The evolution of genome size and distinct distribution patterns of rDNA in *Phalaenopsis* (Orchidaceae)

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*Phalaenopsis* is a horticulturally important genus of c. 63 spp. native to Southeast Asia. Aiming to understand the genome differentiation and phylogenetic relationships in diploid *Phalaenopsis* spp., we investigated the nuclear DNA content by flow cytometry karyomorphology and mapped rDNA loci by fluorescence in situ hybridization to address genome differentiation. Most species have a chromosome complement of 2n = 38 (but have different karyotype compositions). Chromosome reduction is only observed in *Phalaenopsis* section *Aphyllae* (2n = 34 and 36). The genome sizes differ 6.3-fold, from 1C = 1.39 to 8.74 pg. Reconstruction of genome size indicates the genome size for the most recent common ancestor of *Phalaenopsis* subgenus *Parishianae* to be 3.92 pg/1C and for subgenus *Phalaenopsis* to be 2.42 pg/1C. The 45S rDNA sites are polymorphic and correlate positively with genome size, but 5S rDNA sites do not. Our results suggest independent evolutionary processes of genome size increase or decrease in each *Phalaenopsis* lineage. The polymorphism in the number of rDNA loci reveals that complex chromosomal rearrangements, e.g. gain or loss of repetitive DNA or chromosomes, inversion and/or transposition events, may have contributed to create the various karyotypes observed in *Phalaenopsis* spp.

**ADDITIONAL KEYWORDS:** chromosome – fluorescence in situ hybridization – karyotype – molecular phylogenetic analysis – rRNA genes.

**INTRODUCTION**

*Phalaenopsis* Blume consists of c. 63 spp. distributed from the Himalayas, through southern China and Southeast Asia, to northern Australia (Sweet, 1980; Christenson, 2001). *Phalaenopsis* spp. are extensively sold in the floricultural trade as potted plants or cut flowers because of their colourful and long-lasting flowers (US Department of Agriculture, 2015). Phylogenetic analyses of *Phalaenopsis* spp. using nuclear ribosomal DNA internal transcribed spacer (ITS) sequences (Tsai, Huang & Chou, 2006) or ITS sequences combined with plastid DNA sequences (Yukawa et al., 2005) have identified two distinct clades with different numbers of pollinia and biogeographical distributions. Species in the first clade (*Phalaenopsis* subgenus *Parishianae* (H.R.Sweet) Christenson) have four pollinia and the species are distributed throughout the Himalayas and Indochina; species in the second clade (*Phalaenopsis* subgenus *Phalaenopsis*) have two pollinia and are mainly distributed across the islands of tropical Asia and Australia. Although many *Phalaenopsis* spp. are extensively used in breeding programmes, available cytogenetic information is incomplete. Previous cytological studies of *Phalaenopsis* indicated that although all species that had been studied had a chromosome complement of 2n = 2x = 38, there was considerable

variation in chromosome sizes (Sagawa, 1962; Shindo & Kamemoto, 1963; Sagawa & Shoji, 1968). Most Phalaenopsis spp. have a symmetrical karyotype, but a few species, such as P. amboinensis J.J.Sm., P. venosa Shim & Fowlie and P. violacea Teijsm. & Binn., have bimodal karyotypes with heterochromatin blocks that have accumulated at the ends of the large metacentric/submetacentric chromosomes. Such karyotype variation in Phalaenopsis spp. has been considered to be most probably due to differential accumulation of constitutive heterochromatin (Kao et al., 2001).

Knowledge of genome size data is an important consideration when applying various molecular tools to study genetic diversity (Fay, Cowan & Leitch, 2005; Fay et al., 2009). For example, AFLP methods to estimate genetic diversity usually fail for species with large genome sizes as they do not produce a sufficient number of bands to enable genetic diversity to be estimated (Fay et al., 2009). Genome size has also been shown to have considerable biological implications. For example, several studies have identified correlations between genome size and cellular or organ size (Van’t Hof & Sparrow, 1963; Price, Sparrow & Nauman, 1973), such as seed size and leaf size. Genome size is also correlated with the rate of growth and development, which are useful parameters for breeding (Doležel, Doleželova & Novak, 1994; Cerbah and development, which are useful parameters for breeding programmes. Such karyotype variation in Phalaenopsis spp. has been considered to be most probably due to differential accumulation of constitutive heterochromatin (Kao et al., 2001).

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Repetitive DNA sequences (e.g. transposable elements and tandem repeats) are a major component of plant nuclear genomes and may make up to 50–75% of the genome (Kubis, Schmidt & Heslop-Harrison, 1998; Heslop-Harrison & Schwarzacher, 2011). Repetitive DNA sequences show variation in sequence and copy number during evolution (Flavell, 1982) and thus knowledge of repetitive sequences can provide insights into the evolution of plant genomes and add support to taxonomic and phylogenetic studies (Heslop-Harrison & Schmidt, 2012). In higher eukaryotes, ribosomal genes (rDNAs) are organized into two distinct multigene families, coding for 45S rRNA and 5S rRNA, respectively. Both types of rDNAs occur as tandemly repetitive sequences that encode non-translated rRNA involved in ribosome formation (Suzuki, Sakurai & Matsuda, 1996). Typically, repetitive 45S rDNAs with a non-coding intergenic spacer (IGS) between adjacent 45S rDNA units are tandemly arrayed at one or several loci on chromosomes (Roa & Guerra, 2012). The 5S rDNA locus also contains a tandem array of hundreds or even thousands of repeats, usually at a different location from the 45S rDNA locus (Drouin & de Sá, 1995; Garcia et al., 2017). The DNA sequence transcribed for the 5S rRNA is c. 120 base pairs long, and is highly conserved among organisms. A non-transcribed spacer region (5S-NTS) separates adjacent 5S rDNA repeats (Appels & Baum, 1992). Variation in the number and distribution of 5S and 45S rRNA genes as revealed by fluorescence in situ hybridization (FISH) in closely related species has provided valuable information about chromosomal evolution and genome organization (e.g. Adams et al., 2000; Raskina, Belyayev & Nevo, 2004; Lamb et al., 2007; Iovene et al., 2008; for a review, see Gill, Hans & Jackson, 2008). Furthermore, the distribution of rDNA loci also provides useful landmarks for chromosome identification and for tracing karyotype evolution among related species (Jiang & Gill, 1994; Linares et al., 1996; Moscone et al., 1999; Taketa, Harrison & Heslop-Harrison, 1999; Hasterok et al., 2001; Hizume et al., 2002; Boguníć et al., 2011).

