



# Parentage determination of *Vanda* Miss Joaquim (Orchidaceae) through two chloroplast genes *rbcl* and *matK*

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## Abstract

### Background and aims

The popular hybrid orchid *Vanda* Miss Joaquim was made Singapore's national flower in 1981. It was originally described in the *Gardeners' Chronicle* in 1893, as a cross between *Vanda hookeriana* and *Vanda teres*. However, no record had been kept as to which parent contributed the pollen. This study was conducted using DNA barcoding techniques to determine the pod parent of *V. Miss Joaquim*, thereby inferring the pollen parent of the hybrid by exclusion.

### Methodology

Two chloroplast genes, *matK* and *rbcl*, from five related taxa, *V. hookeriana*, *V. teres* var. *alba*, *V. teres* var. *andersonii*, *V. teres* var. *aurorea* and *V. Miss Joaquim* 'Agnes', were sequenced. The *matK* gene from herbarium specimens of *V. teres* and *V. Miss Joaquim*, both collected in 1893, was also sequenced.

### Principal results

No sequence variation was found in the 600-bp region of *rbcl* sequenced. Sequence variation was found in the *matK* gene of *V. hookeriana*, *V. teres* var. *alba*, *V. teres* var. *aurorea* and *V. Miss Joaquim* 'Agnes'. Complete sequence identity was established between *V. teres* var. *andersonii* and *V. Miss Joaquim* 'Agnes'. The *matK* sequences obtained from the herbarium specimens of *V. teres* and *V. Miss Joaquim* were completely identical to the sequences obtained from the fresh samples of *V. teres* var. *andersonii* and *V. Miss Joaquim* 'Agnes'.

### Conclusions

The pod parent of *V. Miss Joaquim* 'Agnes' is *V. teres* var. *andersonii* and, by exclusion, the pollen parent is *V. hookeriana*. The herbarium and fresh samples of *V. teres* var. *andersonii* and *V. Miss Joaquim* share the same inferred maternity. The *matK* gene was more informative than *rbcl* and facilitated differentiation of varieties of *V. teres*.

## Introduction

### DNA barcoding

In angiosperms, plastid genes are generally transmitted through the maternal line (Birky 1994; Mogensen 1996). By sequencing plastid genes, maternal identification is possible. DNA barcoding is an initiative aimed at giving

a genetic identity to all organisms, and is at present most commonly applied to animals and plants. For animals, the mitochondrial gene, *cytochrome c oxidase I (COI)*, has proven to be sufficiently informative (Hebert *et al.* 2003). However, due to the lower mitochondrial substitution rate in plants (cited from Newmaster *et al.* 2006), other plastid gene regions

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have had to be evaluated for use—these include *atpF-atpH* spacer, *matK*, *rbcl*, *rpoB*, *rpoC1*, *psbK-psbI* spacer and *trnK-psbA* spacer (CBOL Plant Working Group 2009). The Consortium for the Barcode of Life (CBOL) has recommended a combination of *rbcl* and *matK* for DNA barcoding of plants (CBOL Plant Working Group 2009). It is important to note that DNA barcoding is not equivalent to molecular taxonomy (Newmaster et al. 2006). The purpose of DNA barcoding is primarily for the expedient identification of new species (Chase et al. 2005; Newmaster et al. 2006). Even so, the chloroplast genes *rbcl* and *matK* have been used in many phylogenetic studies across plant families and in the Orchidaceae (Cameron et al. 1999; Goldman et al. 2001; Hidayat et al. 2005; Barrett and Freudenstein 2008; Freudenstein and Senyo 2008; Cameron 2009; CBOL Plant Working Group 2009).

The chloroplast gene *rbcl* encodes ribulose-1,5-bisphosphate carboxylase oxygenase. It is ~1.4 kb in length. Another chloroplast gene, *matK*, is located within the *trnK* intron. This 1.5-kb gene encodes a maturase required for chloroplast activities such as photosynthesis (Barthet and Hilu 2007). The *matK* region has been shown to have a significantly faster substitution rate than *rbcl*—up to 3-fold faster at the nucleotide level and 6-fold at the amino acid level (Johnson and Soltis 1994; Olmstead and Palmer 1994; Xiang et al. 1998; Muller et al. 2006). *matK* is considered to be one of the most informative loci for determining phylogenetic relationships (Hilu et al. 2003).

### Vanda Miss Joaquim

The precise identification of an orchid hybrid's parentage is of utmost importance to botanists, breeders and enthusiasts alike. The parental characteristics give an indication of a hybrid's growth requirements, flowering habit and other phenotypic aspects. There are occasional instances when the parentage of a particular orchid hybrid is disputed. Some examples include *Vanda* Tan Chay Yan, *Vanda* Mimi Palmer (Chew 2008) and the more exalted *Vanda* Miss Joaquim, Singapore's national flower. We have chosen *V. Miss Joaquim* as our case study to evaluate the feasibility of using DNA barcoding for the identification of the maternal parent of an orchid hybrid. Although some *Vanda* species have been assigned to the genus *Papilionanthe* (Tay 2004), we have chosen to use *Vanda* for ease of reference.

The hybrid *V. Miss Joaquim* arose in the garden of Miss Agnes Joaquim, an Armenian lady residing in Singapore during the late 19th to early 20th century. *Vanda* Miss Joaquim is the result of a cross between *Vanda hookeriana* and *Vanda teres*. Unfortunately, there was no record of which plant contributed the pollen (Ridley 1893).

Nevertheless, H. N. Ridley, then Director of the Singapore Botanic Gardens, described the hybrid in the *Gardeners' Chronicle* in 1893—the Royal Horticultural Society records *V. hookeriana* as the seed parent and *V. teres* as the pollen parent. In herbarium records at the Singapore Botanic Gardens, the *V. Miss Joaquim* specimen, filed by Ridley in 1893, is recorded as *Vanda* Agnes Joaquim and its parentage is described as (*V. teres* × *V. hookeriana*).

In the ensuing years, culture of the plant spread beyond the shores of Singapore, first to Malaysia, then to Hawaii (Yeoh 1963b; Alphonso 1981). *Vanda* Miss Joaquim was selected as Singapore's national flower on 15 April 1981 (Alphonso 1981). It is the only hybrid orchid designated as a national flower. *Vanda* Miss Joaquim is also the first orchid hybrid from Singapore to be registered and the first hybrid *Vanda* in the world to have been registered with the Royal Horticultural Society (Chew 2007).

### The parental species, variants and relevant crosses

*Vanda teres* is native to Burma (Myanmar) and its distribution extends to northeastern India and the Himalayas (Veitch and Sons 1893; Holttum 1953). In its natural habitat, it is 'found in the hot plains and valleys scrambling over the branches of the largest trees and exposed to the full glare of the sun' (Veitch and Sons 1893). In his *Orchids of Malaya*, Holttum (1953) described four varieties of *V. teres*: var. *gigantea*, var. *aurorea*, var. *andersonii* (also spelt *andersonii*) and var. *alba*. In this study, we used three varieties of *V. teres*: var. *aurorea*, var. *andersonii* and var. *alba*. It is not known which variety of *V. teres* gave rise to *V. Miss Joaquim* (Alphonso 1981); however, *V. teres* var. *aurorea* and *V. teres* var. *andersonii* are more common (Yam 1999).

