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SEM AND PHYLOGENETIC ANALYSIS OF NATURALIZED AND CULTIVATED *EPIDENDRUM* IN HAWAII

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ABSTRACT. Naturalized populations of *Epidendrum* L. are found on a rocky hillside in Nuuanu-Pali and Olomana in the Koolau Mountains of Oahu, Hawaii. Scanning electron micrographs were taken to observe polymorphism among the pollinia, petals, leaves, and root tips of two *Epidendrum* specimens (one naturalized specimen from Nuuanu-Pali and one cultivated specimen in the greenhouse). SEM images of pollen from the naturalized *Epidendrum* revealed a length of 830.31 µm and a width of 462.58 µm. Pollen length from the cultivated cultivar, by comparison, was 724.60 µm and the width 276.17 µm. Differing cell structures on the lower surface of the petals were also observed. Polyhedral concave cells with numerous fossae (pits) were seen on the naturalized cultivar and elongated flattened cells on the cultivated one. Transections of the leaf of the naturalized specimen were much thinner (546.33 µm) compared to the thickness of the cultivated cultivar leaf (1505.83 µm), which contained more spongy parenchyma cells. A thinner root tip (1094.19 µm) was seen in the naturalized cultivar, as opposed to 1636.34 µm in the cultivated specimen. To compare relationships between these two specimens along with ten other unknown *Epidendrum* cultivars, we sequenced the plastid *trnL-F* gene region and conducted parsimony analysis among the naturalized *Epidendrum* from Nuuanu-Pali At least six changes separated these specimens into two clades. Shorter and longer plastid simple sequence repeats (cpSSR) from the *rps16-trnK* region support separation of the five *Epidendrum* genotypes evaluated into these two groups, including a naturalized *Epidendrum* from Olomana.

KEY WORDS: *Epidendrum*, scanning electron microscopy, *trnL-F*, *rps16-trnK*

Epidendrum L. species are native to the tropical Americas. They are found from sea level to 10,000 feet elevation, growing as terrestrials, epiphytes or lithophytes. The reed-stem *Epidendrum radicans* Pav. ex Lindl. bears racemes in many shapes and shades of color, such as red, orange, purple or white. Some are marked with yellow on the base of the labellum (Phillips & Hill 1998).

Luer (1975) mentioned that the hybrid *E.* ×obrienianum (*jamiesonis* x *radicans*) registered by Veitch in 1888 in England was brought to Hawaii and subsequently naturalized there. Jan Goo (orchid grower) made many hybrids in the 1940s in Hawaii using *E. radicans*, *E. ibaguense* Kunth, *E. ellipticum* Graham, *E. arachnoglossum* Rchb,f. ex André, and *E. cinnabarinum* Salzm. ex Lindl. Backcrosses were also made using primary hybrids including *E.* ×obrienianum (Sander & Wreford 1961). Unfortunately, many of his hybrids today that are commonly grown as garden plants or in greenhouses have lost their registered names (Rose & Rose 2005).

Scanning electron microscopy and molecular phylogenetic studies were conducted at the University of Hawaii - Manoa, University of Hawaii - Windward Community College, and the Royal Botanic Gardens, Kew, from summer 2006 to fall 2012 to determine the identity of the wild/naturalized *Epidendrum* accessions from Nuuanu-Pali and Olomana on Oahu and compare them to their cultivars commonly grown in the greenhouse.

Materials and methods

Scanning electron microscopy (SEM). – An *Epidendrum* specimen was collected from a rocky hillside in Nuuanu-Pali, Oahu. It had many roots along the stem. The leaf was 1.6 cm wide × 6 cm long. The flower was 3 cm wide × 3.3 cm high (WCC voucher # 934). The greenhouse-grown cultivar at Windward Community College had a leaf 2 cm wide × 10 cm long and a flower 2.6 cm wide × 3 cm high (WCC voucher # 935).

