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Complete Chloroplast Genome Sequence of an Orchid Model Plant Candidate: Erycina pusilla Apply in Tropical Oncidium Breeding

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Abstract

Oncidium is an important ornamental plant but the study of its functional genomics is difficult. Erycina pusilla is a fast-growing Oncidiinae species. Several characteristics including low chromosome number, small genome size, short growth period, and its ability to complete its life cycle in vitro make E. pusilla a good model candidate and parent for hybridization for orchids. Although genetic information remains limited, systematic molecular analysis of its chloroplast genome might provide useful genetic information. By combining bacterial artificial chromosome (BAC) clones and next-generation sequencing (NGS), the chloroplast (cp) genome of E. pusilla was sequenced accurately, efficiently and economically. The cp genome of E. pusilla shares 89 and 84% similarity with Oncidium Gower Ramsey and Phalanopsis aphrodite, respectively. Comparing these 3 cp genomes, 5 regions have been identified as showing diversity. Using PCR analysis of 19 species belonging to the Epidendroideae subfamily, a conserved deletion was found in the rps15-trnN region of the Cymbidieae tribe. Because commercial Oncidium varieties in Taiwan are limited, identification of potential parents using molecular breeding method has become very important. To demonstrate the relationship between taxonomic position and hybrid compatibility of E. pusilla, 4 DNA regions of 36 tropically adapted Oncidiinae varieties have been analyzed. The results indicated that trnF-ndhJ and trnH-psbA were suitable for phylogenetic analysis. E. pusilla proved to be phylogenetically closer to Rodrigueza and Tolumnia than Oncidium, despite its similar floral appearance to Oncidium. These results indicate the hybrid compatibility of E. pusilla, its cp genome providing important information for Oncidium breeding.

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Introduction

The Orchidaceae has great diversity in floral morphology and a rich array of species. It comprises the largest family of flowering plants [1]. The extraordinary variety of orchid floral features and appearances ensure a large consumer demand for orchids. Oncidium, a genus in subtribe Oncidiinae, is a popular and important cut flower. It needs about 3 years to reach sexual maturity under natural conditions [2], its flowering being precisely regulated by temperature [3]. Taiwan, located in tropical and subtropical areas with higher temperature, is one of the Oncidium cultivation and hybridization center of world [4]. To develop new commercial species with competitive advantageous traits such as shorter vegetative stage or tropical growth of Oncidium species continues to be a pressing need.

For orchid breeding, it is important for molecular studies of orchid to help in efforts to create unique flower colors and shapes, as well as disease-resistant cultivars that are of high economic value. In Taiwan, genomics approach by transcriptomic database establishment [5–7] chloroplast genome [8,9], and transformation technology have been applied in orchid research [10,11]. Few transgenic orchids have been obtained and used in orchid breeding [12]. Sweet pepper ferredoxin-like protein (pfp), a new selection marker developed by You et al., was over-expressing in Oncidium orchid “Sherry Baby cultivar OM8” and enhanced Euvinia carotovora resistance of transgenic orchid [13]. Transgenic Phalaenopsis expressing coat protein of Cymbidium mosaic virus (CymMV) enhanced protection against CymMV infection through RNA-mediated resistance [14]. However, several disadvantages that make orchid breeding by either traditional hybridization or gene engineering difficult are: (1) most of the plants grow slowly; (2) there is a wide range of chromosome numbers e.g. n = 6–30 in Oncidiinae [15]; (3) genome sizes are large and have complex polyplody caused by spontaneous or man-made hybridization [16–18]. Many Oncidium genes have been cloned and studied by ectopic expression in Arabidopsis or Eustoma [19–21], however, gain-of-function studies in orchid remains scarce. Therefore, an orchid model plant system is needed for the functional genetic investigation.
Erycina pusilla, is a fast-growing epiphytic orchid with a relatively low chromosome number (n = 6; [22]) and small genome size (1.5 pg per 1C nucleus; [23]). Pollination and production of seed capsules rarely occurs in nature [24]. Currently, advances in cultural techniques and precocious flowering have meant that E. pusilla can be grown rapidly, and will produce flowers and fruit in vitro [25,26]. These characteristics make E. pusilla not only an attractive model plant for functional genomic and flowering studies of Oncidium, but also an excellent parent for traditional hybridization methods. To produce attractive traits and breed new commercial orchid species, E. pusilla has been crossed with several important Oncidiniae orchids, and different hybridization compatibility was found with Oncidium, Rodrigueza and Tolumnia [27]. However, systematic molecular investigations and genomic information on E. pusilla remain unclear.