To date, cytological data of Phalaenopsis have been largely focused on species in Phalaenopsis subgenus Phalaenopsis (Kao et al., 2001; Lin et al., 2001; Lee, 2002, 2007; Chen, 2009; Tseng, 2009) and only P. pulcherrima (Lindl.) J.J.Sm. has been studied cytogenetically in subgenus Parishianae (Peng, 2007). In this study, we examined the nuclear DNA contents of seven Phalaenopsis spp. with the aim of achieving a more comprehensive sampling of taxa in subgenus Parishianae than previously undertaken. We also characterized the chromosomal distribution of 5S rDNA and 45S rDNA loci in 45 Phalaenopsis spp. using FISH. These comprehensive cytogenetic studies and new genome size data reported here, analysed in a molecular phylogenetic framework, provide important insights into the molecular aspects of chromosome evolution in Phalaenopsis and valuable information for breeding programmes.

MATERIAL AND METHODS

PLANT MATERIAL

We selected 45 representative taxa from both subgenera and all sections of Phalaenopsis for this study. The plants were maintained in the greenhouses of the National Museum of Natural Science and voucher specimens were deposited in the herbarium of the National Museum of Nature and Science, Taiwan. Information on chromosome numbers and the vouchers are given in Table 1.

GENOME SIZE MEASUREMENT

Young leaves of each sample were chopped using a sharp razor blade in a Petri dish containing 3 mL of Galbraith's buffer (Galbraith et al., 1983). Then, the suspension of nuclei was filtered through a 30-μm
**Table 1.** The voucher, diploid chromosome number, genome size, and the signals of rDNA FISH experiments in *Phalaenopsis* species. The infrageneric treatment follows Cribb and Schuiteman (2012).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Voucher</th>
<th>2n</th>
<th>1C-value (pg)</th>
<th>Number of rDNA sites</th>
<th>Positions of rDNA sites</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5S rDNA</td>
<td>45S rDNA</td>
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<tr>
<td><strong>Subgenus Parishianae</strong></td>
<td></td>
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<tr>
<td><strong>Section Aphyllae</strong></td>
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<tr>
<td><em>P. honghenensis</em> F.Y.Liu</td>
<td>Yung-I Lee201381</td>
<td>36</td>
<td>5.92 ± 0.16a</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>P. lowii</em> Rchb.f.</td>
<td>Yung-I Lee201382</td>
<td>36</td>
<td>2.46 ± 0.08a</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>P. stobartiana</em> Rchb.f.</td>
<td>Yung-I Lee201383</td>
<td>36</td>
<td>4.25 ± 0.11a</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>P. taenialis</em> (Lindl.) Christenson &amp; Pradhan</td>
<td>Yung-I Lee201384</td>
<td>36</td>
<td>3.56 ± 0.12a</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>P. wilsonii</em> Rolfe</td>
<td>Yung-I Lee201385</td>
<td>36</td>
<td>4.38 ± 0.14b</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

| **Section Esmeraldae**  |                |    |               |  |         |       |         |
| *P. chibae* T.Yukawa   | Yung-I Lee201377 | 38 | 5.47 ± 0.19b  | 2  | 4       | distal   | distal  |
| *P. deliciosa* Rchb.f. | Yung-I Lee201378 | 38 | 4.37 ± 0.14b  | 2  | 2       | distal   | distal  |
| *P. finleyi* Christenson | Yung-I Lee201379 | 34 | 3.03 ± 0.11a  | 2  | 2       | distal   | distal  |
| *P. pulcherrima* (Lindl.) J.J.Sm. | Yung-I Lee201380 | 38 | 6.16 ± 0.18b  | 2  | 8       | proximal | distal  |

| **Section Parishianae** |                |    |               |  |         |       |         |
| *P. appendiculata* Carr | Yung-I Lee201386 | 38 | 3.43 ± 0.07a  | 4  | 4       | distal   | distal  |
| *P. gibbosa* H.R.Sweet | Yung-I Lee201387 | 38 | 5.16 ± 0.16a  | 2  | 6       | proximal | distal  |
| *P. lobbii* (Rchb.f.) Aver. | Yung-I Lee201388 | 38 | 8.74 ± 0.16b  | 2  | 8       | proximal | distal  |
| *P. parishii* Rchb.f.  | Yung-I Lee201389 | 38 | 7.36 ± 0.27b  | 2  | 8       | distal   | distal  |