Pollinia, transverse sections of 2 mm-wide petals and leaves, and transverse sections of 5 mm-long root tips from wild-collected and cultivated accessions were fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 2 hr at 25 C. Tissues were washed twice in 0.1 M sodium cacodylate buffer for 10 minutes each and then post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hr. Specimens ere then dehydrated in a graded ethanol series as follows: 30% (2x for 5 min each), 50% (2x for 5 min each), 70% (3x for 5 min each), 85% (3x for 5 min each), 95% for 5 min, 98% for 10 min ,and 100% before being stored in the refrigerator overnight.

Tissues were then critical-point-dried in a SAMDRI-795 machine. Dried tissues were each mounted on carbon-conductive tape secured on metal stubs for gold-palladium coating using a Hummer 6.2 Sputter Coater. Scanning electron micrographs were taken with a Hitachi S-800 field emission SEM.

Plastid DNA amplification and sequencing. – Total genomic DNA was extracted from silica gel-exsiccated leaves of 12 accessions using the modified Doyle and Doyle (1987) protocol. Samples included one naturalized *Epidendrum* collected from Nuuanu-Pali and 11 cultivated *Epidendrum* plants obtained from four greenhouses (Table 1**)**. Samples were photographed, and living specimens were grown in the climate-controlled greenhouse at Windward Community College as voucher specimens. Target loci *trnL-F* were amplified in 25 µL volumes using standard polymerase chain reaction (PCR) protocols that included the addition of bovine serum antigen (BSA). Primers *trnLc* (5'-CGAAATCGGTAGACGCTACG -3') and *trnLf* (5'-ATITGAACTGGTGACACGAG -3') were used to amplify and sequence *trnL-F* (Taberlet *et al.* 1991). Following sequencing at the Jodrell Laboratory at the Royal Botanic Gardens, Kew, electropherograms were edited using Sequencher 3.1 software (GeneCode Corp., Ann Arbor, Michigan, USA). The resulting *trnL-F* data were analyzed and a dendrogram prepared to include an *Epidendrum ibaguense* nucleotide database available from GenBank for comparison.

Plastid simple sequence repeats (cpSSR).- Plant DNA was isolated using NucleoSpin® Plant II mini spin columns (Macherey-Nagel, Inc., Bethlehem, Pennsylvania). As previously described by Pinheiro *et al*. (2009), primers Epcp-02-Forward 5'-TTCTTGCTTCTTTTTGTGGA -3' and Epcp-02-Reverse 5'- ATTTGTTTGATACGCCATTG -3' were used to amplify the plastid locus Epcp-02 (*rps16-trnK*) from the five genotypes. Accuzyme DNA polymerase (Bioline, Taunton, Massachusetts), which has 3'-5' proofreading exonuclease activity, was used to reduce the rate of erroneous base insertions associated with non-proofreading DNA polymerase.

PCR products were ligated directly from the PCR reactions into the vector PCR-Blunt (Life Technologies, Grand Island, New York). Ligation reaction products were used to transform chemically competent *E. coli* (One Shot TOP10 cells; Life Technologies, Grand Island, New York). Plasmid

TABLE 1. List of silica gel-exsiccated leaves from 12 *Epidendrum* accessions.

