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Confirmation of hybrid origin of *Cyrtanthus* based on the sequence analysis of internal transcribed spacer

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ABSTRACT

The objectives of this study were to create interspecific hybrids between *Cyrtanthus elatus* and *C. sanguineus* and to confirm the hybrid origin of the progeny based on morphological characters and using molecular markers. The tip of the leaves, the shape and size of cells, and stomata distribution in abaxial and adaxial surface of leaf epidermis were analyzed by low temperature scanning electron microscope (LT-SEM). Molecular markers generated from random amplification polymorphic DNA (RAPD) and single nucleotide polymorphisms (SNPs) analysis of internal transcribed spacer 1 and 2 of rRNA gene (ITS 1, 2) were also analyzed to confirm hybrid status. Putative *C. elatus* × *C. sanguineus* hybrid A 3 resembled *C. elatus* and *C. sanguineus* 'Pringle Bay' (PB) × 'Fred Meyer' (FM) B 23 and was similar to *C. sanguineus* at flowering. However, the hybrid origin of hybrid A 3 could not be confirmed based on the morphological characteristics of flowers, the tip of the leaves, and cell structure and surface images in both abaxial and adaxial images of LT-SEM. Based on RAPD markers, putative hybrid A 3 and other putative hybrids that did not flower (A 4, A 5, A 13 and D 39, D 40, and D 42) can be considered as a hybrid based on the presence of both species-specific bands for parental taxa. SNP markers of ITS 1, 2 region also revealed that other putative hybrids of A and D that did not flower during the evaluation can be interspecific hybrids of *C. elatus* and *C. sanguineus*. Therefore, the use of SNP markers of ITS 1, 2 is considered a more accurate tool to characterize hybrid origin than morphological characteristics and RAPD markers.

1. Introduction

The genus *Cyrtanthus* Aiton (Amaryllidaceae) is native to South Africa with waxy tubular white, orange, and red flowers formed as clusters at the tip of scape (Koopowitz, 1986; Reid and Dyer, 1984). *Cyrtanthus elatus* (Jacq.) Traub, *C. makenii* Baker, and *C. sanguineus* Lindl. Walp. among about 50 species in the genus, have been cultivated for many years. Induction of flowering of *C. makenii* following some chilling treatment received outdoors before moving into a 20 °C greenhouse was reported (Mori et al., 1990). *Cyrtanthus sanguineus* flowered following 6 weeks of 5 °C treatment and *C.* 'Orange Gem' × *C. eucallus* R. A. Dyer can be induced to flower after water stress (Lee, personal observation). However, controlled flowering of *C. elatus* and *C. sanguineus* for year-round production with controlled cultural practices such as temperature, photoperiod, and plant growth regulators has not been reported.

A new hybrid with a flower shape intermediate between *C. elatus* and *C. sanguineus* or similar to *C. elatus* Jacq. Traub with relatively easy flowering as observed in *C. sanguineus* would be desirable for pot plant use. Therefore, it would be ideal to combine the free flowering characters inherited from *C. sanguineus* and multiple flowers (4–5 flowers) and strong scape from *C. elatus* by interspecific hybridization. Over the years, self- and cross-pollination of these two species did not yield viable seeds, perhaps due to self-incompatibility in these species. In 2002, when controlled pollinations between *C. sanguineus* and *C. elatus* were made, seeds were obtained from *C. elatus* × *C. sanguineus*. Floral development has not been investigated in many *Cyrtanthus* species, except the report that flower bud abortion occurred at 22 °C in *C. elatus* (Van Nes and vonk Noordegraaf, 1977).

When two hybrid plants flowered in 2011, one putative interspecific hybrid A 3 from *C. elatus* × *C. sanguineus* resembled flowers of *C. elatus* and one intraspecific hybrid B 23 from *C. sanguineus* 'Pringle Bay' (PB) × 'Fred Meyer' (FM) was similar to *C. sanguineus*. Based on the morphological characteristics of leaves during culture and of flowers, it was not possible to determine definitely whether this putative hybrid A 3 was of a hybrid origin although

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Table 1
Species and cross pollination, designation and lane numbers, and the presence of species specific bands, and also morphological characters of tips and mid-ribs in mature leaves.