Chloroplast DNA is useful in evolutionary studies because of its simple structure, highly conserved sequence, and maternal inheritance characters [28]. Several plastid regions, such as psa, atpB, psbB, psbC and rpoC1, have been used to identify phylogenetic relationships in orchid [29,30]. Sequencing of complete plastid genomes of different genera has recently provided useful information regarding RNA editing and loss of introns [31,32]. Chloroplast (cp) genomes of 2 orchids, Phalanopsis aphrodite and Oncidium Gower Ramsey, have been sequenced [8,9]. The availability of chloroplast genome sequences should also help in developing genetic engineering including chloroplast transformation [33,34]. Information on the complete chloroplast genome sequence is not only important for taxonomic classification but also for crop improvement.

To provide information on breeding and molecular aspects of Oncidium, we have sequenced the complete cp genome of E. pusilla using BAC library and next-generation sequencing (NGS). To demonstrate the possibility this orchid as a model, the difference between its cp genome and those of other important Oncidiniae orchids, and different hybridization compatibility was found with Oncidium, Rodrigueza and Tolumnia [27]. However, systematic molecular investigations and genomic information on E. pusilla remain unclear.

Chloroplast-BAC clone identification

Young E. pusilla leaves (200 g) grown in vitro were collected for isolation of high molecular weight DNA [35]. The DNA was partially digested by HhaIII, and the fragments ligated into vector pCC1BAC DH10b (Amplicon, Pullman, WA), which was used for transfection into E. coli. Individual clones were picked up and placed into 384-well plates that contained liquid LB medium with 12.5 mg/L chloramphenicol. The plates were incubated at 37°C overnight and stored at −80°C. Chloroplast specific primers designed by Wu et al. [9] were used to amplify predicted chloroplast regions from a BAC library. The BAC clones containing chloroplast regions of interest were obtained by PCR screening from super pool, plate, row, and spot, as described by Hsu et al. [10]. BAC clones of the chloroplast were identified (clone ID P-5-K16).

Illumina sequencing

BAC plasmids for Illumina sequencing were isolated using the NucleoBond BAC 100 kit (NucleoSpin Blood kit, Macherey-Nagel, Germany). Five micrograms of E. pusilla BAC plasmid were sheared into fragments of 200–600 bp by Bioruptor Next Gen (Diagenode) in 100 μl TE buffer. The purified DNA fragments were treated with T4 DNA polymerase, E. coli DNA polymerase I Klenow fragment and T4 Polynucleotide Kinase. Adapters required for sequencing on the Illumina platform were added to DNA fragments. The ligation products were separated on a 2% agarose gel; those between 270 and 350 bp were excised, eluted from the gel slice, precipitated and resuspended in 15 μl TE, using QIAquick Gel extraction Kit (Qiagen). The adapter-modified DNA fragments were amplified, and the products purified using an Agencourt AMPure XP (Beckman). They were collected in 30 μl of QIAGEN elution buffer (Qiagen). After quantification by Quant-iT dsDNA HS Assay Kit (Invitrogen) and KAPA Library Quantification Kit (KAPABiosystem), the molar concentration was calculated and the quality examined by Expression DNA 1K Analysis Kit (Bio-Rad). The DNA library was then prepared for sequencing.

Bioinformatics

Sequencing was performed on an Illumina GA IIx platform, using a paired-end strategy at a read-length of 75 bases. Nucleotides with low quality scores (<3) were removed from the sequence reads, and any that had a 100% match to the cloning vector sequence or E. coli sequences were also removed from the subsequent assembly process. De novo assembly was conducted using CLC Genomics Workbench (CLC bio, Cambridge, MA). The gaps between the contigs were filled by PCR.

E. pusilla chloroplast genome annotation

The cp genome was annotated using Dual Organellar GenoMe Annotator (DOGMA) [36]. This program uses a FASTA-formatted input file of the complete genomic sequences and identifies putative protein-coding genes by performing BLASTX searches against a custom database of published cp genomes. Both tRNAs and rRNAs were identified by BLASTN searches against the same database of cp genomes. For genes with low sequence identity, manual annotation was performed after identifying the positions of the start and stop codons, as well as the translated amino acid sequence, using the chloroplast/bacterial genetic code. The annotated genome sequences were submitted to NCBI (Accession no: JF_746994).

Plant materials

Orchids were obtained and collected from a local grower in Taiwan. All orchids were maintained in the greenhouse at National Chung Hsing University, Taichung, Taiwan.