*Vanda hookeriana* is a Malaysian species found in wet or swampy sites in Johore and the Kinta Valley in Malaysia (Yeoh 1963a; Dourado 1979). It is also known as the Kinta Weed (Yeoh 1963a; Dourado 1979). *Vanda hookeriana* requires more shade and moisture than other similar species and, in its native habitat, can be found scrambling between small shrubs and bushes (Yeoh 1963a). There are two varieties of *V. hookeriana*: the coloured variety and the *alba* type. We were unable to obtain the *alba* variety for this study as it is extremely rare in the wild (Laycock 1931; Dourado 1979) and may have well disappeared from cultivation (Tay 2004).

*Vanda* Miss Joaquim is a vigorous grower and flowers freely year round. Its leaves have inherited the characteristic kink from *V. hookeriana* but are, on the whole, intermediate between *V. hookeriana* and *V. teres* (Ridley 1893). Additional varieties of *V. Miss Joaquim* include 'Rose Marie', 'Douglas' (Teoh 1981) and 'Singapore Botanic Gardens' (Teoh 1981). In this article,

we deal specifically with the parentage of the ‘Agnes’ variety, which is the varietal name retrospectively given to the original *V. Miss Joaquim* (Alphonso 1981; Teoh 1981). It has been speculated that, based on flower morphology, the variety of *V. teres* that gave rise to *V. Miss Joaquim* is var. *aurorea* (Yam 1999). The coloured variety of *V. hookeriana* has been suggested as the other parent of *V. Miss Joaquim* (Yam 1999).

In this study, we set out to resolve the issue of parentage by tracing the maternal line using DNA sequencing methods. We report here the results obtained by sequencing the *rbcl* and *matK* genes from *V. hookeriana*, *V. teres* and *V. Miss Joaquim*. We also sequenced the *matK* gene of the original herbarium specimens of *V. Miss Joaquim* collected by H. N. Ridley in 1893 and *V. teres* collected by Miss Joaquim in 1893.

## Materials and methods

### Plant specimens used

Fresh samples were collected from the Singapore Botanic Gardens and the National Institute of Education: *V. Miss Joaquim* ‘Agnes’, *V. hookeriana*, *V. teres* var. *alba*, *V. teres* var. *andersonii* and *V. teres* possibly var. *aurorea*. Only the *alba* variety was in flower when we collected the specimens, and therefore our identification of the other two varieties is based first on the fact that the *V. teres* var. *andersonii* was so labelled at the Singapore Botanic Gardens and, second, on Holttum’s description (Holttum 1953) that the leaves of *V. teres* var. *aurorea* are ‘green, not spotted’ and the leaves of *V. teres* var. *andersonii* are ‘green, curved upwards towards stem’. Additional fresh samples were obtained from J. B. Tay—these comprise two subvarieties of *V. teres* var. *alba*, two subvarieties of *V. teres* var. *andersonii* and one specimen of *V. Miss Joaquim* ‘Agnes’.

Herbarium samples were obtained from the SING Herbarium at the Singapore Botanic Gardens: *V. Miss Joaquim* from 1893, collector H. N. Ridley, *V. teres* from 1893, collector Miss Joaquim, *V. hookeriana* collected from Kampar Swamp, Perak, Malaysia, in 1895 by Charles Curtis, and that collected from Kinta Valley, Malaysia, in 1889 by E. Brewster.

All plant samples were deposited at the SING Herbarium and given specimen voucher numbers SING 2010-760 (*V. teres* var. *andersonii*), SING 2011-014 (*V. Miss Joaquim* ‘Agnes’), 2011-015 (*V. teres* var. *aurorea*), SING 2011-016 (*V. teres* var. *alba*) and 2011-017 (*V. hookeriana*).

### Amplification and sequencing

The Finnzymes Phire Plant Direct PCR kit was used for all polymerase chain reactions (PCR). Plant samples were

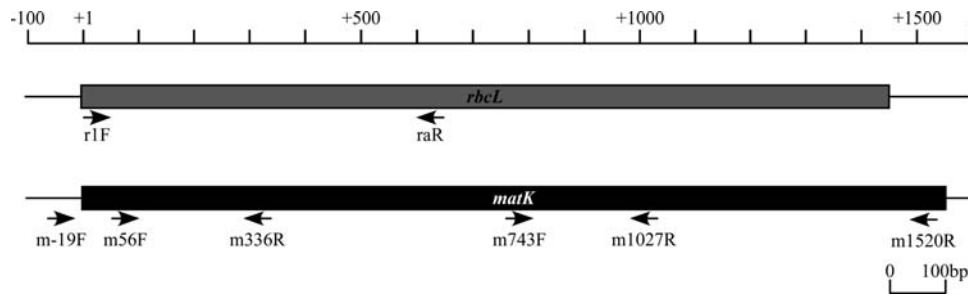
crushed in the dilution buffer provided with the Phire Plant Direct PCR kit. For fresh plant samples, PCR conditions were as follows: denaturation temperature 98 °C, annealing temperature 55 °C for *rbcl*L1F/*rbcl*LaR, *matK*56F/*matK*1027R and *matK*56F/*matK*1520R primer pairs, and extension at 72 °C, for a total of 40 cycles. For herbarium specimens, the nested PCR technique was employed, as previously described (Sambrook and Russell 2001). Primers *matK*-19F and *matK*1520R were used for the first-round nested PCR with denaturation and extension temperatures as described above. Primer annealing was done at 45 °C and the PCR was cycled 50 times. Second-round nested PCR was done using primers *matK*56F and *matK*1027R and cycling conditions were as described above for the primer pair. Primers used for amplification of *rbcl*: *rbcl*L1F 5’-ATG TCA CCA CAA ACA GAA ACT AAA GC-3’ (Molvray et al. 2000) and *rbcl*LaR 5’-CTT CTG CTA CAA ATA AGA ATC GAT CTC-3’ (Kress et al. 2005; Kress and Erickson 2007; Fazekas et al. 2008) (Fig. 1). Primers used for amplification of *matK*: *matK*-19F 5’-CGT TCT GAC CAT ATT GCA CTA TG-3’ (Molvray et al. 2000; Goldman et al. 2001; Pridgeon et al. 2001; Salazar et al. 2003; Whitten et al. 2007; van den Berg et al. 2009), *matK*56F 5’-GGC AAC AAA ACT TCC TAT ATC C-3’, *matK*743F 5’-CTT CTG GAG TCT TTC TTG AGC-3’, *matK*1520R 5’-CGG ATA ATG TCC AAA TAC CAA ATA-3’ (Whitten et al. 2000; Williams et al. 2001; Chase et al. 2009; Neubig et al. 2009), *matK*1027R 5’-CCA GAA AAG AGA AGG AAT AAT TGG-3’ and *matK*336R 5’-GAC TCC AAA ACC TTC TGA TAC C-3’ (Fig. 1). PCR products were purified using Wizard® PCR and Gel Clean-up System (Promega Corporation, Madison, WI, USA), according to the manufacturer’s recommendations.