| **Subgenus Phalaenopsis** |                |    |               |  |         |       |         |
| **Section Polychilos**    |                |    |               |  |         |       |         |
| *P. amboinensis* J.J.Sm. | Yung-I Lee201351 | 38 | 4.88 ± 0.21b  | 2  | 14      | proximal | distal  |
| *P. bastianii* O.Gruss & Roellke | Yung-I Lee201352 | 38 | 2.89 ± 0.14b  | 2  | 2       | proximal | distal  |
| *P. bellina* (Rchb.f.) Christenson | Yung-I Lee201353 | 38 | 6.52 ± 0.24b  | 4  | 8       | distal   | distal  |
| *P. cochlearis* Holttum | Yung-I Lee201354 | 38 | 2.08 ± 0.07b  | 2  | 2       | distal   | distal  |
| *P. corningiana* Rchb.f. | Yung-I Lee201355 | 38 | 3.99 ± 0.19b  | 2  | 8       | distal   | distal  |
| *P. cornu-cervi* Blume & Rchb.f. | Yung-I Lee201356 | 38 | 2.71 ± 0.13b  | 2  | 4       | distal   | distal  |
| *P. fasciata* Rchb.f.   | Yung-I Lee201357 | 38 | 2.89 ± 0.18b  | 2  | 2       | distal   | distal  |
| *P. fuscata* Rchb.f.    | Yung-I Lee201359 | 38 | 2.02 ± 0.10b  | 2  | 4       | distal   | distal  |
| *P. gigantea* J.J.Sm.   | Yung-I Lee201360 | 38 | 2.27 ± 0.12b  | 2  | 2       | distal   | distal  |
| *P. hieroglyphica* (Rchb.f.) H.R.Sweet | Yung-I Lee201361 | 38 | 2.67 ± 0.15b  | 2  | 2       | distal   | distal  |
| *P. lueddemanniana* Rchb.f. | Yung-I Lee201364 | 38 | 2.85 ± 0.15b  | 2  | 2       | distal   | distal  |
| *P. mannii* Rchb.f.     | Yung-I Lee201365 | 38 | 5.72 ± 0.14b  | 2  | 2       | distal   | distal  |
| *P. martiae* Burb.      | Yung-I Lee201366 | 38 | 2.87 ± 0.08b  | 2  | 2       | distal   | distal  |
| *P. micholitizii* Rolfe | Yung-I Lee201367 | 38 | 2.78 ± 0.08b  | 2  | 2       | distal   | distal  |
| *P. modesta* J.J.Sm.    | Yung-I Lee201358 | 38 | 2.27 ± 0.11b  | 2  | 2       | distal   | distal  |
| *P. pallens* Rchb.f.    | Yung-I Lee201369 | 38 | 2.99 ± 0.11b  | 2  | 2       | distal   | distal  |
| *P. pantherina* Rchb.f. | Yung-I Lee201370 | 38 | 2.87 ± 0.13b  | 2  | 2       | distal   | distal  |
| *P. pulchra* (Rchb.f.)  | Yung-I Lee201371 | 38 | 2.32 ± 0.17b  | 2  | 2       | distal   | distal  |

nylon mesh, treated with RNase A (Sigma Chemical Co.), and stained with propidium iodide (PI) (Sigma). Both PI and RNase A were adjusted to a final concentration of 50 μg ml⁻¹. Samples were kept on ice for 30 min and 10 000 nuclei were measured using a flow cytometer (Coulter EPICS Elite ESP, Beckman Coulter) equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. PI fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. Flow histograms were analysed with the FlowMax software (v. 2.4, Partec GmbH). Three individual samples were measured separately for each species and three replicates of each sample were processed. Chicken erythrocyte nuclei (2C = 3 pg) and *Pisum sativum* ‘Minerva Maple’ (2C = 9.56 pg) were used as internal standards.

**Phylogenetic analysis**

The nuclear ribosomal spacer regions, ITS1 and ITS2, and the 5.8S ribosomal gene (ITS) of *Phalaenopsis* spp. were obtained from the NCBI sequence database (National Center for Biotechnology Information, GenBank) for phylogenetic analysis by referring to Carlsward et al. (2006) and Tsai et al. (2006), and the sequence of *Amesiella philippinensis* (Ames) Garay was used as an outgroup taxon (Supporting Information, Table S1). DNA sequences were aligned using CLUSTALW (Thompson et al., 1997) and phylogenetic relationships were analysed using a model-based Bayesian approach using MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003). The 'best-fit' model HKY + I + Γ was selected under the Akaike information criterion test (Akaike, 1974) as implemented in MrModeltest 2.2 (Nylander, 2004). Two separate runs of four Markov chains Monte Carlo (MCMC; Yang & Rannala, 1997) were performed for 3 000 000 generations until the mean deviation of split frequency dropped below 0.01; a tree was sampled every 100th generation. Trees from the first 25% of generations were discarded using the ‘burn-in’ command and the remaining trees were used to calculate an all-compatible consensus topology and posterior probability (PP) values for individual branches. The convergence of each run was estimated using the program Tracer v1.4 (http://beast.bio.ed.ac.uk/Tracer) and the effective sample size values of > 300 were confirmed to have a sufficient level of sampling. The trees obtained in these analyses were drawn with the TreeGraph 2 software (Stöver & Müller, 2010).

### Ancestral genome size reconstruction

The genome size data of this study in combination with the previous investigations (Lin et al., 2001; Chen et al., 2013) were used to reconstruct the ancestral state for genome size in *Phalaenopsis*. The Bayesian method using the software BayesTraits v2 (Page & Meade, 2014) was applied to investigate evolution of genome size of *Phalaenopsis*. In brief, we modelled the genome size evolution of *Phalaenopsis* spp. across the phylogenetic tree inferred from MrBayes analysis using the MCMC method. Given the uncertainty of tree topology and the associated branch lengths, we randomly sampled 3000 trees from the post burn-in phase of MrBayes analysis that were used to guide the BayesTraits analyses (Pagel, Meade & Barker, 2004). We performed this tree-sampling procedure

