Rate heterogeneity in six protein-coding genes from the holoparasite
Balanophora (Balanophoraceae) and other taxa of Santalales

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INTRODUCTION

Rates of nucleotide substitution are known to vary among species and genes, and the factors affecting such variation include population size, life history, metabolic rate and DNA repair efficiency (Britten, 1986; Nickrent and Starr, 1994; Gaut et al., 1996; Laroche et al., 1997; Ohta, 2000; Smith and Donoghue, 2008). Several studies have demonstrated that the substitution rates in nuclear small-subunit ribosomal DNA (18S rDNA) for many holoparasitic and mycoheterotrophic plants are usually high compared with other angiosperms (Nickrent and Starr, 1994; Lemaire et al., 2010). Such rate accelerations are found in the other two intracellular genomes of many holoparasites, including plastid 16S rDNA (Nickrent et al., 1997) and rps2 (dePamphilis et al., 1997) and mitochondrial small-subunit rDNA (Duff and Nickrent, 1997) and matR (Barkman et al., 2004). Among the cases with accelerated substitution rates, the holoparasitic plant Balanophora has one of the highest substitution rates in 18S rDNA among angiosperms (Nickrent and Starr, 1994; Nickrent et al., 2005).

Balanophora (Balanophoraceae) is a root parasite with unisexual flowers, the plants being either dioecious or monocious. The floral organs of Balanophora are highly modified and reduced (Fig. 1A–D). The male flower (Fig. 1D) of Balanophora is composed of a single tepal whorl surrounding the synandrium formed by fused anthers. The female flower (Fig. 1C) completely lacks a perianth and consists only of a single pistil, and the female flowers (pistils) are clustered around numerous club-shaped structures called spadicles (Hansen, 1972) or claviform bodies (Eberwein et al., 2009).

Based on phylogenetic studies using nuclear 18S rDNA and mitochondrial matR regions, the family Balanophoraceae is allied with Santalales (Nickrent et al., 2005; Barkman et al., 2007) and is included in that order in the Angiosperm Phylogeny Group classification (APG III, 2009), but its exact position remains unresolved. Considering that the extreme rate acceleration of 18S rDNA in Balanophora could impose problems for the inference of phylogenetic relationships (see Nickrent et al., 2005), additional gene markers with less rate heterogeneity would be useful in further clarifying phylogenetic relationships of Balanophoraceae. The MADS-box genes

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that have conserved functions for floral homeotic patterning in angiosperms are good candidates for consideration.

The floral homeotic genes for floral patterning under the current ABCDE model have been extensively studied in the two model species, Arabidopsis thaliana and Antirrhinum majus (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Theissen and Saedler, 2001). According to the model, five major classes of floral homeotic genes work in different combinations to specify the identities of sepals, petals, stamens and carpels. Among these, B-class genes are known to be responsible for specifying the identities of petals and stamens. Two major duplication events occurred during the evolution of B-class genes in seed plants (Kramer et al., 1998). The first duplication event was before the origin of the angiosperms and this gave rise to the PISTILLATA/GLOBOSA (PI/GLO) and APELATA/DEFICIENS (AP3/DEF) gene lineages (Kramer et al., 1998; Kramer and Irish, 2000; Theissen et al., 2000). The second major duplication event was in the AP3 lineage after the divergence of Trochodendraceae in extant core eudicots, giving rise to the euAP3 and TM6 gene sub-lineages (Kramer et al., 2006).

A gene duplication event also occurred in the evolution of RNA polymerase II (RPB2) gene which is responsible for transcription. A major duplication occurred shortly before the core eudicot divergence that leads to the RPB2-i and RPB2-d gene lineages (Oxelman et al., 2004; Luo et al., 2007). Because the i copies have been lost several times during the diversification of core eudicots, some lineages may only retain the d copies such as Arceuthobium (Santalaceae s.l. or Viscaceae) of Santalales and Liquidambar (Altingiaceae) of Saxifragales (Luo et al., 2007).