DNA purification and genomic PCR

For chloroplast genomic PCR analysis, total genomic DNA was isolated from leaves using a urea extraction buffer system [37]. The primer designs for Epidendroideae species analysis were based on the various regions of 3 orchid cp genomes. The primer sequences, sequence sizes, and forward primer position in Table 1. Primers designed by Wu et al. [9] were used for Oncidiniae variety analysis (the sequences are shown in Table 1). Genomic PCR was conducted in a final volume of 50 μl containing 2.5 units of Taq DNA polymerase (Violet gene, Taipei, Taiwan), 1.25 mM of each dNTP, and 10 pmol of each primer. The amplification program used was 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The PCR products were sequenced and assembled using VectorNTI Contig Express software.
Analysis of sequence variability of 3 orchid cp genomes and Epidendroideae species

Chloroplast sequences of *E. pusilla*, *Onc*. Gower Ramsey (GenBank accession NC_014056), and *P. aphrodite* (GenBank accession NC_007499) were used for genome comparison. For Epidendroideae species analysis, 5 intergene regions (*atpH-atpI*, *petN-psbM*, *accD-psaI*, *psbE-petL*, and *ips15-trnN*) were obtained by PCR from 19 varieties (Accession no:JN638455–JN638514). Sequences were compared and adjusted using the VectorNTI AlignX software program (vers. 7.0; Invitrogen, Carlsbad, CA; parameters: overlap: 30; identity: 0.95; cutoff score: 40).

**Phylogenetic analysis of Oncidiinae species**

Four cpDNA regions (*trnH-psbA*, *matK*, *trnF-psbM*, and IRb-SSC) of 36 Oncidiinae species were obtained by PCR (Accession no: JN5998910–JN5998996), and from the NCBI database (GQ915119–915130, GU132947–132991, GU136251–136287, GU175342–175358). Alignment of nucleotide sequence was performed using the Clustal X program [38] and adjusted by GeneDoc software. Phylogenetic analysis was conducted using MEGA3.1 [39], and the phylogenetic tree generated using the neighbor-joining method with 1,000 bootstrap trials by means of the neighbor-joining algorithm. Percentages of bootstrap values are indicated on the tree.

**Results**

Comparison of *E. pusilla* chloroplast genome with genomes of 2 other orchid genera

The cp genome of *E. pusilla* is 143,164 bp in size and contains a pair of inverted repeats (IRa and IRb) of 23,439 bp separated by large and small single copy (LSC and SSC) regions of 84,189 and 12,097 bp, respectively (Figure 1). This genome contains 126 different genes that include 73 protein coding genes, 6 pseudogenes, and 19 genes duplicated in the IR region. There are 28 distinct tRNAs and 4 distinct tRNA genes. Fifteen genes contain 1 or 2 introns, and 5 of their introns are within tRNAs. The genome consists of 45.02% protein-coding genes, 46.73% non-coding DNA, which includes the intergenic spacer (IGS) regions, regulatory sequences and introns, 1.94% tRNA and 6.31% rRNA genes. The overall GC and AT content of the cp genome is 36.65% and 63.35%, respectively. The AT content of the LSC and SSC regions is 66.15% and 77.54%, respectively, whereas that of the IR region is 65.21%, including the tRNA gene cluster. The gene order of *E. pusilla* cp genome is very similar to that in the Oncidium cp genome.

To analyze the genome variation in orchids, 5 regions were chosen for PCR analysis in 19 species of the Epidendroideae subfamily (Table 2). In the *atpH-atpI* region, 4 different deletions were found in *E. pusilla*, *Onc*. Gower Ramsey, *Aeonium rigida* and *Aerangis hyalothis*, which varied in size and location (Figure 4A). The first 400 bp was highly diverse in the *petN-psbM* region. Three Calanthe species shared the same deletion. A similar but longer deletion was found in the *ndhB* region in *Onc. Gower Ramsey,* *P. aphrodite*, and *N. nischmii* (Figure 3C). The *ndhE* was only absent in *C. aloifolium* (Figure 3F). Among 3 orchid chloroplast genomes, sequences of ndh genes were most variable. In *E. pusilla* and *Onc. Gower Ramsey*, ndhJ was truncated and ndhF was absent (Figure 3A). All ndhF were absent and ndhD were truncated in three orchid cp genomes (Figure 3B and Figure 3C). The ndhE was only absent in *E. pusilla* (Figure 3D). The ndhA gene sequence was only present in *O. Gower Ramsey* (Figure 3E). The ndhB that located in the IR region was 892 bp in *E. pusilla*, which is 1333 and 1137 bp shorter than in *Onc. Gower Ramsey* and *P. aphrodite*, respectively. These results indicate that deletion and truncation are common in chloroplast-encoded ndh genes of orchid plants.