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**Table 1.** Continued

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Section Phalaenopsis</strong></td>
<td></td>
<td></td>
<td></td>
<td>5S rDNA</td>
<td>45S rDNA</td>
</tr>
<tr>
<td><em>P. amabilis</em> Blume</td>
<td>Yung-I Lee201342 38</td>
<td>1.40 ± 0.06b</td>
<td>4 2</td>
<td>distal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. aphrodite</em> Rchb.f.</td>
<td>Yung-I Lee201343 38</td>
<td>1.40 ± 0.06b</td>
<td>4 2</td>
<td>distal, proximal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. celebrensis</em> H.R.Sweet</td>
<td>Yung-I Lee201344 38</td>
<td>1.77 ± 0.06b</td>
<td>2 2</td>
<td>distal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. equestris</em> (Schauer) Rchb.f.</td>
<td>Yung-I Lee201345 38</td>
<td>1.48 ± 0.06b</td>
<td>2 2</td>
<td>distal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. lindenii</em> Loker</td>
<td>Yung-I Lee201346 38</td>
<td>1.46 ± 0.05b</td>
<td>6 2</td>
<td>distal, proximal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. philippinensis</em> Galamco ex Fowlie &amp; C.Z.Tang</td>
<td>Yung-I Lee201347 38</td>
<td>1.38 ± 0.04b</td>
<td>6 2</td>
<td>distal, proximal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. sanderiana</em> Rchb.f.</td>
<td>Yung-I Lee201348 38</td>
<td>1.41 ± 0.06b</td>
<td>4 2</td>
<td>distal, proximal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. schilleriana</em> Rchb.f.</td>
<td>Yung-I Lee201349 38</td>
<td>1.39 ± 0.05b</td>
<td>6 2</td>
<td>distal, proximal</td>
<td>distal</td>
</tr>
<tr>
<td><em>P. stuartiana</em> Rchb.f.</td>
<td>Yung-I Lee201350 38</td>
<td>1.43 ± 0.06b</td>
<td>6 2</td>
<td>distal, proximal</td>
<td>distal</td>
</tr>
</tbody>
</table>

* Data measured in the present study. In this study, three individual samples were measured separately for each species, and three replicates of each sample were processed. Chicken erythrocyte nuclei (2C = 3 pg) and *Pisum sativum* ‘Minerva Maple’ (2C = 9.56 pg) were used as the internal standard.

b Data measured by Chen et al. (2013).
twice to obtain consistent results from the BayesTraits analyses. The root-to-tip branch lengths were central in BayesTraits analysis to inform tempo and mode of trait evolution (Pagel, 1999). This was undertaken with the drop.tip function of the ape package (Paradis, Claude & Strimmer, 2004) developed in R (R Core Team, 2015). In this study, we applied a log_{10} transformation to the genome size data of Phalaenopsis spp. to model the evolution of this trait in the manner of proportional changes (see Lysák et al., 2009). We first explored two BayesTraits models, corresponding to constant-variance random-walk processes of genome size evolution without (neutral-drift model) and with (directional drift model) a directional trend across the phylogenetic tree, respectively. The former model was specified with a parameter \( \alpha \) for the log_{10}-transformed 1C value at the root of Phalaenopsis spp. and the latter model was introduced with an additional parameter \( \beta \) for a putatively non-zero mean of the random-walk process. We set uniform priors to both \( \alpha \) and \( \beta \), with boundaries at (0, 2) and (−2, 2), respectively. Under each model, two independent MCMC runs were conducted, each run for 10 million iterations (1000 iterations per sample) with the first 10% discarded as burn-in. Traces of independent runs for MCMC convergence were examined using Tracer 1.6 (Rambaut et al., 2013). For model selection, we continued MCMC analyses described above with an additional 10 000 iterations of stepping-stone sampling procedures (Xie et al., 2011) containing 200 stones to acquire marginal likelihood estimates (MLEs) for the two models. The MLE values estimated were used to compute a Bayes factor (BF) (Kass & Raftery, 1995) for interpreting the strength of support for one model over the other. The statistic 2×log\( \text{BF} \) was computed and a value of > 2 was interpreted as positive evidence, supporting an alternative model over a null one.

In this study, the neutral-drift model was chosen from the model selection procedure. Using a null model, we explored three alternative models (each of which specified a specific manner of tree branch transformation). The first two alternative models allowed the investigation of how the tempo of genome size evolution varied across the tree, whereas the third tested the extent to which genome size evolution reflected the phylogenetic tree. Specifically, the first alternative model was introduced with a parameter \( \kappa \); values of \( \kappa > 1 \) and < 1 inflated and shrank the difference between long and short branches of a tree. The second alternative model was introduced with a parameter \( \delta \); values of \( \delta > 1 \) and < 1 stretched individual branches near the root and tips, respectively. The third alternative model was introduced with a parameter \( \lambda \) which had a range between zero and one, and a \( \lambda \) value closer to zero transformed the tree further towards a star phylogeny. We replicated model selection procedures as described above and compared the three alternative models where the relevant parameter was free to be estimated in turn with the null model.

**CHROMOSOME PREPARATION AND FISH**

Chromosome samples were prepared according to the method of Chung et al. (2008) with minor modifications. Young and healthy root tips were harvested and pretreated in 2 mM 8-hydroxyquinoline at 20 °C for 5 h, rinsed with distilled water, then fixed in freshly prepared Carnoy's solution (three parts ethanol to one part glacial acetic acid). Root tips were macerated with 6% cellulose (Onozuka R-10, Yakult Honsha) and 6% pectinase (Sigma) in 75 mM KCl, pH 4.0 at 37 °C for 90 min and then squashed on slides in the same fixative. Slides were air-dried and stored at −80 °C until required. The FISH procedure was essentially the same as that described by Lee, Chang & Chung (2011). The rDNA probes used in this study were: (1) pTA71 containing a repetitive unit of 45S rDNA (c. 9 kb) from Triticum aestivum L. (Gerlach & Bedbrook, 1979) and (2) pTA794 containing a 5S rDNA repeat unit (410 bp) from T. aestivum (Gerlach & Dyer, 1980). Both probes were labelled by nick translation with either digoxigenin-11-dUTP or biotin-16-dUTP (Roche Applied Science). The digoxigenin-labelled probes were detected by anti digoxigenin-rhodamine (Roche Applied Science). The biotin-labelled probes were detected by fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). Chromosomes were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vector Laboratories). All images were captured digitally by a CCD camera attached to an epifluorescence microscope (Axioskop 2, Carl Zeiss AG). The final image adjustments were made with Adobe Photoshop CS2 (version 9.0.2, Adobe Systems Incorporated). For each species, we examined three individual samples, and more than three root tips of each sample were processed. In the FISH experiments, we counted nearly 100 cells and repeated the experiment at least three times for each species.