Compared with plastid and mitochondrial genes, fewer studies exist that have analysed evolutionary rates of nuclear protein-coding genes in parasitic plants. Extreme rate acceleration of 18S rDNA has been found in many heterotrophic taxa including Rafflesia keithii (Rafflesiaceae) and Balanophora spp. (Balanophoraceae), but not all holoparasitic plants, however, display such rate accelerations (Nickrent and Starr, 1994; Nickrent et al., 1998; Young and dePamphilis, 2005; Lemaire et al., 2010). One hypothesis to be tested for the holoparasitic Balanophora is to see if it is a general feature that all genes in the genome evolve more quickly than those from hemiparastic relatives and autotrophic core eudicots.

The highly reduced and modified floral morphology of Balanophora provides an opportunity to examine the conservation and divergence of B-class genes. The B-class genes are expected to show a certain degree of conservation due to the functional constraint. Similarly for the LEAFY homologues, a single-copy gene is responsible for the initiation and patterning of flowers (Blázquez et al., 1997; Moyroud et al., 2009) and RPB2 homologues from Balanophora and other Santalales species. Comparison of the substitution rates of the B-class genes and other nuclear markers could help elucidate whether the rate acceleration is a common feature in the nuclear genome of Balanophora, compared with other santalalean relatives and non-parasitic core eudicots. Moreover, we examined the expression patterns of floral B-class genes in B. laxiflora to determine whether there are underlying genetic patterns that correlate with the unusual floral phenotypes.

MATERIALS AND METHODS

Taxonomic sampling

Eleven members of Santalales representing eight (or six if Santalaceae s.l. is used) families were used in this study: Loranthus kaoi and L. delavayi (Loranthaceae), Chamepereia manillana (Opiilaceae), Thesium chinense (Santalaceae s.l. or Thesiaceae), Santalum album (Santalaceae s.s.), Viscum articulatum and V. aliformosanae (Santalaceae s.l. or Viscaceae), Olax imbricata Roxb. (Olacaceae), Schoepfia jasminodora (Schoepfiaceae) and Balanophora laxiflora and B. fungosa (Balanophoraceae). All plant materials were collected in Taiwan.

Gene sequence determination

The 18S rDNA and mt matR gene sequences were amplified by direct PCR of genomic DNA from the above taxa. Total genomic DNAs were extracted according to standard CTAB extraction methods (Doyle and Doyle, 1987) from fresh leaves or flowers. The PCR conditions and primers for
amplification and sequencing of 18S rDNA followed Nickrent (1994) and the primers used for the matR region are listed in Supplementary Data Table S1.

For LFY, RPB2 and B-class MADS-box genes (PI, euAP3, TM6), coding sequences were determined by analysis of reverse transcriptase PCR (RT-PCR) products generated from mRNA. Total RNA was prepared from young inflorescences or flowers using either Concert Plant reagent (Invitrogen, Carlsbad, CA, USA) or a method developed for Pinus (Chang et al., 1993). The cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Degenerate primers for initial amplification of B-class, LFY and RPB2 gene homologues were based on previous studies (Frohlich and Meyerowitz, 1997; Oxelman et al., 2004; Stellari et al., 2004). PCR reactions were performed with 1 μL of Advantage 2 polymerase mix (Clontech, Palo Alto, CA, USA), 5 μL 10× Advantage 2 PCR buffer, 200 μM of each dNTP, 200 μM of each primer and 1 μL cDNA template in a final volume of 50 μL for the reaction solution. The PCR amplification programme was as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 50 s and a final extension at 72°C for 3 min. The PCR products were then cloned into the pGEM-T Easy Vector system (Promega, Madison, WI, USA) and 30–50 clones were sequenced for each taxon. Complete cDNA sequences of B-class gene homologues of *B. laxiflora* were obtained using 5′RACE kit (Invitrogen) and new gene specific primers were then designed for RT-PCR analysis; these primers are listed in Supplementary Data Table S1.

To determine the gene structure of *Balanophora* B-class genes (BalAP3, BalTM6, BafAP3 and BafTM6), PCR was performed with genomic DNA of *B. laxiflora* and *B. fungosa*. Gene specific primers were designed (see Supplementary Data Table S1) and the PCR amplifications were carried out using Blend Tag (Toyobo, Osaka, Japan) following the manufacturer’s instructions. Intron position was determined by aligning the genomic fragments with the corresponding cDNA sequences.