Purified PCR products were sent to AITBiotech Pte Ltd, Singapore, for sequencing. All samples were sequenced bi-directionally. Sequencing for *rbcl* was done in both directions using the *rbcl*L1F and *rbcl*LaR primers. Sequencing for *matK* was also done bi-directionally using primers *matK*56F and *matK*1027R. To obtain complete sequence coverage of the *matK* amplification product, the primers *matK*743F and *matK*336R were designed and used for sequencing. The larger *matK* fragment amplified using the primers *matK*56F and *matK*1520R was sequenced using primers *matK*56F, *matK*1027R and *matK*1520R.

### Sequence analyses

Sequence data were analysed using Lasergene SeqMan software from DNASTAR, Inc., Madison, WI, USA. Nucleotide and amino acid sequence alignments were performed using the ClustalW algorithm (Thompson et al. 1994) provided by the Lasergene MegAlign software





**Fig. 1 Representation of *rbcl* and *matK* gene structure and corresponding primer positions.** Units on the ruler represent 100-bp increments. Grey and black bars represent the open reading frames (ORFs) of *rbcl* and *matK*, respectively. Primers are represented by arrows: R1F (*rbcl*1F); rAR (*rbcl*aR); m-19F (*matK*-19F); m56F (*matK*56F); m336R (*matK*336R); m743F (*matK*743F); m1027R (*matK*1027R); m1520R (*matK*1520R).

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Vh      GCTGGTGTAAAGATTACAAATTGACTTATTACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACCCCTCAACCCGGAGTTCGCCCTGAAGAAGCAGGG
Vt_Al  GCTGGTGTAAAGATTACAAATTGACTTATTACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACCCCTCAACCCGGAGTTCGCCCTGAAGAAGCAGGG
Vt_An  GCTGGTGTAAAGATTACAAATTGACTTATTACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACCCCTCAACCCGGAGTTCGCCCTGAAGAAGCAGGG
Vt_Au  GCTGGTGTAAAGATTACAAATTGACTTATTACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACCCCTCAACCCGGAGTTCGCCCTGAAGAAGCAGGG
VMJ    GCTGGTGTAAAGATTACAAATTGACTTATTACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACCCCTCAACCCGGAGTTCGCCCTGAAGAAGCAGGG
consensus 1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....110.....120

Vh      GCTCGGTTAGCTGCCGAATCTTCTACTGTTACATGGACAACCTGTGGACTGATGGACTTACCAGCTTGTGATCGTTACAAAGGACGATGCTACCACATCGAGGTCGTTGTTGGGAGGAA
Vt_Al  GCTCGGTTAGCTGCCGAATCTTCTACTGTTACATGGACAACCTGTGGACTGATGGACTTACCAGCTTGTGATCGTTACAAAGGACGATGCTACCACATCGAGGTCGTTGTTGGGAGGAA
Vt_An  GCTCGGTTAGCTGCCGAATCTTCTACTGTTACATGGACAACCTGTGGACTGATGGACTTACCAGCTTGTGATCGTTACAAAGGACGATGCTACCACATCGAGGTCGTTGTTGGGAGGAA
Vt_Au  GCTCGGTTAGCTGCCGAATCTTCTACTGTTACATGGACAACCTGTGGACTGATGGACTTACCAGCTTGTGATCGTTACAAAGGACGATGCTACCACATCGAGGTCGTTGTTGGGAGGAA
VMJ    GCTCGGTTAGCTGCCGAATCTTCTACTGTTACATGGACAACCTGTGGACTGATGGACTTACCAGCTTGTGATCGTTACAAAGGACGATGCTACCACATCGAGGTCGTTGTTGGGAGGAA
consensus .....130.....140.....150.....160.....170.....180.....190.....200.....210.....220.....230.....240

Vh      AATCAATATATTGCTTATGTAGCTTATCCTTTAGACCTTTTGAAGAAGGTTCTGTACTAACATGTTTACTTCCATTGTGGTAAATGATTTGGTTTCAAAGCCCTGGAGCTCTACGT
Vt_Al  AATCAATATATTGCTTATGTAGCTTATCCTTTAGACCTTTTGAAGAAGGTTCTGTACTAACATGTTTACTTCCATTGTGGTAAATGATTTGGTTTCAAAGCCCTGGAGCTCTACGT
Vt_An  AATCAATATATTGCTTATGTAGCTTATCCTTTAGACCTTTTGAAGAAGGTTCTGTACTAACATGTTTACTTCCATTGTGGTAAATGATTTGGTTTCAAAGCCCTGGAGCTCTACGT
Vt_Au  AATCAATATATTGCTTATGTAGCTTATCCTTTAGACCTTTTGAAGAAGGTTCTGTACTAACATGTTTACTTCCATTGTGGTAAATGATTTGGTTTCAAAGCCCTGGAGCTCTACGT
VMJ    AATCAATATATTGCTTATGTAGCTTATCCTTTAGACCTTTTGAAGAAGGTTCTGTACTAACATGTTTACTTCCATTGTGGTAAATGATTTGGTTTCAAAGCCCTGGAGCTCTACGT
consensus .....250.....260.....270.....280.....290.....300.....310.....320.....330.....340.....350.....360

Vh      CTGGAAGATCTCGGAATTCGCCCTTCTTATTCCAAAACCTTCCAAAGTCCGGCTCATGGCATCCAAAGTGAAGAGAGATAAATGAACAAGTATGGTCGTCCTCCATGGGATGCTACTATT
Vt_Al  CTGGAAGATCTCGGAATTCGCCCTTCTTATTCCAAAACCTTCCAAAGTCCGGCTCATGGCATCCAAAGTGAAGAGAGATAAATGAACAAGTATGGTCGTCCTCCATGGGATGCTACTATT
Vt_An  CTGGAAGATCTCGGAATTCGCCCTTCTTATTCCAAAACCTTCCAAAGTCCGGCTCATGGCATCCAAAGTGAAGAGAGATAAATGAACAAGTATGGTCGTCCTCCATGGGATGCTACTATT
Vt_Au  CTGGAAGATCTCGGAATTCGCCCTTCTTATTCCAAAACCTTCCAAAGTCCGGCTCATGGCATCCAAAGTGAAGAGAGATAAATGAACAAGTATGGTCGTCCTCCATGGGATGCTACTATT
VMJ    CTGGAAGATCTCGGAATTCGCCCTTCTTATTCCAAAACCTTCCAAAGTCCGGCTCATGGCATCCAAAGTGAAGAGAGATAAATGAACAAGTATGGTCGTCCTCCATGGGATGCTACTATT
consensus .....370.....380.....390.....400.....410.....420.....430.....440.....450.....460.....470.....480