**RESULTS**

**CHROMOSOME NUMBERS**

The results of the genome size and chromosome number analyses are presented in Table 1 and Figure 1. Most Phalaenopsis spp. showed a consistent chromosome number of \( 2n = 2x = 38 \), except for species in Phalaenopsis section Aphyllae (H.R.Sweet) Christenson and one species in section Esmeraldae Rchb.f. In section Aphyllae, P. honghenensis F.Y.Liu (Fig. 2A), P. lowii Rchb.f. (Fig. 2B), P. stobartiana

Figure 1. Ancestral genome size reconstruction of diploid *Phalaenopsis* spp. and evidence for genome expansion and contraction across the phylogenetic tree. The phylogenetic tree topology was generated from Bayesian inference using the nuclear internal transcribed spacer. The tree is rooted with *Amesiella philippinensis* and the values above branches indicate node support (posterior probabilities ≥ 0.95). Ancestral genome sizes for the most recent common ancestor (MRCA) of each major *Phalaenopsis* clade are shown. Closed circles indicate the haploid genome size (1C values in pg) for extant species; dashed lines indicate the ancestral genome sizes for the MRCA of subgenus *Parishianae* (red) and subgenus *Phalaenopsis* (blue) clades. For each *Phalaenopsis* sp., the increase/decrease in genome size relative to the MRCA of its clade is shown.
Figure 2. Location of 5S and 45S rDNA sites on somatic chromosomes of *Phalaenopsis* spp. determined by fluorescence *in situ* hybridization. (A) *P. honghenesis*, (B) *P. lowii*, (C) *P. stobartiana*, (D) *P. taenialis*, (E) *P. wilsonii*, (F) *P. chiba*, (G) *P. deliciosa*, (H) *P. finleyi*, (I) *P. pulcherrima*, (J) *P. appendiculata*, (K) *P. gibbosa*, (L) *P. lobbii*, (M) *P. parishii*, (N) *P. amboinensis* and (O) *P. bastianii*. Arrows indicate the 45S rDNA signals (green) and arrowheads indicate the 5S rDNA signals (red). Scale bars = 10 μm.
Rchb.f. (Fig. 2C), P. taenialis (Lindl.) Christenson & Pradhan (Fig. 2D) and P. wilsonii Rolfe (Fig. 2E) had 2n = 2x = 36 and P. finleyi Christenson (section Esmeraldae) had 2n = 2x = 34 (Fig. 2H). The karyotypes of most Phalaenopsis spp. were symmetrical and uniform in size. The exception to this was encountered in Phalaenopsis section Polychilos (Breda) Rchb.f., in which a lineage containing P. amboinensis J.J.Sm. (Fig. 2N), P. bellina (Rchb.f.) Christenson (Fig. 3A), P. corningiana Rchb.f. (Fig. 3C), P. sumatrana Korth. & Rchb.f. (Fig. 4B), P. tetraspis Rchb.f. (Fig. 4C), P. venosa Shim & Fowlie (Fig. 4D) and P. violacea Teij. & Binn. (Fig. 4E) possessed asymmetrical and bimodal karyotypes. In these species, larger chromosomes were usually metacentric or submetacentric, whereas smaller chromosomes were subtelocentric or acrocentric.

### Genome size

Combining the genome size data generated in this study with previous investigations (Lin et al., 2001; Chen et al., 2013) means that data are now available for 56 out of 63 species (c. 89%) in Phalaenopsis. This improved sampling relative to previous studies shows that genome size in Phalaenopsis ranges from 1C = 1.38 pg in P. philippinesis Golamco ex Fowlie & C.Z.Tang to 1C = 8.74 pg in P. lobbi (Rchb.f.) Aver. (Table 1). The genome sizes in subgenus Parishianae were relatively high (mean 1C value = 4.95 pg), whereas those for species belonging to subgenus Phalaenopsis were lower (mean 1C value = 2.88 pg). To verify whether the genome sizes increased or decreased independently in Phalaenopsis spp., we reconstructed the genome size for the most recent common ancestor (MRCA) of each clade. The results of our analysis indicated that the evolution of genome size in Phalaenopsis showed no directional trend and no heterogeneous tempos in different parts of the phylogenetic tree. The ancestral genome size for the MRCA of subgenus Parishianae was reconstructed as 3.92 pg/1C, whereas that for the MRCA of subgenus Phalaenopsis was reconstructed as 2.42 pg/1C (Fig. 1).

### Mapping 5S and 45S rDNA loci

The 5S and 45S rDNA sites are summarized in Table 1 and described in detail below.

### Subgenus Parishianae

The species of subgenus Parishianae were characterized by possessing two to eight 45S rDNA sites and two to four 5S rDNA sites per chromosome complement.

### Section Aphyllae

In P. honghenensis, four distal 45S rDNA sites were detected (Fig. 2A), whereas in the other species of section Aphyllae analysed, i.e. P. lowii (Fig. 2B), P. stobartiana (Fig. 2C), P. taenialis (Fig. 2D) and P. wilsonii (Fig. 2E), only two distal 45S rDNA sites were observed. All these species had two proximal 5S rDNA sites, except for P. lowii which had two distal 5S rDNA sites.

### Section Esmeraldae

P. deliciosa Rchb.f. (Fig. 2G) and P. finleyi (Fig. 2H) had two distal 45S rDNA sites and two distal 5S rDNA sites, P. chiba T.Yukawa (Fig. 2F) had four distal 45S rDNA sites and two distal 5S rDNA sites, and P. pulcherrima (Fig. 2I) had eight distal 45S rDNA sites and two proximal 5S rDNA sites.

### Subgenus Phalaenopsis

The species of subgenus Phalaenopsis were grouped into two sections and varied in the number of rDNA sites from two to 14 45S rDNA sites and two to six 5S rDNA sites per chromosome complement.