**Phylogenetic analyses**

Phylogenetic analyses were conducted with Bayesian inference (BI) using MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) and with maximum likelihood using Genetic Algorithm for Rapid Likelihood Inference (GARLI) version 0.951 (Zwickl, 2006). The model of DNA substitution for later incorporation was estimated using Modeltest 3-06 (Posada and Buckley, 2004), and the GTR + I + G model was selected as the fittest for the 18S rDNA, PI, AP3/TM6 and RPB2 data-sets according to the Akaike information criterion. For the mt matR dataset, the fittest TIM + G model suggested by Modeltest is not implemented in MRBAYES, and therefore we chose the closely related GTR + G model instead. Sequence(s) of *Akebia* (Lardizabalaceae) was chosen as out-group in all analyses [following Shan et al. (2006) and Jaramillo and Kramer (2007)]. Trees were sampled every 500 generations from 2.5 million generations of Markov chain Monte Carlo searches in BI analysis. The first 500 trees were discarded and the remaining trees were used to calculate posterior probabilities. Maximum likelihood bootstrap percentages (MLBP) (Felsenstein, 1985) were evaluated from 200 iterations using GARLI version 0.951 (Zwickl, 2006).

**Branch length estimates and rate heterogeneity test**

Branch lengths representing the number of non-synonymous (dN) and synonymous (dS) substitutions per site were estimated for protein-coding genes in our study using HyPhy package (Pond et al., 2005). The Muse-Gaut (MG94) model of codon substitution was adopted to estimate dN and dS rates independently in each lineage. Likelihood ratio tests (Yang, 1998) were implemented to determine the significance of rate heterogeneity for dN and dS of protein-coding genes among holoparasitic *Balanophora*, hemiparasitic Santalales and autotrophic core eudicots. Both *B. laxiflora* and *B. fungosa* sequences were included to represent ‘Balanophora’ in the analysis. Each of the tests contained two hypotheses for the categories compared: (1) that they share the same rate parameters (null hypothesis) or (2) that they have different parameters (alternate hypothesis). If the likelihood log estimate of alternative hypothesis is significantly higher than that of the null hypothesis (*P* < 0.05), it is assumed that the two categories have significantly different evolutionary rates. Rate correlations between dN and dS were analysed using Spearman’s rank correlation test under the statistical program R (R Development Core Team, 2008).

**Gene expression analysis**

Different fresh floral tissues of *B. laxiflora* were dissected and frozen in liquid nitrogen for gene expression assays. Tepals, synandria and pollen were collected from open male flowers, and floral bracts were collected from floral buds and mature flowers. The bracts that occur basally on the inflorescence and parts of the inflorescence axis were collected from young male and female inflorescences. Female flowers and spadicles were prepared in a mixture since they are difficult to separate. Total RNA was extracted from the dissected tissues separately and was reverse transcribed as described above. Gene-specific primers of *B. laxiflora* used in this study are listed in Supplementary Data Table S1. Fifty nanograms of RNA was used in each PCR reaction and the amplification was performed with 94°C for 2 min, followed by 34 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 50 s and a final extension at 72°C for 3 min using Platinum Taq DNA Polymerase (Invitrogen). The amplified fragments were further confirmed by direct sequencing.

**Scanning electron microscopy**

Male inflorescences of *B. laxiflora* were cut into small pieces and preserved in 70% ethanol and then dehydrated in an ethanol series to 100% ethanol. The samples were then critical-point dried, mounted onto pin stubs, coated with gold and examined using a Hitachi S-520 scanning electron microscope.
RESULTS

Sequence data

In this study we identified 25 B-class, two LFY, nine 18S rDNA, nine RPB2 (d-copy; Supplementary Data Fig. S1) and nine mitochondrial matR gene homologues from two Balanophora and nine hemiparasitic species (Supplementary Data Table S2). All these taxa have one PI-like gene and one TM6-like gene except for V. articulatum, which has two PI homologues and two TM6 homologues. However, only two euAP3 genes were obtained in our screening for Balanophoraceae and other Santalales, i.e. BalAP3 from B. laxiflora and BafAP3 from B. fungosa. All the B-class gene homologues obtained display the conserved amino acid domains M, I, K and C and the typical motifs of the C-terminal region for PI/AP3 subgroups (Kramer et al., 1998; see Supplementary Data Fig. S2).