Vh      AAACCAAATGGGATTATCTGCAAAAACCTACGGTAGAGCGGTTTATGAATGCTTAAGGGGTGGACTGATTTTACTAAGGATGATGAAAACGTAATTCACAACCATTTATGCGTTGG
Vt_Al  AAACCAAATGGGATTATCTGCAAAAACCTACGGTAGAGCGGTTTATGAATGCTTAAGGGGTGGACTGATTTTACTAAGGATGATGAAAACGTAATTCACAACCATTTATGCGTTGG
Vt_An  AAACCAAATGGGATTATCTGCAAAAACCTACGGTAGAGCGGTTTATGAATGCTTAAGGGGTGGACTGATTTTACTAAGGATGATGAAAACGTAATTCACAACCATTTATGCGTTGG
Vt_Au  AAACCAAATGGGATTATCTGCAAAAACCTACGGTAGAGCGGTTTATGAATGCTTAAGGGGTGGACTGATTTTACTAAGGATGATGAAAACGTAATTCACAACCATTTATGCGTTGG
VMJ    AAACCAAATGGGATTATCTGCAAAAACCTACGGTAGAGCGGTTTATGAATGCTTAAGGGGTGGACTGATTTTACTAAGGATGATGAAAACGTAATTCACAACCATTTATGCGTTGG
consensus .....490.....500.....510.....520.....530.....540.....550.....560.....570.....580.....590.....600
    
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**Fig. 2 Nucleotide sequence alignment of a 600-bp fragment spanning the +43 to +642 region of the 1441-bp *rbcl* open reading frame, using the *Phalaenopsis aphrodite* (AY16449.1) *rbcl* sequence as reference.** Vh, *V. hookeriana*; Vt\_Al, *V. teres* var. *alba*; Vt\_An, *V. teres* var. *andersonii*; Vt\_Au, *V. teres* var. *aurea*; VMJ, *V. Miss Joaquim*.

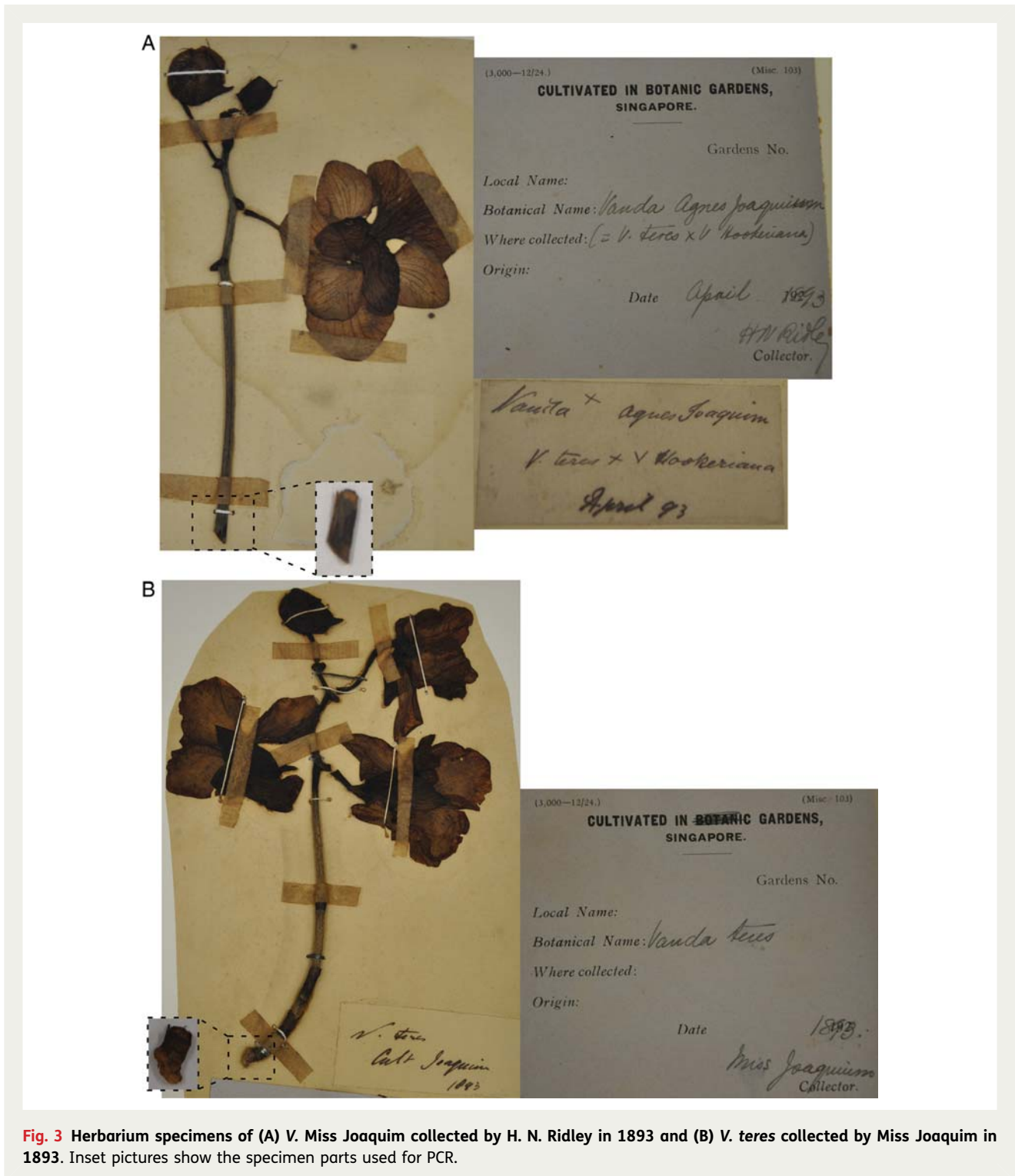
from DNASTAR, Inc., Madison, WI, USA. DNA bases showing similarity or identity were highlighted using the BoxShade software available on <http://mobyle.pasteur.fr/cgi-bin/portal.py?form=boxshade>.

## Results

### *rbcl*

We amplified and sequenced the *rbcl* gene from all five fresh plant samples (see Materials and methods). Polymerase chain reaction for each sample was done in duplicate and each PCR product was sequenced in

both directions using primers *rbcl*1F and *rbcl*aR. As the amplification product was ~600-bp, no internal primers were necessary to obtain a full sequence in either direction. Replicate sequences for each sample were aligned and a consensus sequence was obtained. An alignment of consensus nucleotide sequences from all samples was done and no nucleotide variation was observed (Fig. 2). As no base variation was observed, a third replicate for PCR and sequencing was not necessary. This was also the reason why PCR of the *rbcl* gene from the herbarium specimens was not attempted.



### *matK*

Amplification of the *matK* gene from the five fresh plant samples was done in triplicate. For the herbarium specimens (Fig. 3), the nested PCR technique was employed

and replicate amplification experiments were attempted. Repeated attempts at amplifying *matK* from all herbarium material for *V. hookeriana* failed. Only one successful nested PCR product was obtained for *V. Miss Joaquim*.