No.	Plant labels	Leaf fwt (g)	Flower color and size (cm)	Leaf size (cm) and stem color
1	*Epi.* Clem (*Epi.* C)	6	dull orange, yellow callus. W: 2.5, L: 3	W: 2, L: 6.5
2	*Epi.* Inge (*Epi.* I)	6	purplish red, yellow callus. W: 2.6, L: 3	W: 2, L: 10
3	*Epi.* Clay 6	6	bright red, yellow callus, red dots. W:2.6, L: 3	W: 1.6, L: 6.2
4	*Epi.* Clay 3	6	pure yellow. W: 2.2, L: 2.4. Pedicel: 2.7	W: 4.5, L: 1.8. Purple stem
5	*Epi.* Clay 7	6	pure yellow. W: 2.2, L: 2.4. Pedicel: 2.5	W: 2, L: 7.5
6	*Epi.* Clay 1	6	brownish orange. W: 2.9, L: 3	W: 2, L: 4.4
7	*Epi.* 'Pali' (*Epi.* P)	6	bright red, yellow callus, red dots. W:3, L:3.3	W: 1.6, L: 6
8	*Epi.* Clay 2	6	possibly yellow/red	W: 1.9, L: 5.7
9	*Epi.* Clay 8	6	possibly red/orange	W: 1.3, L: 6
10	*Epi.* Clay 5	4.545	possibly red	W: 1.3, L: 4.6
11	*Epi.* Clay 4	3.468	possibly red/purple	W: 1.2, L: 4.5
12	*Epi.* small white (*Epi.* sm12)	6	white w/light purple base. W: 1.5, L: 1.7	W: 1.9, L: 7.5

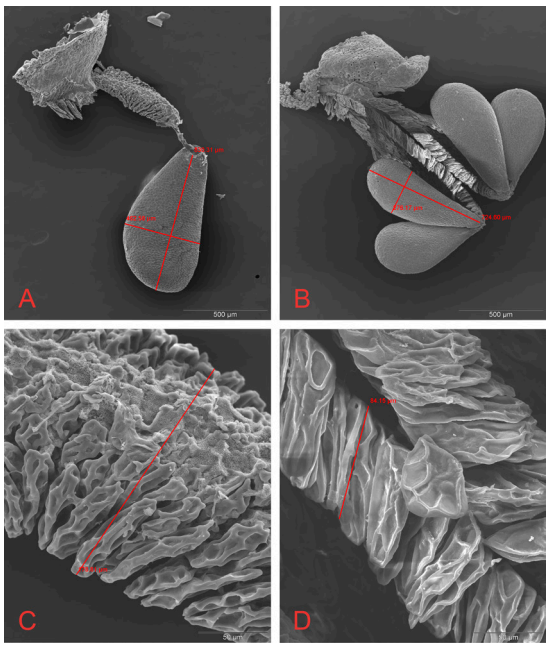

Figure 1. SEM images of pollinia (60x) and stipes (500x) of *E.* P and *E.* I. A. Pollen size of *E.* P (830.31 µm long and 462.58 µm wide); B. Pollen size pf *E.* I (724.60 µm long and 276.17 µm wide); C. Stipe of *E.* P (deeply folded and rugose, 178.81 µm wide); D. Stipe of *E.* I (84.15 µm wide).

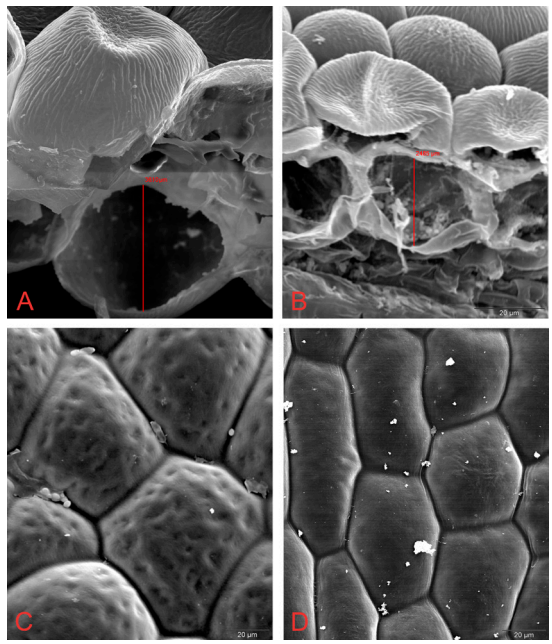

Figure 2. SEM images of the upper layer (1,300x) and the lower layer (800x) of petals of *E.* P and *E.* I. A. Bulbous cells on the upper layer of the petal of *E.* P (36.10 µm high); B. Bulbous cells on the upper layer of the petal of *E.* I (24.85 µm high); C. Polyhedral convex cells with numerous fossae seen on the lower layer of the petal of *E.* P; D. Elongated flattened cells on the lower layer of the petal of *E.* I.