Genomic DNA sequences of BalAP3 and BafAP3 were determined by direct PCR of the genomic DNA using several pairs of primers (see Supplementary Data Table S1) amplifying partial genomic sequences. All exon–intron structure and sequences can be identified, except for intron 4 sequences in all four B-class genes of Balanophora, because we could not obtain a PCR product using flanking primers of intron 4. After comparing cDNA sequences with other known euAP3 homologues, an additional 39-bp new exon was found at the C-domain region (which we denote as 6a) between the ordinary exons 6 and 7 of B-class genes in both B. laxiflora and B. fungosa (Supplementary Data Fig. S3). An approx. 0.5-kb region between exons 2 and 3 of LFY and an approx. 1.7-kb region between exons 12 and 24 of RPB2 genes were amplified. The two LFY homologues from Balanophora and the nine RPB2 homologues from Balanophora and other Santalales are quite conserved and can be easily aligned to other eudicot homologues. All of the sequences obtained and gene accessions used in the phylogenetic analyses are provided in Supplementary Data Table S2.

Molecular phylogenetics

The BI phylogenetic trees resulting from analysis of 18S rDNA, mt matR, PI, AP3 and RPB2 sequences are shown in Fig. 2. These are generally congruent with the relationships of angiosperm phylogenies inferred from previously published data based on multiple plastid, mitochondrial and nuclear rDNA gene datasets (Solís et al., 1999; Solís et al., 2000). In general, the PI and AP3 trees received higher nodal support compared with the 18S rDNA and mt matR trees. All of the trees consistently showed that Santalales including Balanophoraceae formed a monophyletic group, which received high BI support (1.0 on the PI, AP3 and RPB2 tree) and low to moderate maximum likelihood support (MLBP = 66% on RPB2, 55% on PI, 87% on the AP3 tree) in RPB2 and B-class gene trees (Fig. 2C–E), and still lower for 18S rDNA (BI = 0.87, MLBP < 50%) and mt matR (BI = 0.89, MLBP < 50%) gene trees (Fig. 2A, B). In contrast, the relationships of Balanophora to other members of Santalales is not resolved in any of the five gene trees. With 18S rDNA, matR and RPB2, Balanophora is related to Olax, Schoepfia and/or Loranthus (Fig. 2A–C). With PI (Fig. 2D), Balanophora is sister to Olax and this clade is sister to the remaining Santalales, whereas with AP3/TM6 it is sister to all other Santalales except Olax. The relationships of the other santalalean taxa also differed among the four gene trees. Some of the nodes received relatively high support (BI > 0.95, MLBP > 90%, marked as black dots on Fig. 2), but none of those nodes in Santalales showed incongruent phylogenetic relationships among the four gene trees.

Rates of molecular evolution

Extensive rate variation was detected among the six Balanophora gene sequences (18S rDNA, matR, LFY, PI, AP3 and RPB2) (Fig. 3). Balanophora 18S rDNA showed an extraordinarily high substitution rate compared with hemiparasitic Santalales and non-parasitic species. Long branches for Balanophora sequences were also detected on mitochondrial matR and some of the other gene trees constructed from sites with synonymous substitutions. Therefore we performed likelihood ratio tests to examine rate heterogeneity in non-synonymous (dN) and synonymous (dS) substitutions for the three B-class genes (PI, euAP3, TM6), as well as LFY, RPB2 and mt matR, among holoparasitic Balanophora, hemiparasitic Santalales and autotrophic core eudicots.

The matR, PI, TM6 and RPB2 sequences of other Santalales showed significantly lower rates of dN and dS in all the comparisons to Balanophora or other core eudicots (Table 1). In contrast, Balanophora sequences generally showed an increase in dN or dS; however, only Balanophora matR showed significant rate accelerations (>10-fold). On the other hand, the B-class genes and LFY of Balanophora showed varying substitution rates compared with the core eudicots. Among these genes, significant rate accelerations were only found in dS of Balanophora PI, TM6 and RPB2 genes, and dN of Balanophora euAP3. In other comparisons, Balanophora B-class gene sequences have only slight increases in substitution rates. It is worth noting that the dN and dS values of Balanophora B-class and RPB2 genes are all less than two times higher than the core eudicot sequences, far less than seen with mt matR. Furthermore, Balanophora LFY sequences not only did not show a significant rate increase in dS, they actually showed significantly lower dS values compared with the core eudicots (0.290 vs. 0.732, Table 1). The analyses showed that dN and dS are significantly correlated within euAP3, TM6, LFY, RPB2 and matR among the groups, but the rates are poorly correlated within PI sequences.