For *V. teres*, two separate PCR reactions using primers matK-19F and matK1520R were successful and these were re-amplified using the internal nested primers matK56F and matK1027R. A total of three amplification products were finally obtained. All amplification products were sequenced in both directions using primers matK56F and matK1027R. As the amplification product was 1-kb long, internal primers matK743F and matK336R were used to obtain complete sequence coverage of the products. Replicate sequences from each sample were aligned to obtain a consensus nucleotide sequence, and these consensus sequences were used in the final alignment (Fig. 4). The nucleotide sequence alignment (Fig. 4) reveals base variations at 10 sites within the 1-kb sequence length aligned. The alignment was trimmed to a 720-bp region (Fig. 4) within which the 10 base variations are clustered. Codon positions of the base variants are summarized in Table 1. Translations of the nucleotide sequences were performed *in silico* and the predicted amino acid sequences were aligned (Fig. 5). The amino acid sequence alignment indicates that the variable nucleotides translate into sequence substitutions at the amino acid level (Fig. 5). From the nucleotide and protein sequence alignments, it is apparent that the sequence of *V. teres* var. *andersonii* is identical to the sequence of *V. Miss Joaquim*. In addition, the *matK* sequence of the fresh sample of *V. Miss Joaquim* is identical to that of the herbarium specimens for *V. Miss Joaquim* and *V. teres*. The maximum number of nucleotide base variations between samples is seven base substitutions between *V. hookeriana* and *V. teres* var. *andersonii*.

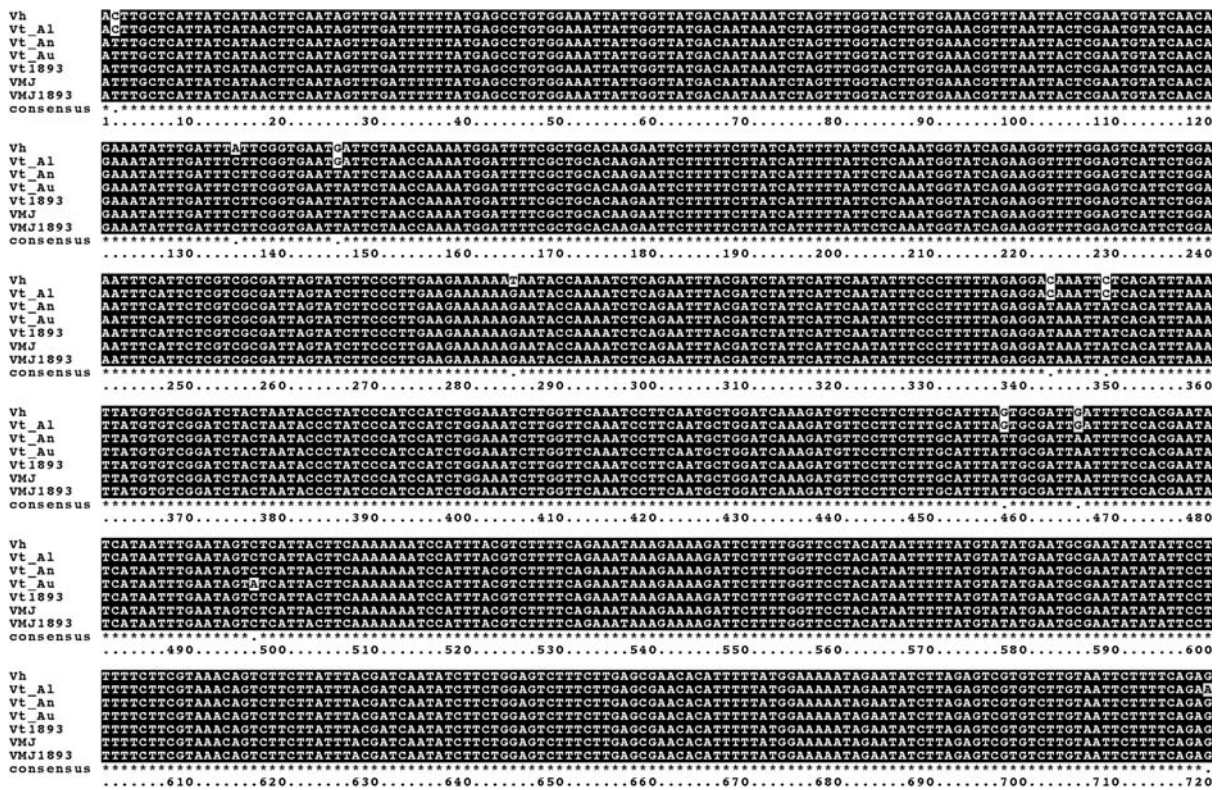
To obtain a better sampling of specimens, additional fresh samples of *V. teres* var. *alba*, *V. teres* var. *andersonii* and *V. Miss Joaquim* ‘Agnes’, along with our existing samples of *V. hookeriana*, *V. teres* var. *alba* and *V. teres* var. *aurora*, were amplified over an ~1.5-kb extended region demarcated by primers matK56F (this study) and matK1520R (Whitten *et al.* 2000; Williams *et al.* 2001; Chase *et al.* 2009; Neubig *et al.* 2009). This region encompasses the standard barcode regions defined by the 1R\_KIM and 3F\_KIM primers (Fazekas *et al.* 2008), and the matK390F and matK1326R primers (Cuénoud *et al.* 2002). Amplification products were sequenced once using the primers matK56F, matK1027R and matK1520R (Fig. 1). The three sequences obtained for each amplification product were assembled to form contigs. These contigs were then aligned to give an interspecific *matK* alignment (Fig. 6). Additional variation was observed only for *V. hookeriana* and *V. teres* var. *alba* in this extended amplification product. The results obtained confirm our earlier finding that the *matK* sequence of *V. teres* var. *andersonii* is identical to that of *V. Miss Joaquim* ‘Agnes’.

## Discussion

During this study, we only observed flowering of the *V. Miss Joaquim* (Fig. 7E) and *V. teres* var. *alba* (Fig. 7B) plants. *Vanda hookeriana*, *V. teres* var. *andersonii* and *V. teres* var. *aurora* did not flower. In order to identify the plants, we relied heavily on leaf morphology (Fig. 7) and the plant labelling of *V. hookeriana* and *V. teres* var. *andersonii* at the Singapore Botanic Gardens. Identification of *V. hookeriana* (Fig. 7A) was straightforward as it unmistakably possessed the characteristic leaf kink. *Vanda hookeriana* stem and leaves were also clearly less stout than in *V. teres*, a growth form that seems less vigorous. Our *V. teres* var. *andersonii* (Fig. 7C) has leaves that are curved towards the stem, matching Holttum’s description (Holttum 1953). In addition, it possesses purple spots, which although not mentioned by Holttum to be present in *V. teres* var. *andersonii*, is specifically mentioned as being absent from the variety *aurora* (Holttum 1953). Thus, our identification of *V. teres* var. *aurora* (Fig. 7D) relies on the absence of spots; the slight curvature of the leaves towards the stem is less pronounced than that in *V. teres* var. *andersonii*; and it is the other most common variety besides *V. teres* var. *andersonii*. Other *V. teres* varieties are unlikely as *V. teres* var. *gigantea* was mentioned by Holttum as having ‘straight purple-spotted leaves’ (Holttum 1953).

