinate fronds) and have never been placed in the *D. repens* complex (Kato & Tsutsumi 2008, Nooteboom 2013). There is no evidence of gene flow between these two species and *D. repens* complex in our study since there is no shared haplotype (Fig. 6).

Two well-supported clades (X and Y) were discovered in the cpDNA analysis of the *D. repens* complex. Within each of the two clades, a low level of variation was observed and suggests a recent diversification within this complex. Clade X not only has a wide distribution including China, Indonesia, Japan, Malaysia, Taiwan, the Philippines and Vietnam, but also has diverse ploidy levels including diploids, triploids and tetraploids. Two diploid accessions (*Wade0873* and *Wade1125*) each shared their haplotypes with other triploid and tetraploid accessions, indicating that these two diploids might be maternal donors of those polyploids. Clade Y is restricted to Indonesia, the Philippines and Taiwan and all accessions were identified as tetraploid. The lack of diploid specimens in clade Y might be the result of insufficient sampling, with only two diploid accessions in the phylogenetic analysis. Although we cannot exclude the possibility that the alleles in clade Y originated from hybridization with another species outside the complex, this scenario is less likely since we have included most of the related species outside the complex.

Overall, the phylogenetic structure of this study does not support the lumping of 53 taxa into a single species by Nooteboom (1994) but suggests there are at least two diploid ancestral lineages. A more comprehensive sampling in the future including all the species previously placed under the genus *Humata* may further clarify the circumscription and the number of diploid ancestral lineages of the *D. repens* complex. Additional data from more variable markers are also needed to better resolve the relationship among each of the haplotypes.

### Recurrent hybridization resulted in morphological variation

Morphological analyses reveal several important characters in the *D. repens* complex. The ratio of length and width of stipe scale, leaf length and basal pinnae length, and leaf length and leaf width earn the highest loadings in the analysis at regional scale. The number of pairs of petioled pinnae, leaf division, and indusium width load heavily in the analysis at global scale. These characters represent the shape of the stipe scales, fronds and indusia. When these characters are applied to the individuals from a small region, such as Taiwan, several forms can be recognized (Table 5, Fig. 2). This subdivision was mainly congruent with the PCA plot although an overlap between some forms was found (Fig. 3a). Because only the fertile fronds were measured in this study, the degree of frond dimorphism could not be represented and this might explain the overlapping between some forms in the PCA plot. Although these forms are not completely separated in the PCA plot, most of them are supported in the cpDNA analysis (Fig. 6). For example, morphological forms A, B, C, D, E, G and I all correspond to a unique haplotype. The only two exceptions are the morphological form F and H. The single accession of morphological form F (i.e. *Wade0788*) shares the haplotype with form D, indicating that the morphological difference between these two forms might result from having different paternal lineages. The two accessions of morphological form H (*Wade0948*, *Wade0951*) have an identical haplotype with forms D and G, respectively. This result suggests that these 2 accessions may have different origins although they shared the same morphology.