Expression in the six protein-coding genes of Balanophora laxiflora

To find out whether the six protein-coding genes identified in B. laxiflora are actually expressed, RT-PCR was carried out using gene-specific primers on RNA samples. The results showed that all six protein-coding gene products were present in inflorescences (Fig. 4). The three B-class homoelogous genes displayed differential expression patterns in different parts including both reproductive and vegetative organs (Fig. 4B). BalAP3 is expressed at high levels in male and female inflorescences, and the expression can be detected in all parts examined. Similarly, BalTM6 is also expressed in
Fig. 2. Phylogenetic relationships of Santalales and Balanophora spp. inferred from Bayesian analysis (BI) of 18S rDNA, matR, RPB2, PI and AP3 gene sequences. Numbers above the branches are Bayesian posterior probabilities, and values below are maximum likelihood branch support determined by GARLI. Mitochondrial matR trees are based on a 1968 nucleotide alignment, and nuclear gene trees are based on 1739 (18S rDNA), 765 (PI), 864 (AP3), 549 (LFY), 1719 (RPB2) nucleotide alignments, respectively. See Supplementary Data Fig. S1 for RPB2 gene tree showing the obtained RPB2 sequences belonging to the d clade.
FIG. 3. Rate divergence of nuclear and mitochondrial genes in Balanophora. BI trees based on \( d_N \) and \( d_S \) for protein-coding genes or rDNA gene. All protein-coding gene trees are constrained to the same scale, whereas the rDNA tree is on a different scale (see notes in figure).
male and female inflorescences, but the expression is low in the pollen and floral bracts compared with BalAP3. In contrast, BalPI is not expressed in the female inflorescence, and it is mainly restricted to the tepals, synandria and the floral bracts of male inflorescences.

Surface morphology of bracts and tepals in Balanophora laxiflora

Surface morphology was examined in tepals, floral bracts and basal bracts in B. laxiflora. There are up to 14 bracts (sometimes referred to as ‘leaves’) at the bases of the male and female inflorescences of B. laxiflora (Fig. 1A, B). Each of the male flowers has two small subtending floral bracts (Fig. 1B, D). The epidermal cells of these floral bracts are oval in shape and the surface is papillate covered with short ridges (Fig. 5A). Although the basal bracts are showy, their epidermal cells are flattened and elongated with smooth surfaces on both adaxial and abaxial sides (Fig. 5E, F). In contrast, the epidermal cells of mature tepals are oval in shape and their surface is smooth on the adaxial side (Fig. 5D). They also show irregular cuticular sculpturing on their abaxial sides (Fig. 5B, C), similar to what was observed on the floral bracts (Fig. 5A). No stomata were observed on either side of the tepals, floral bracts or basal bracts.