We were unable to find any sequence variation in the 600-bp region of the *rbcl* gene sequenced. This finding is in accordance with many other studies that have shown that *rbcl* has a slower rate of nucleotide substitution compared with other loci used for phylogenetic analyses. It is possible that sequencing a larger fragment of *rbcl* may reveal some sequence variations. We found *matK* to be a very useful locus for delineating the relationships between the plants studied. Sequence information obtained from *matK* alone facilitated the identification of the pod parent of *V. Miss Joaquim*. The *matK* sequences alone were sufficient to differentiate *V. teres* to the variety level. It is interesting to note that *V. teres* var. *alba* has a sequence that is more similar to *V. hookeriana* than any of the other *V. teres* varieties (Fig. 4 and Table 1). *Vanda teres* var. *alba* differs from *V. hookeriana* by three bases and from the other two *V. teres* varieties by seven bases. Only one base substitution distinguishes *V. teres* var. *aurora* from *V. teres* var. *andersonii* (Fig. 4 and Table 1). We found that the nucleotide substitution rate throughout all three codon positions in *matK* was fairly consistent, with a frequency of 0.4 for codon 1 and 0.3 for positions 2 and 3 (Table 1). Despite our small data set, our finding is in agreement with other reports that the substitution rate is





**Fig. 4** Nucleotide sequence alignment of a 720-bp region spanning +105 to +826 of the 1548-bp *matK* open reading frame. Vh, *V. hookeriana*; Vt\_Al, *V. teres* var. *alba*; Vt\_An, *V. teres* var. *andersonii*; Vt\_Au, *V. teres* var. *aurea*; VMJ, *V. Miss Joaquim*.

**Table 1** Variable sites in the *matK* sequence from Vh, *V. hookeriana*; Vt\_Al, *V. teres* var. *alba*; Vt\_An, *V. teres* var. *andersonii*; Vt\_Au, *V. teres* var. *aurea*; Vt1893, *V. teres* herbarium specimen from 1893; VMJ, *V. Miss Joaquim*; VMJ1893, *V. Miss Joaquim* herbarium specimen from 1893. VMJ was used as the anchor sequence and variable nucleotides are highlighted in bold.

Strain	Nucleotide position relative to translation start point (+1)									
	+106	+239	+250	+389	+447	+453	+562	+570	+601	+824
VMJ	T	C	T	G	T	A	T	A	C	G
VMJ1893	T	C	T	G	T	A	T	A	C	G
Vh	C	A	G	T	C	C	G	G	C	G
Vt_Al	C	C	G	G	C	C	G	A	C	A
Vt_An	T	C	T	G	T	A	T	A	C	G
Vt_Au	T	C	T	G	T	A	T	A	A	G
Vt1893	T	C	T	G	T	A	T	A	C	G
Codon position	1	2	1	2	3	3	1	3	1	2

comparable between all codon positions (Johnson and Soltis 1994; Hilu and Liang 1997; Xiang *et al.* 1998; Hilu *et al.* 2003; Barthet and Hilu 2008). The high

rate of amino acid substitution in *matK* seen in our study has also been noted in previous studies (Olmstead and Palmer 1994; Barthet and Hilu 2008).

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Vh      LAHYHNFNSLI F Y E P V E I I G Y D N K S S L V L V K R L I T R M Y Q O O K Y L I V S V N D S N Q N G F S L H K N
Vt_Al   LAHYHNFNSLI F Y E P V E I I G Y D N K S S L V L V K R L I T R M Y Q O O K Y L I S S V N D S N Q N G F S L H K N
Vt_An   FAHYHNFNSLI F Y E P V E I I G Y D N K S S L V L V K R L I T R M Y Q O O K Y L I S S V N Y S N Q N G F S L H K N
Vt_Au   FAHYHNFNSLI F Y E P V E I I G Y D N K S S L V L V K R L I T R M Y Q O O K Y L I S S V N Y S N Q N G F S L H K N
VMJ     FAHYHNFNSLI F Y E P V E I I G Y D N K S S L V L V K R L I T R M Y Q O O K Y L I S S V N Y S N Q N G F S L H K N
consensus *****
1.....10.....20.....30.....40.....50.....60

Vh      SFSYHFYSQMVSEGFVILEISFSSRLVSSLEEKRIIPKSONLRSIHSIFPFLEDKLSHLN
Vt_Al   SFSYHFYSQMVSEGFVILEISFSSRLVSSLEEKRIIPKSONLRSIHSIFPFLEDKLSHLN
Vt_An   SFSYHFYSQMVSEGFVILEISFSSRLVSSLEEKRIIPKSONLRSIHSIFPFLEDKLSHLN
Vt_Au   SFSYHFYSQMVSEGFVILEISFSSRLVSSLEEKRIIPKSONLRSIHSIFPFLEDKLSHLN
VMJ     SFSYHFYSQMVSEGFVILEISFSSRLVSSLEEKRIIPKSONLRSIHSIFPFLEDKLSHLN
consensus *****
.....70.....80.....90.....100.....110.....120

Vh      YVSDLLIPYPIHLEILVQILQCWIKDVPSLHLLVRLIFHEYHNLNSLITSKKSIIYVFSEIK
Vt_Al   YVSDLLIPYPIHLEILVQILQCWIKDVPSLHLLVRLIFHEYHNLNSLITSKKSIIYVFSEIK
Vt_An   YVSDLLIPYPIHLEILVQILQCWIKDVPSLHLLRLIFHEYHNLNSLITSKKSIIYVFSEIK
Vt_Au   YVSDLLIPYPIHLEILVQILQCWIKDVPSLHLLRLIFHEYHNLNSLITSKKSIIYVFSEIK
VMJ     YVSDLLIPYPIHLEILVQILQCWIKDVPSLHLLRLIFHEYHNLNSLITSKKSIIYVFSEIK
consensus *****
.....130.....140.....150.....160.....170.....180

Vh      KRFFWFLHNFYVYECEYIFLFLRKQSSYLRSISSGVFLERTHFYGKIEYLRVVS CNSFQR
Vt_Al   KRFFWFLHNFYVYECEYIFLFLRKQSSYLRSISSGVFLERTHFYGKIEYLRVVS CNSFQR
Vt_An   KRFFWFLHNFYVYECEYIFLFLRKQSSYLRSISSGVFLERTHFYGKIEYLRVVS CNSFQR
Vt_Au   KRFFWFLHNFYVYECEYIFLFLRKQSSYLRSISSGVFLERTHFYGKIEYLRVVS CNSFQR
VMJ     KRFFWFLHNFYVYECEYIFLFLRKQSSYLRSISSGVFLERTHFYGKIEYLRVVS CNSFQR
consensus *****
.....190.....200.....210.....220.....230.....240
    