Analysis of 5 regions in 19 Epidendroideae species

To analyze the genome variation in orchids, 5 regions were chosen for PCR analysis in 19 species of the Epidendroideae subfamily (Table 2). In the *atpH-atpI* region, 4 different deletions were found in *E. pusilla*, *Onc. Gower Ramsey*, *Aeonium rigida* and *Aerangis hyalothis*, which varied in size and location (Figure 4A). The first 400 bp was highly diverse in the *petN-psbM* region. Three Calanthe species shared the same deletion. A similar but longer deletion was found at the same position in *Goeudorum densiflorum* and *Phaus nischmii*. *Goeudorum densiflorum* contained one more insertion (Table 2 and Figure 4B). Three deletions at different locations of the *petN-psbM* region were found in *Onc. Gower Ramsey*, *Cymbidium aloifolium* and *Aer. Hyalothis* (Table 2, and Figure 4B). In the *accD-psal* region, *Onc. Gower Ramsey* and *Geo. densiflorum* shared the same 536 bp deletion. *Aer. Hyalothis*, which belongs to the Angraceae subtribe of Vandaecea, contained 2 deletions (Table 2, and Figure 4C). *E. pusilla*, *Bletilla formosa* and

### Table 1. Primers for Epidendroideae genes and Oncidiinae phylogenetic analysis.

| Sequence     | Sequence     | Position | bp  |
|--------------|--------------|----------|-----|
| *atpH-atpI*  | 3740         | 1204     |     |
| *petN-psbM*  | 28958        | 933      |     |
| *accD-psaI*  | 58330        | 928      |     |
| *psbE-petL*  | 64478        | 946      |     |
| *ips15-trnN* | 114398       | 762      |     |
| *trnH-psbA*  | 142336       | 1413     |     |
| *matK*       | 1875         | 936      |     |
| *trnF-ndhJ*  | 49294        | 946      |     |
| *IrB-SSC*    | 118168       | 1390     |     |

Primer sequences, annealing position of the forward primer in *E. pusilla*, and the PCR amplification length are presented. The first 5 sets of primer were used for Epidendroideae analysis. The last 4 sets of primer were used for Oncidiinae phylogenetic analysis [9].

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Figure 1. Gene map of *Erycina pusilla* chloroplast genome. Genes on the outside of the map are transcribed clockwise whereas genes on the inside of the map are transcribed counterclockwise. Colors indicate genes with different functional groups.

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Dendrobium equitant was each contained one unique deletion (Table 2, and Figure 4C). Analysis of the psbE-petL intergene sequences showed 5 different deletions located in a disorderly fashion within E. pusilla, Onc. Gower Ramsey, Cyn. aloifolium and Era. corneli (Table 2, and Figure 4D). In the rps13-trnN region, a deletion found in all 4 Cymbidieae species, including E. pusilla, Onc. Gower Ramsey, Cyn. aloifolium and Geo. densiflorum (Table 2, and Figure 4E). With the exception of Aca. rigid, which contained another 295 bp deletion, the other species all shared similar rps13-trnN sequences (Table 2, and Figure 4E).

Phylogenetic analysis of 36 Oncidiinae species

To investigate the relationship between the molecular study and orchid breeding, 36 important Oncidium species were suitable for phylogenetic analysis (Table 3). Based on the variation in the 3 orchid cp genomes (Figure 2) and previous studies [9], 4 primers (Table 1) were chosen for PCR amplification and phylogenetic analysis. Using the matK gene, which is in a highly conserved region, Millossia (Mssia) was grouped with Beallara (Bilba), and Zeledenia (Zln) was grouped with Tolumnia (Tol). Other species could be grouped by genus, but species could not be separated. Erycina was phylogenetically close to Tolumnia and Zeledoncidium, but distant from Oncidium and Odontocidium (data not shown). The primers for amplifying the variable regions, IRb-SSC, could only partially divide the Oncidium genus from the others, which was not suitable for this analysis.

With the exception of members of the Millossia and Beallara genera, other species could be separated well using trnF-trnH region, although the bootstrap scores were low (Figure 5A). We therefore combined 2 regions, trnF-trnH and trnH-psbA, for phylogenetic analysis (Figure 5B). The combined analysis gave a similar, but more distinguishable, result when compared with that using the trnF-trnH region alone. The Millossia genus was separated from Beallara, Onc. ornithorynchum and other Oncidium species were separated into 2 different groups. Phylogenetic analysis showed that E. pusilla, Rodrigueza, and Tolumnia were grouped together, distinct from the Oncidium, Odontocidium and Beallara group.