### Table 1: The estimated dN, dS values and P-values of the likelihood ratio tests between taxa

|          | mt matR | PI | TM6 | AP3 | LFY | RPB2 |
|----------|---------|----|-----|-----|-----|------|
| dN values |         |    |     |     |     |      |
| Core eudicots | 0.015   | 0.078 | 0.072 | 0.083 | 0.037 | 0.009 |
| Balanophora  | 0.134   | 0.091 | 0.088 | 0.118 | 0.039 | 0.010 |
| Other Santalales* | 0.011   | 0.059 | 0.056 | – | – | 0.005 |
| P-values of dN comparison | <0.01 | <0.001 | <0.001 | – | – | <0.001 |
| Core eudicots vs. other Santalales* | <0.01 | <0.001 | <0.001 | – | – | <0.001 |
| Balanophora vs. other Santalales* | <0.001 | <0.001 | <0.001 | – | – | <0.001 |
| Balanophora vs. core eudicots | <0.001 | 0.14 (n.s.) | 0.05 (n.s.) | <0.001 | 0.85 (n.s.) | 0.61 (n.s.) |
| dS values |         |    |     |     |     |      |
| Core eudicots | 0.021   | 0.378 | 0.315 | 0.369 | 0.732 | 0.293 |
| Balanophora  | 0.220   | 0.563 | 0.560 | 0.450 | 0.290 | 0.572 |
| Other Santalales* | 0.013   | 0.237 | 0.308 | – | – | 0.266 |
| P-values of dS comparison | <0.01 | <0.001 | <0.001 | – | – | <0.001 |
| Core eudicots vs. other Santalales* | <0.001 | <0.001 | <0.001 | – | – | <0.001 |
| Balanophora vs. other Santalales* | <0.001 | <0.001 | <0.001 | – | – | <0.001 |
| Balanophora vs. core eudicots | <0.001 | <0.001 | <0.001 | 0.10 (n.s.) | <0.001 | <0.001 |
| dN/dS |         |    |     |     |     |      |
| Core eudicots | 0.714   | 0.206 | 0.228 | 0.225 | 0.050 | 0.030 |
| Balanophora  | 0.609   | 0.161 | 0.157 | 0.262 | 0.135 | 0.017 |
| Other Santalales* | 0.846   | 0.248 | 0.181 | – | – | 0.018 |
| Correlation coefficient of dN and dS | 0.881 | –0.272 | 0.739 | 0.653 | 0.614 | 0.672 |
| P-values of dN and dS correlation | <0.01 | <0.06 (n.s.) | <0.01 | <0.01 | <0.01 | <0.01 |

n.s., not significant; –, data not available.
* Santalales here includes hemiparasitic taxa and excludes Balanophoraceae.
DISCUSSION

Rate variation in Balanophora species and other Santalales

It has previously been shown that Balanophora and other taxa of Balanophoraceae have significantly increased rates of nucleotide substitution in the 18S rDNA (Nickrent et al., 2005) and matR (Barkman et al., 2007) genes. In this study, we demonstrated that extreme rate acceleration is not ubiquitous in the Balanophora genome. The six B-class genes of Balanophora identified, three each from B. laxiflora and B. fungosa, show more modest nucleotide substitution rates as compared with rates for 18S rDNA and matR. Non-synonymous (d_N) and synonymous (d_S) substitutions per site were both estimated for protein-coding genes. In general, a lower d_N : d_S ratio indicates a conserved sequence or an increased rate of substitutions at silent sites, whereas a higher d_N : d_S ratio indicates a relaxation of purifying selection. Therefore the d_S value is an indication of non-selective substitution rate, i.e. similar to the background substitution of a genome (Yang, 1998). In our results, both PL and TM6 display significant d_S rate accelerations compared with d_N rate, whereas the euAP3 paralogues are significantly elevated in d_N rate (but not d_S rate), suggesting that Balanophora euAP3 has evolved under a relaxed purifying selection, but they do not show congruent patterns among B-class genes.

In general, positive correlations between d_N and d_S are found in nuclear genes of mammals, prokaryotes and Drosophila (Graur, 1985; Li et al., 1985; Wolfe and Sharp, 1993; Akashi, 1994). In plants, d_N and d_S of the nuclear paralogues of Arabidopsis thaliana (Zhang et al., 2002) and the plastid genes of Geraniaceae (Guisinger et al., 2008) are also reported to be positively correlated. Our result also found significant correlations between d_N and d_S in the four protein-coding genes (except for PL, see Table 1), suggesting that the intensity of selective constraints on both d_N and d_S are similar in these gene regions. The reason for the incongruence of PL data requires further investigation.

Although the rates of sequence evolution detected from d_N and d_S in Balanophora are generally higher than those of other plants, the d_N : d_S ratios of the six protein-coding genes examined for the three groups (Balanophora, hemiparasitic Santalales and core eudicots) are all <0.3, with the exception of mitochondrial matR (Table 1), suggesting the B-class, LFY and RPB2 genes are being subjected to strong purifying selection. However, functional constraints alone cannot explain the rate heterogeneity in Balanophora since rDNA should be also under strong selection against nucleotide changes. The 18S rDNA of Balanophora has at least a 5-fold higher substitution rate compared with other flowering plants and it is even more extreme (10-fold higher) for its matR gene.