DNA purification was performed using NucleoSpin® Plasmid mini spin columns (Macherey-Nagel, Inc., Bethlehem, Pennsylvania). Sequence analysis was conducted at University of Hawaii's Advanced Studies in Genomics, Proteomics and Bioinformatics using a 3700 xl DNA Analyzer (Applied Biosystems, Foster City, California).

Results and discussion

Scanning electron microscopy of pollen from the naturalized *Epidendrum* from Nuuanu-Pali (*E.* P) showed a length of 830.31 µm and a width of 462.58 µm (Fig. 1A). Length of the pollen from the cultivated cultivar (*E.* I), by comparison, was 724.60 µm, and the width was 276.17 µm (Fig. 1B). A more deeply folded and rugose stipe was observed in *E.* P (Fig. 1C). Its width was also greater (178.81 µm), compared to 84.15 µm in *E.* I (Fig. 1D).

Similar bulbous cell structures were found on the upper layer of petals from both *E.* P and *E.* I. The heights of *E.* P and *E.* I bulbous cells were 36.10 µm and 24.85 µm, respectively (Fig. 2A, B). Differing cell structures occurred on the lower surface of the petals. Polyhedral convex cells with numerous fossae (pits) belong to *E.* P (Fig. 2C), whereas elongated flattened cells are seen on *E.* I (Fig. 2D).

The two cultivars showed no detectable differences in leaf and/or root tip cell structures other than a much thinner transverse section of *E.* P leaf tissue (546.33 µm) (Fig. 3A**)** compared to a 1505.83 µm thickness in *E.* I (Fig. 3B). Differences in the thickness of leaf tissues were determined by differences in the size of the cells as seen in each of their vascular bundles. Vascular bundle heights of *E.* P and *E.* I were 119.68 µm and 189.26 µm, respectively (Fig. 3C, D). A thinner root tip (1094.19 µm) was also observed in the naturalized *E.* P, as opposed to the thicker root tip (1636.34 µm) of the cultivated *E.* I (Fig. 4A, B). The sizes of their respective parenchyma cells are 21.76 µm × 23.03 µm for *E.* P and 41.11 µm × 31.90 µm for *E.* I. The greater thickness of leaf and root tissues of the cultivated cultivar might be attributed to the abundant and continuous water received during

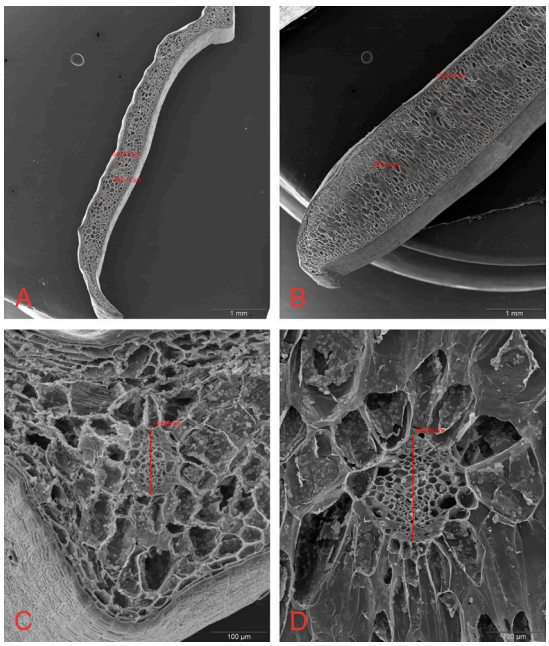

FIGURE 3. SEM images of x-sections of leaves (20x) and midribs (200x) of *E.* P and *E.* I. A. Leaf thickness of *E.* P (546.33 µm); B. Leaf thickness of *E.* I (1505.83 µm); C. Vascular bundle height of *E.* P (119.68 µm); D. Vascular bundle height of *E.* I (189.26 µm).