Discussion

Advantages of next generation sequencing and BAC libraries for chloroplast genome sequencing

In Taiwan, analysis of orchid genomic sequence have provided valuable information for investigating molecular mechanisms of orchid flowering development, perspectives, and disease resistance pathway [4,19–21]. In the other hand, total cp genomes are useful for evolutionary studies [28]. Total DNA or chloroplast DNA had been used on several occasions as basic materials to obtain cp genomes [8,40–42], but in such studies the possibility of DNA contamination could not be entirely ruled out [43,44]. Other studies show that DNA fragments can be transferred between chloroplasts, mitochondria, and nuclear genomes during evolution [45–47]. Sequences of mitochondria and chloroplasts of rice and maize share high percentage sequence homology [48,49]. There are 68 kb cpDNA sequences (42.4% of the cp genome) in the mtDNA of V. vinifera [50]. To reduce the possibility of DNA contamination in our study, we applied BAC library screening by using chloroplast genes as probes for sequencing the complete chloroplast genomes.

For cp genome sequencing, a shotgun library [31] and a PCR-based method were used [9,51]. The PCR-based method relies on the sequence conservation of the chloroplast genome. The products were further validated with Sanger sequencing. Recently, NGS has become a powerful tool for genome sequencing as it is time-saving, low in cost and uses high-throughput technology [40,52]. Various chloroplast genomes, such as 6 woody bamboos belonging to the BEP clade with controversial internal relationships [52] have been successfully sequenced by NGS. However, using NGS sequencing and a BAC library has not previously been used to sequence a complete orchid cp genome.

E. pusilla cp genome BAC clones were identified in our study by using PCR screening. By using chloroplast specific primers [9], the BAC library could be screened using PCR [10], which is easier and faster than traditional hybridization methods [53]. Meanwhile, we also identified BAC plasmids with mitochondria clones which contained chloroplast homologous sequences. Several single nucleotide polymorphisms (SNP), insertion/deletion and homology sequences were found between the mitochondria and chloroplast sequences in E. pusilla (data not shown). Using Illumina sequencing and chloroplast specific BAC plasmids, the possibility of reassembly errors caused by homologous sequences between chloroplast and mitochondria or nuclear DNA could be excluded. Combining a BAC library screening using a PCR approach and Illumina sequencing, we obtained an accurate chloroplast genome sequence efficiently and economically. The chloroplast genome studies will support the identification by phylogenetically close relatives of Erycina and will help in their breeding and genetic improvement.

Figure 2. Comparison of chloroplast genomes of E. pusilla, Onc. Gower Ramsey, and P. Aphrodite. Deletions or insertions longer than 70 bp in P. aphrodite or Onc. Gower Ramsey in comparison with E. pusilla are labeled as white triangles or black triangles individually. Highly diverse sequence regions larger than 500 bp are labeled with black blocks. Numbers indicate the longest length of comparative deletions, insertions, or diverse sequence retions of three species orchids.

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Characteristics of *E. pusilla* chloroplast genome

The gene order of the 3 orchid chloroplast genomes was very similar. Unlike other monocot plants, such as maize, rice, and wheat (NC_001320, NC005973, NC_002762, NC_001666), the 3 orchid cp genomes contained the *ycf2* gene, which is similar to dicot plants, such as tobacco, *Arabidopsis*, and Lotus (NC_001879, NC_000932, NC_002694) [8]. The 153 bp longer *ycf2* in *P. aphrodite* (Figure 2) is also found in other dicot plants. The function of the short 153 bp *ycf2* in *E. pusilla* and *Onc.* Gower Ramsey needs further exploration.