When the individuals from all around the world are considered, the situation is more complicated. On the PCA plot for the global analysis, no obvious grouping can be found, indicating a continuous morphological variation (Fig. 3b). The delimitation of each form in material from Taiwan becomes inapplicable because some intermediate forms were found in other Asiatic regions and are included in the analysis. Although samples from outside Taiwan are relatively few in the molecular phylogenetic analysis, the results still shed some light into the formation of the continuous morphological variation. Comparing cpDNA and nDNA phylogeny, multiple alleles of most polyploid individuals are found in different clades which indicates hybrid origin of those individuals (Fig. 8). Moreover, the alleles of three individuals (*Wade0548, Wade0591, Wade0843*) were distributed in three
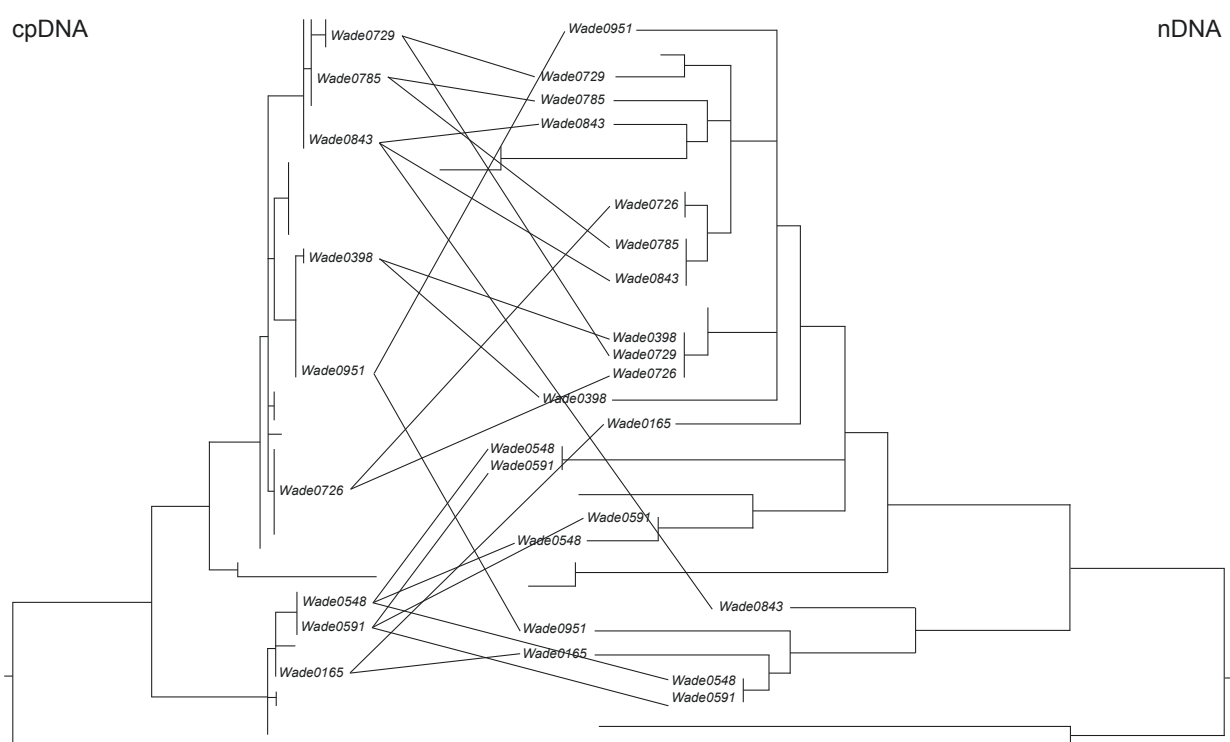


**Fig. 8**  Combination of cpDNA and nDNA phylogeny of the *Davallia repens* complex. The phylogenies were simplified from Fig. 6 and 7.

different clades, providing the evidence for recurrent hybridization. As a result, we propose that recurrent hybridization events has resulted in the continuous morphological variation of the *D. repens* complex, as was hypothesized by Nooteboom (1998).

### *Cytogeography of D. repens complex*

Tryon & Lugardon (1991) mention that the highly variable spore size observed in *D. repens* complex might imply its cytological complexity. In this study, multiple approaches, including chromosome number count, flow cytometry and spore size measurement, show three different ploidy levels, i.e., diploid, triploid and tetraploid. The number and distribution of diploids and polyploids (triploid and tetraploid) are very different. In our herbarium specimens analysis, ploidy level of 44 specimens was estimated and only 11 of them are diploids. The polyploids are widely distributed on Caroline Island, China, Indonesia, Japan, Madagascar, Malaysia, New Caledonia, Sri Lanka, Taiwan, the Philippines and Vietnam, whereas the diploids were only found in Malesia and South Pacific regions, including Admiralty Islands, Indonesia, Malaysia, Papua New Guinea, Solomon Islands, the Philippines and Vanuatu. The Malesia region then not only harbours more than 90 % of the species of *Davalliaceae* (Nooteboom 1992, 1994), but also contains a high morphological variation of *D. repens* complex (this study). Since diploids are generally considered to thought to be the primitive cytotype (Ebihara et al. 2005, Grusz et al. 2009, Rousseau-Gueutin et al. 2009, Dyer et al. 2012), the Malesian region is not only the diversity hotspot for *Davalliaceae*, but also the centre of origin of the *D. repens* complex.