```

**Fig. 5** *In silico* translation and amino acid alignment of *matK* from Vh, *V. hookeriana*; Vt\_Al, *V. teres* var. *alba*; Vt\_An, *V. teres* var. *andersonii*; Vt\_Au, *V. teres* var. *aureora*; VMJ, *V. Miss Joaquim*.

The sample size of this study was restricted by the difficulty in finding plants in bloom or that were clearly labelled. In particular, while it was easy to find plants labelled *V. teres*, only two plants were found labelled *V. teres* var. *andersonii*. Additional samples of *V. hookeriana*, *V. teres* var. *alba*, *V. teres* var. *andersonii* and *V. Miss Joaquim* ‘Agnes’ were obtained to address sampling effects. Amplification and sequencing of the extended 1.5-kb fragment of the *matK* gene, encompassing the standard barcode regions, from these additional plant samples and our existing specimens confirmed our earlier conclusions derived using the *matK*56F and *matK*1027R primers. We therefore conclude that the sequences obtained using the *matK*56F and *matK*1027R primers were indicative of the variation observed within the standard barcode regions defined by the 1R\_KIM, 3R\_KIM, *matK*390F and *matK*1326R primers.

The herbarium specimens served to confirm that *V. teres* was the pod parent of the original *V. Miss Joaquim* described in 1893. We have shown that the fresh sample of *V. teres* var. *andersonii* is identical in sequence to the herbarium specimen. In addition, the fresh sample of *V. Miss Joaquim* ‘Agnes’ is also identical in sequence to the herbarium specimen of *V. Miss Joaquim* from 1893. This suggests that the contemporary and herbarium samples of *V. Miss Joaquim* ‘Agnes’ share the same inferred maternal parent. The flowers

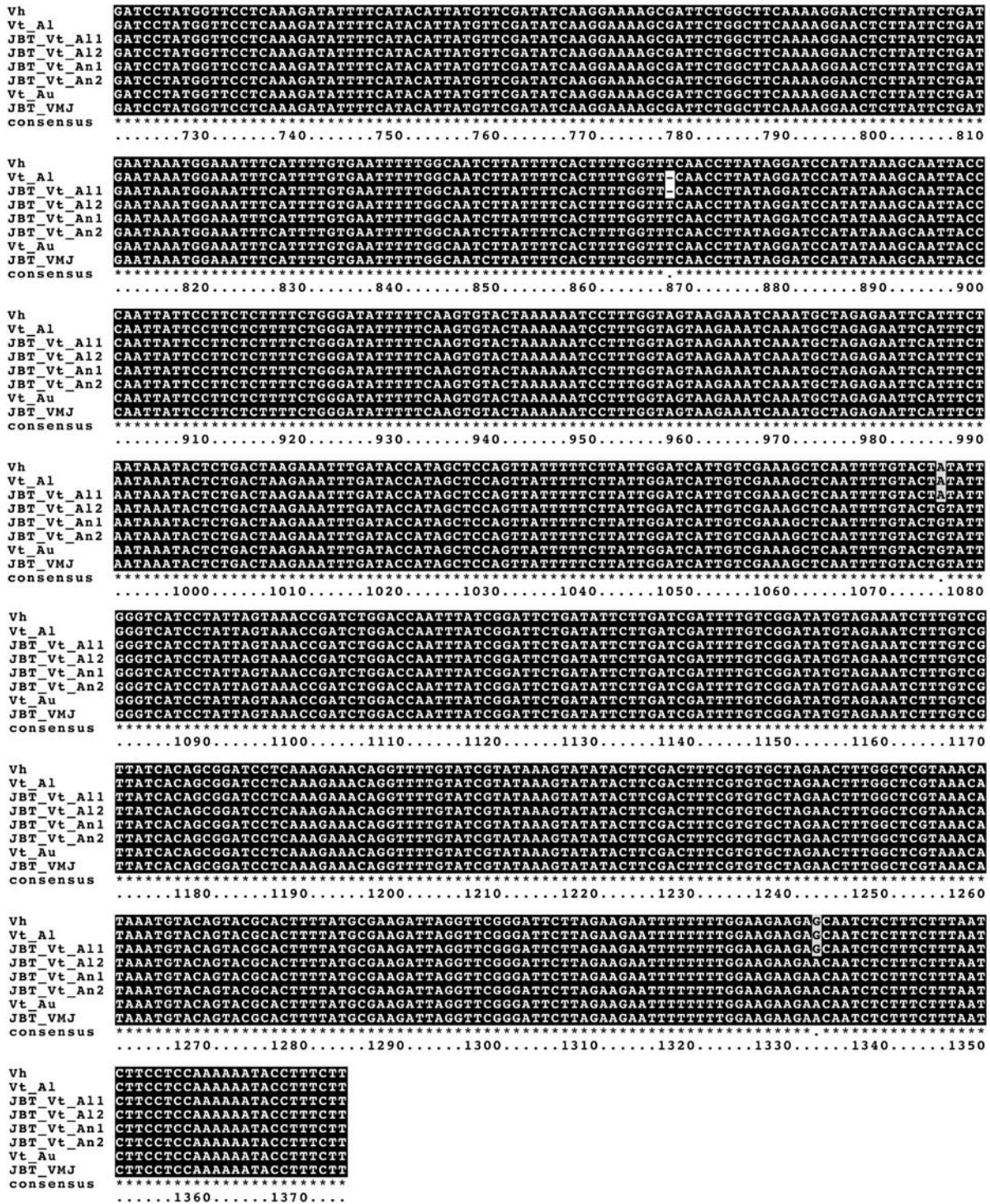
of the present-day *V. Miss Joaquim* remain morphologically similar to that described by Ridley (1893) and depicted by the Royal Horticultural Society in 1897 (Royal Horticultural Society Orchid Committee 1897; Royal Horticultural Society 2010) (Fig. 8).

Identification of plants can be challenging, if not impossible, when labels are lost or destroyed. We have shown that *matK* can be used to identify and distinguish terete *Vanda* species and varieties. In orchid hybridization, records of parentage are occasionally lost, ambiguous or erroneous. Due to the complexity of many orchid hybrids, sequencing nuclear genes could potentially be a long and difficult process. We have shown that DNA barcoding using the chloroplast gene *matK* is a useful technique for identification and/or confirmation of parentage, particularly when the maternal parent is disputed. We have shown that the direct PCR technique can be used to obtain amplification products from herbarium specimens. Due to the lack of high-molecular-weight DNA, the nested PCR technique may be employed to produce a higher yield, yet specific amplification product.

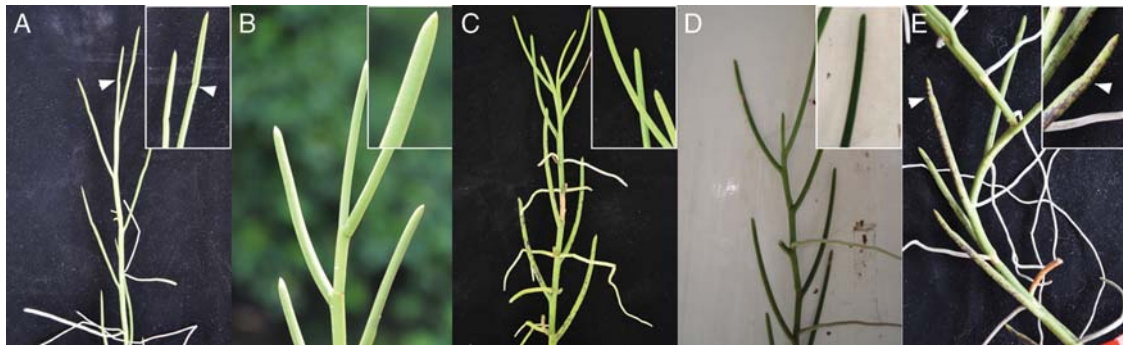
### Conclusions and forward look

In conclusion, we have answered a 118-year-old question of the pod parent of Singapore’s national flower, *V. Miss Joaquim*. We have shown through DNA amplification and sequencing of the original 1893 herbarium