Unusual acceleration of substitution rate in genes or genomes of certain species or lineages have been documented in several plants, mostly for mitochondria and plastids. Mitochondrial-specific increases in substitution rate were found in some Plantago (Cho et al., 2004), Pelargonium hortorum (Parkinson et al., 2005) and several Silene spp. (Sloan...
et al., 2009). The nuclear and plastid genomes of these taxa are largely unaffected and possess typical substitution rates. On the other hand, plastid genes in parasitic plants sometimes show rate variations due to different degrees of selective constraints on photosynthetic genes. For example, the synonymous rate of *Cascuta obtusiflora* plastid genes is five to eight times higher than the rates in *C. exaltata* because of a higher functional constraint for photosynthesis in the former (McNeal et al., 2007). Such rate increases are usually found to be lineage-specific and probably affecting the whole plastid genome, as in the case of Orobanchaceae (Young and dePamphilis, 2005).

The pattern of rate variation in 18S rDNA is more complicated since there is no clear relationship between elevated substitution rates and functional constraints. Although the extreme rate acceleration cases of 18S rDNA are all found in heterotrophic taxa such as *Rafflesia keithii* (Rafflesiacaeae) and *Balanophora* spp., not all holoparasitic plants display such rate accelerations (Nickrent and Starr, 1994; Nickrent et al., 1998; Young and dePamphilis, 2005; Lemaire et al., 2010). *Cynomorium coccineum* (Cynomoriiaceae) and *Orobanchaceae* show no significantly accelerated substitution rates in 18S rDNA (dePamphilis et al., 1997; Wolfe and dePamphilis, 1997; Lemaire et al., 2010). Similarly, in mycoheterotrophic plants, rate accelerations in 18S rDNA can be found in *Rhizanthella gardneri* (Orchidaceae) and *Thissmia aseroe* (Thissmiaceae); others like *Monotropa uniflora* (Ericaceae) and *Corallorhiza maculata* (Orchidaceae) lack such rate increases (Lemaire et al., 2010).

It was suggested that the functionality of 18S rDNA with accelerated substitution rates is retained in those heterotrophic plants, given the pattern of compensatory mutations, and that few mutations occur in the functionally and structurally important regions of the ribosomal molecule (Nickrent and Starr, 1994; dePamphilis, 1995; Lemaire et al., 2010). Therefore the increased substitution rate in 18S rDNA might reflect an overall elevated mutation rate in the nuclear genome in these plants. Several possible causes of the elevated substitution rates have been proposed, such as defective DNA repair efficiency, rapid generational time, higher speciation rates and small effective population size (Nickrent and Starr, 1994; Nickrent et al., 1998; Lemaire et al., 2010). However, such a hypothesis would suggest an overall elevated substitution rate in the entire genome for that species.

Here we provided evidence of rate heterogeneity in nuclear genomes of the two *Balanophora* spp. The substitution rates in 18S rDNA in *B. laxiflora* and *B. fungosa* are five times higher than other flowering plants, whereas the synonymous substitutions in *P1*, *AP3/TM6*, *LFY* and *RPB2* show a comparable rate (0.5–2.0 times higher) to the autotrophic core eudicots. These results suggest that the molecular evolutionary factors affecting substitution rates in 18S rDNA might be different from nuclear protein-coding genes, at least for the B-class, *LFY* and *RPB2* genes demonstrated in this study. Three models of rate variation have been proposed for explaining nucleotide substitution patterns: (1) gene-specific, (2) organism-specific and (3) genome-specific (Nickrent et al., 1998). Our preliminary data on the six protein-coding genes indicate that the rate variation in *Balanophora* probably evolved in a gene-specific manner. The mechanism for such rate heterogeneity in *Balanophora* is unknown and a thorough screening for other nuclear genes will reveal if it is ubiquitous in the *Balanophora* genome. It also remains to be determined if a rate discrepancy exists between 18S rDNA and other nuclear genes for the heterotrophic plants that are known to have highly elevated 18S rDNA substitutions.