### Section Polychilos

Most species of section Polychilos showed constancy in 45S rDNA and 5S rDNA distributions, with two distal 45S rDNA and two distal 5S rDNA sites (Figs 2O, 3B, E, G–O, 4A, F). A few species, i.e. P. cornucervi Blume & Rchb.f. (Fig. 3D) and P. fuscata Rchb.f. (Fig. 3F), showed duplication of 45S rDNA loci, having four distal 45S rDNA sites. In section Polychilos, massive duplication of 45S rDNA loci could only be detected in the phylogenetic lineage containing P. amboinensis (Fig. 2N), P. bellina (Fig. 3A), P. corningiana (Fig. 3C), P. sumatrana (Fig. 4B), P. tetraspis (Fig. 4C), P. venosa (Fig. 4D) and P. violacea (Fig. 4E). These species had between eight and 14 distal 45S rDNA sites. The highest number of sites was observed in P. amboinensis, in which among the
Figure 3. Location of 5S and 45S rDNA sites on somatic chromosomes of Phalaenopsis spp. determined by fluorescence in situ hybridization. (A) *P. bellina*, (B) *P. cochlearis*, (C) *P. corningiana*, (D) *P. cornu-cervi*, (E) *P. fasciata*, (F) *P. fuscata*, (G) *P. gigantea*, (H) *P. hieroglyphica*, (I) *P. lueddemanniana*, (J) *P. mannii*, (K) *P. mariae*, (L) *P. micholitzii*, (M) *P. modesta*, (N) *P. pallens* and (O) *P. pantherina*. Arrows indicate the 45S rDNA signals (green), and arrowheads indicate the 5S rDNA signals (red). Scale bars = 10 μm.
Figure 4. Location of 5S and 45S rDNA sites on somatic chromosomes of *Phalaenopsis* spp. determined by fluorescence *in situ* hybridization. (A) *P. pulchra*, (B) *P. sumatrana*, (C) *P. tetraspis*, (D) *P. venosa*, (E) *P. violacea*, (F) *P. viridis*, (G) *P. amabilis*, (H) *P. aphrodite*, (I) *P. celebensis*, (J) *P. equestris*, (K) *P. lindenii*, (L) *P. philippinensis*, (M) *P. sanderiana*, (N) *P. schilleriana* and (O) *P. stuartiana*. Arrows indicate the 45S rDNA signals (green), and arrowheads indicate the 5S rDNA signals (red). Scale bars = 10 μm.
14 sites, four were detected at the two distal ends of one pair of chromosomes (Fig. 2N). In this section, most species showed two distal 5S rDNA sites, except for P. bellina with four distal sites (Fig. 3A), and P. amboinensis and P. venosa with two proximal sites (Figs 2N, 4D).

Section Phalaenopsis

Most species of section Phalaenopsis showed constancy in 45S rDNA distribution, with two distal 45S rDNA sites (Fig. 4G–O). In contrast, two to six 5S rDNA sites were detected, in which two distal sites were detected in the lineage containing P. celebensis H.R.Sweet (Fig. 4I), P. equestris (Schauer) Rchb.f. (Fig. 4J) and P. lindenii Loker (Fig. 4K); four sites were detected in the lineage containing P. amabilis Blume (four distal sites; Fig. 4G), P. aphrodite Loker (two distal sites and two proximal sites; Fig. 4H) and P. sanderiana Rchb.f. (four distal sites; Fig. 4M); four distal sites and two proximal sites were detected in the lineage containing P. philippinensis (Fig. 4L), P. schilleriana Rchb.f. (Fig. 4N) and P. stuartiana Rchb.f. (Fig. 4O).

DISCUSSION

Karyotypes

A consistent somatic chromosome number of 2n = 38 in Phalaenopsis and other groups of the Aeridineae has been reported in previous studies (Woodard, 1951; Storey, 1952; Sagawa, 1962; Shindo & Kamemoto, 1963; Storey, Kamemoto & Shindo, 1963; Kamemoto, 1965; Jones, 1967; Tara & Kamemoto, 1970; Arends & Van der Laan, 1986; Aoyama, 1993). Here we report a reduction of chromosome number (to 2n = 34 or 36) in section Aphyllae (P. honghenensis, P. lowii, P. stobartiana, P. taenialis and P. wilsonii) and in section Esmeraldae (P. finleyi). From the phylogenetic analysis (Fig. 1), it is clear that P. finleyi is closely related to section Aphyllae, and they form a monophyletic lineage sharing the character of chromosome number reduction. This agrees with the classification of Christenson (2001), who considered that P. finleyi should belong to section Aphyllae based on morphological features. Although Cribb & Schuiteman (2012) placed P. finleyi in section Esmeraldae, our cytological data suggest that section Aphyllae should include P. finleyi.

The karyomorphology in Phalaenopsis reflects the close phylogenetic relationships of those species that have undergone increases in genome size (Fig. 1). In subgenus Parishianae, the species with larger genome sizes, e.g. P. honghenensis (Fig. 2A), P. pulcherrima (Fig. 2I), P. gibbosa (Fig. 2K), P. lobbii (Fig. 2L) and P. parishii (Fig. 2M), possess symmetrical karyotypes.

Several terminal and intercalary DAPI-positive bands are evident on their large and symmetrical chromosomes. The increase in genome size by adding repetitive DNA more or less equally to all chromosomes of the complement has also been observed in Cypripedium L. (Leitch et al., 2009), Lathyrus L. (Narayan & Durrant, 1983), Nicotiana L. (Narayan, 1987) and Vicia L. (Raina, 1990). In contrast, in section Polychilos of subgenus Phalaenopsis, the lineage comprising species that have all undergone genome size increases, i.e. P. amboinensis (Fig. 2N), P. bellina (Fig. 3A), P. corningiana (Fig. 3C), P. sumatrana (Fig. 4B), P. tetraspis (Fig. 4C), P. venosa (Fig. 4D) and P. violacea (Fig. 4E), except for P. modesta, all possesses bimodal and asymmetrical karyotypes. In these species, large DAPI-positive bands are mainly located in the terminal regions of two large metaphasic chromosomes and/or the long arms of large subtelocentric chromosomes. Only a few or no bright DAPI-positive bands were observed on small chromosomes of these species. In Oxalis L. (de Azkue & Martinez, 1988), Cypella Klatt and Hesperoxiphion Baker (Kent, 1990), Paphiopedilum Pfitzer (Cox et al., 1998) and Cephalanthera Rich. (Moscone et al., 2007), complex chromosomal rearrangements, such as Robertsonian fusions and fissions, and/or unequal gains or losses of repetitive DNA to specific chromosomes and chromosome regions have been shown to contribute to the changes of chromosome size and karyotype symmetry.