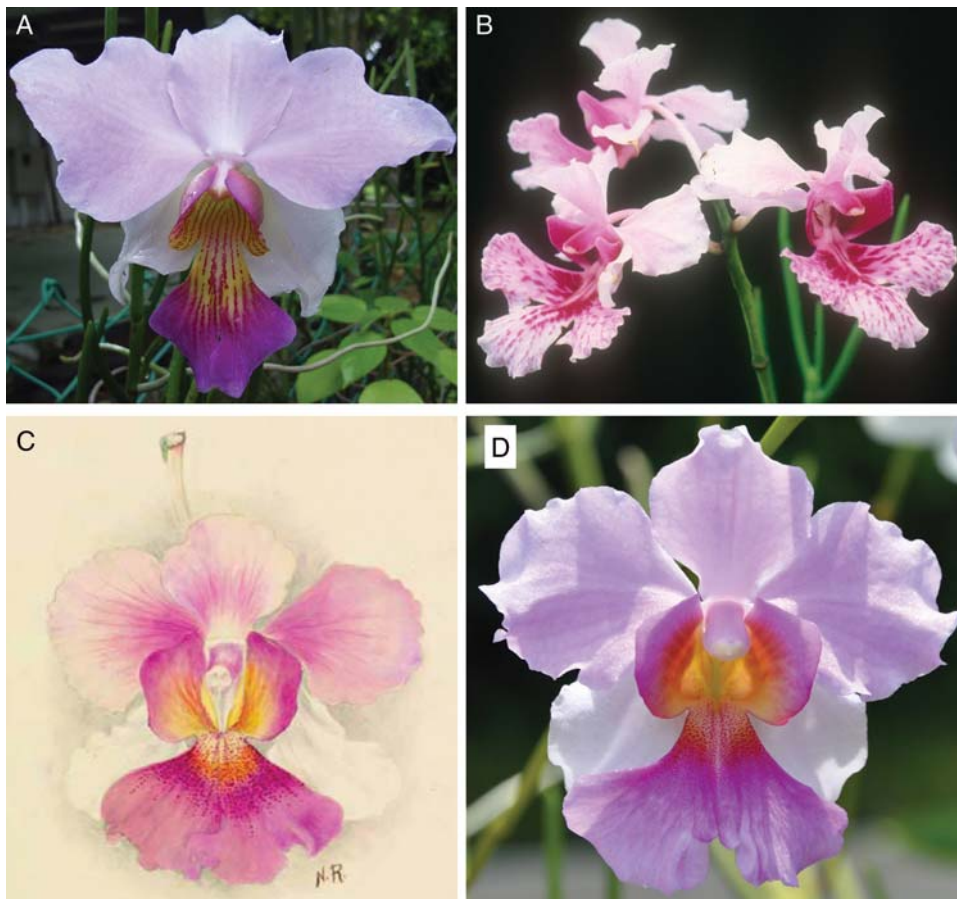




**Fig. 6** Nucleotide sequence alignment of a 654-bp region downstream of the *matK*56F/*matK*027R fragment spanning +827 to +1480 of the 1548-bp *matK* open reading frame. The aligned region between +105 and +826, within the *matK*56F/*matK*1027R fragment, has been omitted due to space constraints. Vh, *V. hookeriana*; Vt\_A1, *V. teres* var. *alba*; JBT\_Vt\_A11, *V. teres* var. *alba* subvar. 1 from J. B. Tay; JBT\_Vt\_A12, *V. teres* var. *alba* subvar. 2 from J. B. Tay; JBT\_Vt\_An1, *V. teres* var. *andersonii* subvar. 1 from J. B. Tay; JBT\_Vt\_An2, *V. teres* var. *andersonii* subvar. 2 from J. B. Tay; Vt\_Au, *V. teres* var. *auorea*; JBT\_VMJ, *V. Miss Joaquim* ‘Agnes’ from J. B. Tay.



**Fig. 7** Pictures of fresh plant specimens used for PCR. (A) *V. hookeriana*; (B) *V. teres* var. *alba*; (C) *V. teres* var. *andersonii*; (D) *V. teres* var. *aurea*; and (E) *V. Miss Joaquim* 'Agnes'. Inset pictures show an enlarged view of leaves and white arrows indicate kinks in leaves.



**Fig. 8** Pictures of flowers of specimens used in this study. (A) *V. teres* var. *andersonii* (J. B. Tay); (B) *V. hookeriana* (courtesy of the Singapore Botanic Gardens Library); (C) *V. Miss Joaquim* painted by Nelly Roberts in 1897 (Royal Horticultural Society 2010); (D) present-day *V. Miss Joaquim*.



specimens and present-day plants that the pod parent of *V. Miss Joaquim* is *V. teres* var. *andersonii* while the pollen parent, by exclusion, is *V. hookeriana*. In the course of our investigation, it was shown that *V. teres* var. *andersonii* and *V. teres* var. *aurorea* differed in their *matK* sequence by one nucleotide. The *rbcl* sequence of the various *Vanda* specimens did not show any sequence variation. This result concurs with observations in Leguminosae (Lavin *et al.* 2005) that the *matK* gene is suitable for orchid barcoding and parentage determination as it gives a much greater resolution at the infrafamilial level.

Further work can be done to confirm the role of *V. hookeriana* as the pollen parent of *V. Miss Joaquim* by using random amplification of polymorphic DNA. Additional specimens of *V. Miss Joaquim* and the various *V. teres* varieties can also be sequenced to minimize the probability of biased results due to sampling effects. The option of sequencing the entire chloroplast genome (Nock *et al.* 2011) for better resolution can also be considered, now that next-generation sequencing methods have made whole-genome sequencing much more cost effective.

### Accession numbers

All sequences obtained from fresh plant specimens have been deposited in the GenBank database under accession numbers HQ439013–HQ439022.

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### Contributions by the authors

Both authors conceptualized the project and experimental strategy. G.S.K. performed the experiments and prepared the manuscript. T.F.C. edited the manuscript.

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### Conflicts of interest statement

None declared.

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