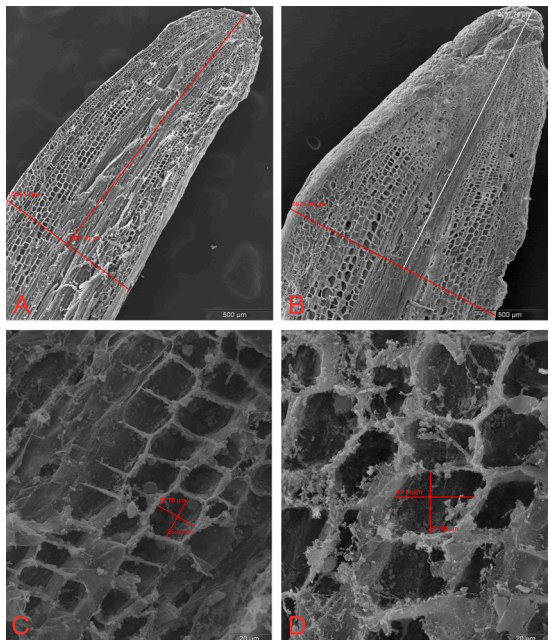

FIGURE 4. SEM images of 5 mm longitudinal section of root tips (50x) and parenchyma cells (700x) of *E.* P and *E.* I. A. Root tip tissue of *E.* P (1094.19 µm); B. Root tip tissue of *E.* I (1636.34 µm); C. Parenchyma cell size of *E.* P (21.76 µm x 23.03 µm); D. Parenchyma cell size of *E.* I (41.11 µm x 31.90 µm).

its growth in a climate-controlled greenhouse equipped with a sprinkler system.

Morphological differences in cell structures found on the stipes and lower surfaces of petals indicate that the naturalized *E.* P and the cultivated *E.* I might have genetically divergent origins. A single *trnL-F* tree is shown in Figure 5. Parsimony analysis among the naturalized *E.* P and the other 11 greenhouse-cultivated *Epidendrum* accessions showed that at least six changes separate these specimens into two separate groups. *Epidendrum* Clay 1, *E.* Clay 3, *E.* Clay 7, *E.* Clem, *E.* P, and *E.* sw12 belong to one group of 4 units in length. *Epidendrum* Clay 2, *E.* Clay 4, *E.* Clay 5, *E.* Clay 6, *E.* Clay 8, and *E.* I belong to another group of 2 units in length. The naturalized *E.* P belongs to the first group, and the cultivated *E.* I belongs to the second group. Sequence data seem to support the morphological dissimilarities seen in the SEM images of these two corresponding genotypes. Within the first group, *E.* Clay 1, *E.* Clay 3, and *E.* sw12 are differentiated into subgroups of 1 unit in length. These three specimens are closely related *Epidendrum* hybrids of white-yellow flowers. In the second group, *E.* Clay 5 is differentiated further into a subgroup of 1 unit in length. It also appears to be a hybrid, with flower color undetermined at this time.

Locus Epcp-02 (*rps16-trnK*) was previously reported to contain a microsatellite in *Epidendrum* species that varies in the number of cytosines from C_8 to C_{11}. Interestingly, in this study a range from C8 in *E.* IB, to C_{16} in *E.* O was observed. Using this simple sequence repeat (SSR), *E.* I and *E.* IB (shorter mono tandem repeats C_{10} and C_8, respectively), could be distinguished from the longer mono tandem repeats C_{16} (*E.* O) and C_{14} (*E.* RP, and *E.* C) (Fig. 6). *Epidendrum* I and *E.* IB have the same single-nucleotide polymorphism (SNP) guanines at positions 65 and 63, respectively, which distinguish these lines from the other genotypes. This finding supports categorization of the genotypes based on long (C_{14-16}) and short (C_{8-10}) mono tandem repeats. *Epidendrum* I can be distinguished from *E.* IB as well the other genotypes by cytosine at base pair position 204.