Our study comprising a comprehensive sampling of taxa demonstrates that the amplification of repetitive DNA (e.g. constitutive heterochromatin) could be specific to chromosomes and/or chromosome regions in the different phylogenetic groups which have independently undergone genome size expansion, resulting in changes in chromosome size and symmetry, as also reported by Kao et al. (2001) in their karyological study of Phalaenopsis.

GENOME SIZE EVOLUTION IN PHALAENOPSIS

The present study has revealed that Phalaenopsis possesses a relative large range of genome sizes compared with many other genera in Orchidaceae (Cypripedium being an exception; Leitch et al., 2009), with maximum and minimum C-values differing 6.3-fold. The reconstruction of ancestral genome sizes inferred the genome size for the MRCA of subgenus Parishianae to be 3.92 pg/1C and for subgenus Phalaenopsis to be 2.42 pg/1C (Fig. 1) with decreases and/or increases in genome size inferred in the different lineages in each subgenus during diversification and speciation.

In subgenus Parishianae, the genome sizes of most species are higher than the reconstructed ancestral state, suggesting a general trend towards genome size expansion (Fig. 1). A feature of the growth habit of
many species in subgenus Parishianae is that they are deciduous during the harsh dry season. Nuclear DNA content has been shown to affect cellular and organismal characters (e.g. nuclear volume, cell size, cell cycle, generation time and growth rate) and thus can influence life form, development, phenology and the ecological performance of plant species, particularly in species with larger genomes (reviewed by Leitch & Bennett, 2007; Leitch & Leitch, 2012; Greilhuber & Leitch, 2013).

In subgenus Parishianae, speciation is predicted to have occurred mostly in the Himalayas and Indochina, whereas in subgenus Phalaenopsis it is more likely to have occurred in Malesia (Yukawa et al., 2005). These two regions differ significantly in their climate with Indochina being characterized by distinct dry and rainy seasons and Malesia having a more constant climate, with high precipitation throughout the year. Since genome size has been shown to be broadly correlated with guard cell size (Beaulieu et al., 2008), the species with larger genome sizes in subgenus Parishianae are predicted to have larger guard cells that may show a slower response to water stress compared with those species with smaller genome size and this may contribute to the reason why they shed leaves during the dry season. In relation to this, it is perhaps worth noting that P. pulcherrima (section Esmeraldae) is the only terrestrial Phalaenopsis spp. with an evergreen habit among the species with larger genome sizes in subgenus Parishianae. Compared to terrestrial species, the epiphytes are likely to be under considerable water stress and it has been suggested that orchid species with larger genome sizes may be restricted to a terrestrial rather than epiphytic habit (Leitch et al., 2009). In P. pulcherrima, a shift in life-style to a terrestrial habit could represent an adaptive strategy to enable continuous growth year-round in Indochina with its distinct dry/rainy seasons. Possessing evergreen leaves ensures the supply of carbohydrates year-round that would be advantageous for enabling species to grow quickly in the tropics. In savanna tree species, evergreen species showed greater leaf mass fraction and relative growth rate than deciduous species (Tomlinson et al., 2014).

In subgenus Phalaenopsis, the species with larger genome size were found in the P. amboinensis–P. bellina–P. venosa–P. violacea–P. corningiana–P. sumatrana–P. tetraspis lineage (except for P. modesta J.J.Sm.) and P. manni Rchb.f. in section Polychilos compared with the ancestral genome size reconstructed for the MRCA for the subgenus, indicating genome size expansion. Species of the P. amboinensis–P. bellina–P. venosa–P. violacea–P. corningiana–P. sumatrana–P. tetraspis lineage are restricted to individual Malesian islands (Andaman and Nicobar Islands, Borneo, Sumatra and Sulawesi). It has been suggested that species with larger genome sizes may have a more restricted ecological distribution (Knight & Ackerly, 2002; Knight & Beaulieu, 2008). However, species with smaller genome sizes also occur in these Malesian islands (Christenson, 2001). It is not clear whether the species with larger genome sizes occupy a specific ecological niche in these islands or whether other factors such as genetic drift are involved in driving genome expansion (Lynch & Conery, 2003; Whitney et al., 2010).

Apart from the genome increases noted above, the evolution of other Phalaenopsis spp. is predicted to have been accompanied by genome decreases (Fig. 1). This is particularly notable in section Phalaenopsis (subgenus Phalaenopsis), species of which have the smallest genome sizes so far reported and with little variation (1.38–1.77 pg/1C). In this lineage, the genome sizes of all species are lower than the reconstructed ancestral state (2.42 pg/1C), suggesting genome downsizing. Another example of this section is seen in subgenus Parishianae. Here, although most species have larger genomes (mean 1C value = 4.95 pg) than that of the MRCA (3.92 pg/1C), the genome sizes of a few species in this subgenus, i.e. P. appendiculata (section Parishianae), P. lowii (section Aphyllae), P. finleyi (section Aphyllae) and P. taeialis (section Aphyllae), are lower, indicating genome downsizing. In section Aphyllae, the reduction of genome size may be due in part to the loss of one or two pairs of chromosomes (e.g. P. finleyi 2n = 34, P. lowii 2n = 36). In angiosperms, genome size evolution has been shown to include both increases and decreases (Soltis et al., 2003), which are predominantly driven by changes in the abundance of repetitive DNA sequences, e.g. tandem repeats and transposable elements which are ubiquitous in plant genomes (Heslop-Harrison & Schmidt, 2012). The amplification of repeat families, typically retroelements, and the lack of DNA removal mechanisms may result in the genome size expansion in plants (Kelly et al., 2015; Leitch & Leitch, 2012), whereas DNA removal via recombination-based processes such as unequal homologous recombination and/or illegitimate recombination are considered to be important for genome downsizing (Bennetzen, Ma & Devos, 2005; Bennetzen & Wang, 2014). Since all Phalaenopsis spp. are considered to be diploid, variation in genome size is caused by changes in the amount of DNA per chromosome (chromosome size), without significantly altering the number of chromosomes per genome (polyploidization). Further studies are needed to compare the composition of repetitive DNA families in different lineages of Phalaenopsis.