Results of spore number count and gametophyte cultivation further indicate that all these polyploids reproduce by apogamy (Table 1). Since no sexual polyploid has been found, we propose that apogamy is the major mechanism for hybrid stabilization in the *D. repens* complex. Previous studies have shown a difference in habitat preference between different ploidy levels (Soltis & Soltis 2000, Shinohara et al. 2006, Huang et al. 2007, Libor & Milan 2008). In this study, some polyploid genotypes might have adapted to a cooler habitat and thus were able to disperse northward (in the northern hemisphere) or southward (in the southern hemisphere). The apogamous reproductive mode further enhances the chance of successful colonization of new habitats for these genotypes through the long distance dispersal of single spores (Schneller et al. 1998, Page 2002, Trewick et al. 2002, Pangua et al. 2003, De Groot et al. 2012).

## CONCLUSION

This study provides the first insight in the evolution of the *D. repens* complex, showing that hybridization, polyploidization and apogamy play an important mechanism in generating the genetic and morphological diversity of the *D. repens* complex. By integrating the results of this study, we propose the following scenario for the evolution of the *D. repens* complex: In the early evolutionary stage of the species complex, there were at least two ancestral lineages, distributed in the Malesian region (corresponding to the X and Y clade). By means of hybridization, these two lineages diversified into numerous morphological forms and genetic combinations, that were stabilized by means of apogamy. Those polyploidized progenies further adapted to new habitats and eventually shaped the extant distribution.

Future study involving a more comprehensive sampling especially from the Malesia region may further clarify the evolution of the *D. repens* complex. With that, more detailed and interesting questions such as: 'What is the true circumscription of the *D. repens* complex?'; 'Is there gene flow between *D. angustata* or *D. pectinata* and the *D. repens* complex?'; 'If not, by what mechanism did these species become isolated?'; 'How many diploid ancestors contributed to the formation of the complex?' and 'When and how many times have the hybridization events happened?' can be answered.

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**Appendix**   GenBank accessions. Information is presented as follows: taxon name, collection number, GenBank accession numbers for four plastid loci: *atpB-rbcL / rbcL-accD / rps16-matK / matK*; *pgiC* (number of haplotypes).