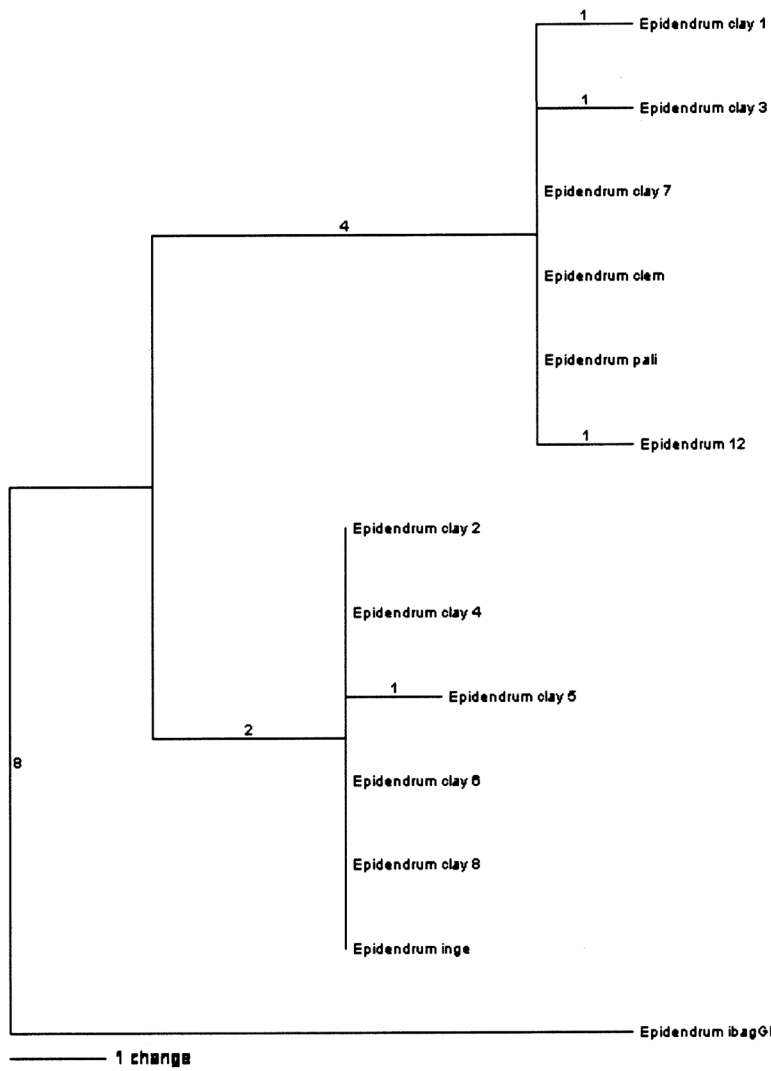

Figure 5. Most-parsimonious tree obtained in a cladistic analysis of plastid (*trnL-F)* DNA sequences plus one structural character of the root. Numbers above branches are number of changes.

In combination, the SSR and polymorphism associated with Locus Epcp-02 (*rps16-trnK*) can be used to distinguish related *Epidendrum* genotypes. The evaluation of additional genotypes will be required to determine if the cytosine at base pair 204 of *E.* I is unique to this genotype and if long (C14 – 16) and short (C8 – 10) SSRs correlate with established morphological differences that may distinguish these groups. Further study will be done when the following specimens (*E. radicans, E. evectum*, and *E.* ×obrienianum) become available.