**VARIATION IN NUMBERS AND CHROMOSOMAL LOCATIONS OF rDNA**

In Phalaenopsis, the number of SS and 45S rDNA sites can vary from two to six and two to 14, respectively (Table 1). In subgenus Parishianae, polymorphisms in
the number and location of 45S rDNA sites are most extensive in the species with larger genome sizes (e.g. *P. pulcherrima*, *P. gibbosa*, *P. lobbii* and *P. parishii*). In *P. lobbii* and *P. parishii*, it is particularly notable that one pair of chromosomes has four strong 45S rDNA sites at both distal ends (Fig. 2L, M). The strong FISH signals indicate high copy numbers of the 45S rDNA repeats, and their presence at both chromosome ends could serve as excellent markers for chromosome identification in these species. Furthermore, in section *Polychilos*, duplication of 45S rDNA sites also occurs in the lineage with large genome sizes and bimodal karyotypes, i.e. *P. amboinensis* (Fig. 2N), *P. bellina* (Fig. 3A), *P. venosa* (Fig. 4D), *P. violacea* (Fig. 4E), *P. corningiana* (Fig. 3C), *P. sumatranana* (Fig. 4B) and *P. tetraspis* (Fig. 4C). In this lineage, the multiple 45S rDNA sites are only detected at the distal end of small chromosomes that have little or no constitutive heterochromatin. The increased genome size associated with a larger cell volume may require higher rDNA transcriptional activity to maintain optimal cellular metabolisms through the amplification of rDNA repeats or by up-regulating transcription of individual rDNA copies (Prokopowich, Gregory & Crease, 2003). However, the transcriptional activity of rDNA genes may be regulated epigenetically by dosage effect, such that only a subset of rDNA genes are functional. It is also possible that some rDNA loci are non-functional because of the insertion of transposable elements (Weber et al., 2013). In contrast to the above pattern, *P. manii*, also in section *Polychilos*, has a larger genome size but without a bimodal karyotype, and with only two distal 45S rDNA sites (Fig. 3J). Thus, duplication of 45S rDNA sites may not necessarily accompany genome expansion or perhaps elimination of 45S rDNA sites occurred during species divergence. Together, these examples illustrate how the evolutionary events associated with rDNA array mobility have occurred independently in the different lineages of section *Polychilos*.

Variations in the number, position and size of rDNA sites between related species have been attributed to chromosome rearrangements, such as translocations, inversions, duplications, deletions and transpositional events (Schubert & Wobus, 1985; Raskina et al., 2004, 2008; Altinkut et al., 2006). These processes may act alone or in combination to generate novel rDNA loci, together with *in situ* amplification of pre-existing minor rDNA arrays via unequal crossing over or retrotransposition that can also lead to the formation of chromosome landmarks detectable by FISH (Hall & Parker, 1995; Datson & Murray, 2006; Eickbush & Eickbush, 2007; Lan & Albert, 2011).

An analysis of the number of 5S rDNA sites in section *Phalaenopsis* showed they reflected phylogenetic relationships (Table 1; Fig. 1). Although some species in this section have only two 5S rDNA sites, the *P. amabilis–aphrodite–sanderiana* lineage has four 5S rDNA sites (Fig. 4G, H, M) and the *P. philippinensis–P. schilleriana–P. stuartiana* lineage has six 5S rDNA sites (Fig. 4L, N, O). Nevertheless, overall the number and distribution of 5S rDNA loci detected by FISH in *Phalaenopsis* are generally less polymorphic than the 45S rDNA loci, a pattern also observed across many plant species studied with the exception of a few monocots, i.e. *Alstroemeria* L. (Baeza, Scharer & Budahn, 2007), *Iris* L. (Martinez et al., 2010), *Paphiopedilum* (Lan & Albert, 2011) and *Tulipa* L. (Mizuochi, Marasek & Okazaki, 2007). In *Paphiopedilum*, 5S rDNA sites were shown to be hemizygous, suggesting that gene conversion via double-strand break repair events may be involved in the diversification of 5S rDNA distribution in that genus (Lan & Albert, 2011). Nevertheless, in section *Phalaenopsis*, polymorphic 5S rDNA sites did not display hemizygosity, suggesting that the increased number of 5S rDNA sites in some species is more likely to be caused by transpositional events.

In conclusion, our results demonstrate variations in genome size, karyotype symmetry and rDNA sites in *Phalaenopsis* lineages, suggesting that evolutionary events leading to genome expansion and downsizing have occurred independently in the different lineages. Increases in genome size have been accompanied either by equal or by unequal additions of repetitive DNA to chromosomes and this has resulted in changes in chromosome size and karyotype symmetry. The diversification of rDNA loci, in terms of their number and distribution patterns, suggests that the occurrence of chromosomal rearrangements, the amplification of DNA sequences and their transposition have contributed to the mobility of rDNA loci in different *Phalaenopsis* lineages.

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**REFERENCES**


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. GenBank accession numbers of nrITS sequences obtained from the NCBI sequence database (National Center for Biotechnology Information, GenBank) for phylogenetic analysis in this study.