The products of the *ndh* genes catalyze the transfer of electrons from NADH to plastoquinine, which adjusts the redox level of the photosynthetic electron transporters [54]. Most *ndh* genes in the chloroplast genome of *E. pusilla*, *Onc.* Gower Ramsey, *P. aphrodite* and the other 14 Oncidiinae species were deleted or truncated [8,9]. The non-functional *ndh* genes are also found in crassulacean acid metabolism (CAM) and C3 plants, such as *Pinus thunbergii*, *Keteleeria davidiana*, *Ephedra equisetina* and *Welwitshia mirabilis*, which belong to autotrophic, heterotrophic, gennospermae, or monocot species, respectively [55–57]. Furthermore, in *Erodium* genus, 11 plastid-encoded *ndh* genes were intact in *Ero. texanum* and *Ero. carsimum*, but were deleted in *Ero. chrysonum*. No morphologic or biological features are associated with *ndh* gene loss in *Erodium* [58]. These results indicate that the loss-of-function of chloroplast encoding the *ndh* genes might not affect photosynthesis. The ancestral plastid *ndh* genes of orchids are presumed to have been transferred to the nucleus [8]. The orchid nuclear genome sequences, which are still unavailable, are needed to clarify the horizontal gene transfer questions of *ndh* genes in orchids.

Figure 3. Structure of *ndh* genes in 3 orchid cp genomes. Numbers indicate the position in the chloroplast genome. The angled dashed lines indicate the gaps. Different colors indicate different *ndh* genes, a color key is shown at the bottom of each part of the Figure. Accession number of *O. sativa* Japonica is NC_001320. doi:10.1371/journal.pone.0034738.g003
Table 2. Summary of gene patterns in Epidendroideae subfamily.

| Tribe/Subtribe/Species | atpH-atpI | petN-psbM | accD-psaI | psbE-petL | rps15-trnN |
|------------------------|-----------|-----------|-----------|-----------|-----------|
| Cymbidieae              |           |           |           |           |           |
| Oncidiinae             |           |           |           |           |           |
| Erycina pusilla        | ●         | ●         | ●         | ●         | ▲         |
| Onc. Gower Ramsey      | ●         | ●         | ▲         | ●         | ▲         |
| Cyrtopodiinae          |           |           |           |           |           |
| Cymbidium aloifolium   | ○         | ●         | —         | ●         | ▲         |
| Eulophiinae            |           |           |           |           |           |
| Geodorum densiflorum   | —         | △★        | ▲         | —         | ▲         |
| Arethuseae             |           |           |           |           |           |
| Arethusinae            |           |           |           |           |           |
| Arundina graminifolia  | —         | ○         | —         | —         | —         |
| Coelogyninae           |           |           |           |           |           |
| Bletilla formosana     | ○         | —         | ●         | —         | —         |
| Podochileae            |           |           |           |           |           |
| Eriinae                |           |           |           |           |           |
| Eria corneri           | ○         | —         | ○         | ●         | ○         |
| Vandeae                |           |           |           |           |           |
| Aeridinae              |           |           |           |           |           |
| Acampe rigida          | ●         | ○         | ○         | ●         | ●         |
| Doritis pulcherrima    | —         | —         | ○         | ○         | —         |
| Phalaenopsis aphpodite | ○         | ○         | ○         | ○         | ○         |
| Angraecinae            |           |           |           |           |           |
| Aerangis hyaloids      | ●         | ●         | ●         | ○         | —         |
| Unplaced subtribes     |           |           |           |           |           |
| Collabiinae            |           |           |           |           |           |
| Spathoglottis plicata  | ○         | —         | ○         | —         | ○         |
| Calanthe rosea         | ○         | —         | ○         | ○         | ○         |
| Calanthe alismaefolia  | ○         | ▲         | ○         | ○         | ○         |
| Calanthe discolor      | ○         | ▲         | ○         | ○         | ○         |
| Calanthe sylvatica     | ○         | ▲         | ○         | ○         | —         |
| Phaius mishmensis      | —         | △         | ○         | ○         | ○         |
| Phaius takeoi          | ○         | ○         | ○         | ○         | ○         |
| Dendrobiinae           |           |           |           |           |           |
| Dendrobium equitans    | ○         | ○         | ●         | ○         | ○         |

Within each detected region, different species that share the same sequences are labeled with white circles; unique deletions are labeled as black circles. The same deletions found in different species are labeled as triangles of the same color. Black stars indicate insertions. ‘—’ indicates that no PCR product was obtained.