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Epcp02-EpiI      1 TTCTTGCTTCTTTTTGTGGAACCCCCCCCCC------AACAAGGGGGGGATAACCTTTCT
Epcp02-EpiIB     1 TTCTTGCTTCTTTTTGTGGAACCCCCCCC--------AACAAGGGGGGGATAACCTTTCT
Epcp02-EpiO      1 TTCTTGCTTCTTTTTGTGGAACCCCCCCCCCCCCCCCAACAAGGGGGGGATAACCTTTCT
Epcp02-EpiRP     1 TTCTTGCTTCTTTTTGTGGAACCCCCCCCCCCCCC--AACAAGGGGGGGATAACCTTTCT
Epcp02-EpiC      1 TTCTTGCTTCTTTTTGTGGAACCCCCCCCCCCCCC--AACAAGGGGGGGATAACCTTTCT

Epcp02-EpiI     55 TCTTGTATTTGTATTGTACCTTTCTTTTTTTGATTAAAGAAAGCCGCTATTTTTTTTATC
Epcp02-EpiIB    53 TCTTGTATTTGTATTGTACCTTTCTTTTTTTGATTAAAGAAAGCCGCTATTTTTTTTATC
Epcp02-EpiO     61 TCTTGTATTTATATTGTACCTTTCTTTTTTTGATTAAAGAAAGCCGCTATTTTTTTTATC
Epcp02-EpiRP    59 TCTTGTATTTATATTGTACCTTTCTTTTTTTGATTAAAGAAAGCCGCTATTTTTTTTATC
Epcp02-EpiC     59 TCTTGTATTTATATTGTACCTTTCTTTTTTTGATTAAAGAAAGCCGCTATTTTTTTTATC

Epcp02-EpiI    115 TCTATCTTTTCCTTATTTTTTTCCGCTCAAAGTTTGATTTTTTATGTTGTGCTAATTCAA
Epcp02-EpiIB   113 TCTATCTTTTCCTTATTTTTTTCCGCTCAAAGTTTGATTTTTTATGTTGTGCTAATTCAA
Epcp02-EpiO    121 TCTATCTTTTCCTTATTTTTTTCCGCTCAAAGTTTGATTTTTTATGTTGTGCTAATTCAA
Epcp02-EpiRP   119 TCTATCTTTTCCTTATTTTTTTCCGCTCAAAGTTTGATTTTTTATGTTGTGCTAATTCAA
Epcp02-EpiC    119 TCTATCTTTTCCTTATTTTTTTCCGCTCAAAGTTTGATTTTTTATGTTGTGCTAATTCAA

Epcp02-EpiI    175 CGCAAATTTCTATATAATTTCTTGAAATTCGTTTTTTAAAAAGTCCATTTATATTGACAA
Epcp02-EpiIB   173 CGCAAATTTCTATATAATTTCTTGAAATTTGTTTTTTAAAAAGTCCATTTATATTGANNN
Epcp02-EpiO    181 CGCAAATTTCTATATAATTTCTTGAAATTTGTTTTTTAAAAAGTCCATTTATATTGACAA
Epcp02-EpiRP   179 CGCAAATTTCTATATAATTTCTTGAAATTTGTTTTTTAAAAAGTCCATTTATATTGACAA
Epcp02-EpiC    179 CGCAAATTTCTATATAATTTCTTGAAATTTGTTTTTTAAAAAGTCCATTTATATTGACAA

Epcp02-EpiI    235 TGG-CGTATCAAACAAAT
Epcp02-EpiIB   233 NNGGCGTATCAAACAAAT
Epcp02-EpiO    241 TGG-CGTATCAAACAAAT
Epcp02-EpiRP   239 TGG-CGTATCAAACAAAT
Epcp02-EpiC    239 TGG-CGTATCAAACAAAT
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FIGURE 6. Sequence alignment of the chloroplast locus Epcp-02 (*rps16–trnK*) from four cultivated cultivars: *Epidendrum* Inge (*E.* I), *E. ibaguense* (*E.* IB), *E.* Red Purple (*E.* RP), *E.* Clem (*E.* C), and a naturalized *Epidendrum* from Olomana (*E.* O). Forward and reverse primer sequences are underlined.

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