*Davallia angustata*: *Wade2208*, KJ468928 / KJ468983 / KJ469038 / KJ468841.
*Davallia corniculata*: *Wade2080*, KJ468927 / KJ468982 / KJ469037 KJ468840.
*Davallia fejensis*: *K013078*, *pgiC*: KJ468873(1) / KJ468874(2).
*Davallia ophioglossa*: *Wade1151*, KJ468919 / KJ468974 / KJ469029 / KJ468832.
*Davallia pectinata*: *Nitta643*, KJ468885 / KJ468940 / KJ468995 / KJ468798. *Wade0785*, KJ468896 / KJ468951 / KJ469006 / KJ468809; *pgiC*: KJ468871(1) / KJ468872(2).
*Davallia repens*: *100371*, KJ468875 / KJ468930 / KJ468985 / KJ468788. *101619*, KJ468876 / KJ468931 / KJ468986 / KJ468789. *101981*, KJ468877 / KJ468932 / KJ468987 / KJ468790. *Kuo295*, KJ468878 / KJ468933 / KJ468988 / KJ468791; *pgiC*: KJ468848(1) / KJ468849(2). *Kuo2069*, KJ468879 / KJ468934 / KJ468989 / KJ468792. *Kuo2070*, KJ468880 / KJ468935 / KJ468990 / KJ468793. *Kuo3424*, KJ468881 / KJ468936 / KJ468991 / KJ468794. *Lu16409*, KJ468882 / KJ468937 / KJ468992 / KJ468795. *Lu16812*, KJ468883 / KJ468938 / KJ468993 / KJ468796. *Lu17812*, KJ468884 / KJ468939 / KJ468994 / KJ468797. *Wade0142*, KJ468886 / KJ468941 / KJ468996 / KJ468799. *Wade0165*, KJ468887 / KJ468942 / KJ468997 / KJ468800; *pgiC*: KJ468869(1) / KJ468870(2). *Wade0398*, KJ468888 / KJ468943 / KJ468998 / KJ468801; *pgiC*: KJ468854 / KJ468855. *Wade0475*, KJ468889 / KJ468944 / KJ468999 / KJ468802. *Wade0548*, KJ468890 / KJ468945 / KJ469000 / KJ468803; *pgiC*: KJ468864(1) / KJ468865(2) / KJ468866(3). *Wade0591*, KJ468891 / KJ468946 / KJ469001 / KJ468804; *pgiC*: KJ468861(1) / KJ468862(2) / KJ468863(3). *Wade0613*, KJ468892 / KJ468947 / KJ469002 / KJ468805. *Wade0714*, KJ468893 / KJ468948 / KJ469003 / KJ468806. *Wade0726*, KJ468894 / KJ469004 / KJ468807; *pgiC*: KJ468846(1) / KJ468847(2). *Wade0729*, KJ468895 / KJ468950 / KJ469005 / KJ468808; *pgiC*: KJ468852(1) / KJ468853(2). *Wade0785*, KJ468897 / KJ468952 / KJ469007 / KJ468810; *pgiC*: KJ468856(1) / KJ468857(2) / KJ468858(3). *Wade0788*, KJ468898 / KJ468953 / KJ469008 / KJ468811; *pgiC*: KJ468859(1) / KJ468860(2). *Wade0789*, KJ468899 / KJ468954 / KJ469009 / KJ468812. *Wade0796*, KJ468900 / KJ468955 / KJ469010 / KJ468813. *Wade0841*, KJ468901 / KJ468956 / KJ469011 / KJ468814. *Wade0843*, KJ468902 / KJ468957 / KJ469012 / KJ468815; *pgiC*: KJ468843(1) / KJ468844(2) / KJ468845(3). *Wade0844*, KJ468903 / KJ468958 / KJ469013 / KJ468816. *Wade0849*, KJ468904 / KJ468959 / KJ469014 / KJ468817. *Wade0871*, KJ468905 / KJ468960 / KJ469015 / KJ468818. *Wade0873*, KJ468906 / KJ468961 / KJ469016 / KJ468819. *Wade0885*, KJ468907 / KJ468962 / KJ469017 / KJ468820. *Wade0948*, KJ468908 / KJ468963 / KJ469018 / KJ468821. *Wade0951*, KJ468909 / KJ468964 / KJ468919 / KJ468822; *pgiC*: KJ468867(1) / KJ468868(2). *Wade0967*, KJ468910 / KJ468965 / KJ469020 / KJ468823. *Wade1056*, KJ468911 / KJ468966 / KJ469021 / KJ468824; *pgiC*: KJ468850(1) / KJ468851(2). *Wade1063*, KJ468912 / KJ468967 / KJ469022 / KJ468825. *Wade1113*, KJ468913 / KJ468968 / KJ468826. *Wade1124*, KJ468915 / KJ468970 / KJ469025 / KJ468828. *Wade1125*, KJ468916 / KJ468971 / KJ469026 / KJ468829. *Wade1148*, KJ468917 / KJ468972 / KJ469027 / KJ468830. *Wade1149*, KJ468918 / KJ468973 / KJ469028 / KJ468831. *Wade1320*, KJ468920 / KJ468975 / KJ469030 / KJ468833. *Wade1470*, KJ468921 / KJ468976 / KJ469031 / KJ468834. *Wade1472*, KJ468922 / KJ468977 / KJ469032 / KJ468835. *Wade1860*, KJ468923 / KJ468978 / KJ469033 / KJ468836. *Wade1866*, KJ468924 / KJ468979 / KJ469034 / KJ468837. *Wade1961*, KJ468925 / KJ468980 / KJ469035 / KJ468838. *Wade2005*, KJ468926 / KJ468981 / KJ469036 / KJ468839. *Wu974*, KJ468929 / KJ468984 / KJ469039 / KJ468842.
*Davallia sessilifolia*: *Wade1115*, KJ468914 / KJ468969 / KJ469024 / KJ468827.