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Analysis of 5 regions in 19 Epidendroideae species

In the petN-psbM region, 3 species belonging to the Collabiinae shared a deletion of the same size (Table 2, and at the same position, Figure 4B). However, a 20 bp longer deletion was found in Geo. densiflorum and Pha. mishmensis, which belong to the Eulophiinae and Collabiinae subtribes, respectively. Therefore, the petN-psbM deletion found in the three Collabiinae species might not specific to the Collabiinae subtribe.
Four species belongs to Cymbidieae tribe including *E. pusilla*, *Onc. Gower Ramsey*, *Cym. aloifolium*, and *Geo. Densiflorum* shared the same deletion in the *rps15-trnN* region that is located between the SSC and the IR ([Table 3, Figure 3E](#f4)). SSC/IR is one of the most variable loci and could be an evolution marker [59]. To examine whether this deletion is commonly exits in Cymbidieae tribe, SSC to IR sequences of Epidendroideae were download from the NCBI database and further analyzed [60,61]. The deletion in *rps15-trnN* was conserved in all 77 Cymbidieae tribe, including species belonging to Oncidiinae, Crytropodiinae, Eulophiinae, and Maxillarieae subtribes (Figure S1). Similar to *P. aphrodite*, other species belonging to the Vandae tribe, Podochileae tribe, Collabiniinae subtribe, or Dendrobieae subtribe contained no such deletion. Together, these findings indicate the deletions in the *rps15-trnN* were commonly exits in Cymbidieae tribe.

**Phylogenetic analysis of Oncidiinae species**

Due to the unique morphology, *E. pusilla* has rendered taxonomic classification and denomination a continual challenge [62]. In 2001, *E. pusilla* was finally named according to the molecular systematics which were performed based on the *ITS*, *matK*, and *trnL-F* DNA regions [63]. The fast-growing feature of *E. pusilla* makes it a good parent for breeding. To supply molecular information for *Oncidium* breeding, 36 Oncidiinae species including tropical-adaption commercial hybrids were further analysis. Though the *matK* region was a good marker for Oncidiinae phylogeny investigation [63]. In our study, the *matK* regions within 36 Oncidiinae commercial species were too conserved to separate well. The *matK* regions might not a good marker to make phylogenetic inferences within commercial hybrids. Another primer for the IRb-SSC region failed to produce a PCR product.
for many species, which also made phylogenetic analysis using a 4 DNA region combination difficult in this study. However, the phylogenetic analysis using the two regions trnF–ndhJ and trnH–psbA were able to demonstrate a good resolution within 36 Oncidiinae including commercial hybrids. E. pusilla is located close to the Tolumnia and Rodriguezia, while Oncidium, Oncidesa, and Oncidium belong to another group (Figure 4). The phylogenetic result is able to explicate the hybridization compatibility of E. pusilla [27]. No fruit set and seed production in Ionocidium Popcorn ‘Haruri’ and Oncidesa Little Dragon crossed with E. pusilla. Fruit can be obtained by crossing E. pusilla with Rod. lanceolata but only few seed would be germinated. However, fruits and progeny could be germinated successfully by crossing E. pusilla with several species belonging to the Tolumnia genus including Tol. Genting Angel. Our phylogenetic analysis using trnF–ndhJ and trnH–psbA thus provides a reference for the hybridization compatibility of E. pusilla. For traditional hybridization breeding, this is important information to select new hybrid parents systematically and create new commercial species efficiently.

Previously, orchid taxonomy has been based on floral traits and morphological features. However, classification is changed frequently because the characteristics of orchid are easily affected by interspecific or intergeneric crossing and changes in environment. E. pusilla and Zelenkocidium Little Angel used to be taxonomically grouped into the Oncidium because of their similar floral appearances. Currently, molecular taxonomy has started to reveal