**B-class genes in Santalales and the novel exon in Balanophora AP3**

Despite the fact that many *PI* and *TM6* homologues were successfully identified from the selected members of Santalales including *Balanophora*, only two eu*AP3* homologues were found in the two *Balanophora* spp. examined, and no eu*AP3* homologues were detected in other members of Santalales. Different pairs of degenerate primers, including those most commonly used in previous studies (Kramer et al., 1998; Stellari et al., 2004) and primers designed specifically for the *Balanophora* eu*AP3* genes (Bal*AP3* and Bas*AP3*), were employed under several PCR conditions. The lack of eu*AP3* sequences from these taxa of Santalales indicates that their homologues are either too divergent to be detected by PCR-based screening or their genes might express at a low levels, thus escaping detection using RT-PCR.

Most of the B-class genes in angiosperms consist of seven exons and six introns. Our alignment of eu*AP3* homologues showed that *AP3* from both *Balanophora* species have relatively conserved M, I and K domains. Exons 1–5 in the *Balanophora* B-class genes have the same length as those in *Vitis*, but the length of the C-domain is quite variable (Supplementary Data Fig. S3). An additional *AP3* exon in both *Balanophora* species is found between the C1 and C2 domains, which are known to involve transcriptional activation (Riechmann and Meyerowitz, 1997). However, we were unable to find other cases of similarly modified C-domains in other plants, and thus the issue of its origin is unclear at this time. Moreover, an intensive screening of Santalales and/or other members of Balanophoraceae is required to understand better the evolution of these B-class genes and to make inferences on their function.

**Differential expression of B-class genes in Balanophora laxiflora**

The expression patterns of *Balanophora AP3* and *TM6* suggest a largely redundant role for these two genes since they are co-expressed at comparable levels in all organs, except that *TM6* expression is lower in pollen and floral bracts (Fig. 4B). Given the fact that *AP3* has distinct C-terminal sequences, it is reasonable to propose a certain degree of functional differentiation from *TM6* in *Balanophora*. In contrast, *PI* might have a conserved B-class gene function since it has strong expressions in tepals and synandria, but not in any reproductive organs of the female *Balanophora* plant. This result is congruent with the floral ABCDE model prediction, since no perianth and androecium structures can be found in the female plant of *B. laxiflora*. The tepals in male *B. laxiflora* have cobblestone-like epidermal cells that are similar to those seen in the petals of other plants which have short cuticular ridges on the surface (Kay and Daoud, 1981). Similar cuticular modification can also be found in the epidermal cells of floral bracts in the male *B. laxiflora*. Su & Hu — Rate heterogeneity of holoparasitic Balanophora 1145 Downloaded from https://academic.oup.com/aob/article-abstract/110/6/1137/111647 by guest on 29 July 2018
It remains to be determined if the expression of PI in the floral bracts of *Balanophora* is correlated with epidermal cell morphology in these organs (tepals, synandria and floral bracts).

**Phylogenetic utility of nuclear RPB2 and floral B-class genes in Balanophoraceae**

Although 18S rDNA and *matR* are widely used markers in reconstructing angiosperm phylogeny, the extreme rate acceleration of nucleotide substitution in these genes in *Balanophora* presents problems for accurate phylogenetic reconstruction. Our results clearly showed that *Balanophora* is a member of Santalales based on both RPB2 and B-class gene phylogenies, even though the exact relationships are still problematic owing to low support for some nodes. We demonstrated that the evolutionary rates of the nuclear protein-coding genes are relatively conserved compared with 18S rDNA and *matR*; thus they represent promising markers for further phylogenetic analyses. Future work will be to increase the sampling of the highly diverse Olacaceae s.l. (Malecot and Nickrent, 2008).

**Conclusions**

For the holoparasite *Balanophora*, nucleotide substitution rates in the B-class genes, *LFY* and *RPB2* homologues show that these nuclear protein-coding genes are relatively conserved compared with 18S rDNA and mitochondrial *matR*. These sequences are thus potential candidates for further phylogenetic analyses of Balanophoraceae. There is rate heterogeneity between 18S rDNA, mitochondrial *matR* and the five nuclear protein-coding genes, hinting at an unusual and currently unexplored mechanism of sequence evolution for the mitochondrial and nuclear ribosomal genes of *Balanophora*.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used in this study. Table S2: listing of the GenBank accession numbers for all sequences used in this study. Figure S1: RPB2 gene tree showing the obtained RPB2 sequences belonging to the clade. Figure S2: conserved C-terminal of Santalales B-class gene homologues. Figure S3: genomic structure of *Balanophora* AP3 and TM6 homologues.

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