| Genus   | Variety               | Ovary Parent         | Pollen Parent         |
|---------|-----------------------|----------------------|-----------------------|
| Ada     | keiliana              | Ada keiliana         | Ada keiliana          |
| Beallara| Eurostar              | Beallara Tahoma Glacier | Oncidium schroederianum |
|         | Marflitch ‘Howard Dream’ | Miltassia Charles | Odontioda Fremar |
|         | Peggy Ruth Carpenter ‘Morning Joy’ | Beallara Tahoma Glacier | Miltonidium Purple Queen |
|         | Smile Eri              | Beallara Tahoma Glacier | Odontioda (Torona X Ingera) |
|         | Tahoma Glacier ‘Sugar Sweet’ | Bratonia Cartagena | Oncidium Alaskan Sunset |
| Comparettia | ignea               | Comparettia ignea | Comparettia ignea |
|         | macroplectron         | Comparettia macroplectron | Comparettia macroplectron |
| Degarmoara | Flying High       | Miltassia Jet setter | Odontoglossum McNabianum |
| Erycina | pusilla               | Erycina pusilla      | Erycina pusilla       |
| Huangara | Niu Boy               | Leomesezia Lava Burst | Macradenia multiflora |
| Ionocidium | Popcorn ‘Haruri’    | Ionopsis utricularioides | Ionopsis utricularioides |
| Ionopsis | utricularioides       | Ionopsis utricularioides | Ionopsis utricularioides |
| Macradenia | multiflora           | Macradenia multiflora | Macradenia multiflora |
| Miltassia | Olmec                | Brassia Rex          | Miltonia Minas Gerais |
| Odontocidium | Golden Gate        | Odontoglossum bictoniense | Odontocidium Tiger Hambuhren |
|         | Wildcat ‘Garfield’   | Odontocidium Rustic Bridge | Odontocidium Crowborough |
| Odontoglossum | Margarete Holm     | Odontoglossum bictoniense | Odontoglossum Hans koch |
|         | Violetta von Holm    | Odontoglossum bictoniense | Odontoglossum Bic-ross |
| Oncidesa | Little Dragon        | Gomesa echinata      | Oncidium chirophorum |
| Oncidium | Gower Ramsey         | Oncidium Goldiana    | Oncidium Guinea Gold |
|         | Gower Ramsey ‘Lemon Heart’ | Oncidium Goldiana | Oncidium Guinea Gold |
|         | Gower Ramsey ‘Sunkist’ | Oncidium Goldiana | Oncidium Guinea Gold |
|         | ornithorhynchum      | Oncidium ornithorhynchum | Oncidium ornithorhynchum |
| Sherry Baby ‘Tricolor’ | Oncidium Jamie Sutton | Oncidium Honolulu |
| Sweet Sugar Million ‘Coin’ | Oncidium Aloha | Oncidium varicosum |
| Tsiku Marguerite ‘Romantic Fantasy’ | Oncidium Twinkle | Oncidium sotoanum |
| Twinkle  | Oncidium chirophorum | Oncidium sotoanum |
| Rodriguezia | lanceolata       | Rodriguezia lanceolata | Rodriguezia lanceolata |
|         | venusta              | Rodriguezia venusta  | Rodriguezia venusta  |
| Tolumnia | calochila            | Tolumnia calochila   | Tolumnia calochila   |
|         | Fire Ring            | —                    | —                    |
| Genting Angel | Tolumnia Irene Gleason | Tolumnia Linda |
| Jairak Firm ‘Brown White’ | — | — |
| Zelenchia | midas                | Zelenchia midas      | Zelenchia midas      |
| Zelenkocidium | Little Angel        | Zelenkoca onusta     | Oncidium flexuosum   |

*—* indicates that no parents information were obtained.

![Table 3. Parents of 36 varieties of Oncidiinae.](https://www.plosone.org/figure/Table3)
The Application of Orchid Model Plant, *E. pusilla*
more precise phylogenetic relationship and many species of orchid have been renamed and reclassified. For example, Onc. Little Angel was reclassified as *Zelenkocidium* Little Angel. Onc. Midas, the hybrid of *Zelenkoa onusta* and *Oncidium flexuosum*, was renamed as *Zeleninia Midas*. According our results, *Zeleninia* and *Zelenkocidium* are located close to *Tolumnia* and distant from *Oncidium* in the phylogenetic tree in the current study (Figure 4) thus validating previous results. Beyond the species that we have examined, there might be other species that should be moved to *Zeleninia* or *Zelenkocidium* from *Oncidium* despite having a similar floral appearance to *Oncidium*. The phylogenetic tree showed *Odontoglossum* Violetta was phylogenetically distant from *Odontoglossum* and *Oncidium*, and *Onc. orthorhynchum* was much closer to *Erycina* and *Tolumnia* than *Oncidium* and *Odontoglossum*. We therefore suggest that the taxonomy of *Onc. Violetta* and *Onc. orthorhynchum* should be further checked and compared with the parent. Similar misplacements might occur in many commercial *Oncidium* species, a possibility that requires further investigation. Accurate Orchid taxonomy is not only desirable for evolutionary studies, but is important for orchid breeding.

**Supporting Information**

**Figure S1** Sequences of Oncidiinae (yellow), Cypripedioideae (red), Eulophiinae (cyan), and Maxillariae subtriche (pink) of Cymbidiae tribe, Vandeae tribe (green), Podochilae tribe (orange), Sobraliae subtriche (blue), Collabinae subtriche (gray), and Dendrobieae subtriche (purple) were downloaded from NCBI and analyzed by using VectorNTI AlignX software program. (TIF)

**Author Contributions**

Conceived and designed the experiments: MTC CSL. Performed the experiments: ICP DCL FHW. Analyzed the data: HD NDS. Contributed reagents/materials/analysis tools: CC MCS MTC CSL. Wrote the paper: ICP CSL.

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