Species/hybrids[a]	Designation (Fig. 4)[b]	Lane numbers in Fig. 4	Morphological characteristics[c]			
			Tip		Vein	
			Female	Male	Female	Male
C. elatus	CE	1, 42	1, 42			1, 42
C. sanguineus	CS	24		24	24	
C. elatus × *C. sanguineus*	A	2–15	3, 5	2		2, 5
	D	39–41	39, 40			39
C. sanguineus PB × FM	B	16–23		16–23		16–23
C. sanguineus FM × PB	C	25–36	34	25–34, 36		25–34, 36
C. herrei	CH	43				

[a] *C. sanguineus* from Fred Meyer (FM) and Pringle Bay (PM).
[b] Designation of cross pollination and lane number in Fig. 4.
[c] Morphological characteristics of sample number 1, 3, and 5, for example, resemble *C. elatus* and of sample number of 17–23, for example, resemble *C. sanguineus*.

hybrids showed mosaic morphological patterns either close to each parental species, or intermediate with extreme characters (Rieseberg and Ellstrand, 1993).

Morphological characters are often used to establish that specific plants have a hybrid origin. Characters such as the shape of the tip of the leaf in *Tsuga* (Roh et al., 2007), can be used to identify the species. However, the shapes of the apices of needles from young plants could not be used as a possible key character to identify *T. dumosa* (D. Don) Eichler in Engler & Prantl. and *T. chinensis* var. *forrestii* (Downie) Silva. The shape of the epidermis cells was used to separate taxa in the genus *Lilium* (Lee, 1989). When the anatomy of leaf cross sections of the mid-rib in the genus *Lilium* was investigated, *L. hansonii* Leich. had no apparent raised mid-rib and *L. tsingtauense* Gilg. had a raised mid-rib. However, no differences were apparent on the surface image and undulation of cell shape between these two species (Lee, 1989).

Molecular markers generated by random amplification polymorphic DNA (RAPD) and Single nucleotide polymorphisms (SNPs) of the nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) have been used to verify and determine whether seedlings are of hybrid origin in *Pulsatilla tongkangensis* (Lee et al., 2010), *Arisaema* (Lee et al., 2011), and many other plants (Roh et al., 2007, 2008). Single nucleotide polymorphisms (SNPs) and DNA sequence variations occurring with a single nucleotide (Brooks, 1999) have been useful to study the genetic variation and hybridization among species, and sequence analysis of cpDNA, and nuclear internal transcribed spacers (ITS) were used in *Anemone* and related genera (Ehrendorfer and Samuel, 2001; Roh et al., 2007; Schuettpelz et al., 2002). However, the information obtained from the molecular markers and the morphological characteristics were not congruent, particularly when the parental species were not pure lines as was observed in *Clematis* (Yuan et al., 2010) or true to the species type but could not be identified as in *P. tongkangensis* (Lee et al., 2010).

In *Cyrtanthus*, morphological characteristics of seed propagated from *C. sanguineus* when two clonal germplasm taxa were cross-pollinated did not show any variations (Lee, unpublished data). However, it is not known how interspecific hybrids between *C. elatus* × *C. sanguineus* might vary morphologically. The objectives of this study were to obtain interspecific hybrids obtained from cross-pollination between *C. elatus* × *C. sanguineus* and to verify the hybrid origin of the progeny based on leaf morphology and molecular markers generated from RAPD and SNPs of ITS 1, 2 region.

2. Materials and methods

2.1. Plant materials

Eighteen cross pollinations between *C. elatus* and *C. sanguineus* were made in July, 2005 following emasculation one day prior to anthesis of female plants by applying pollen collected two days after anthesis. Clonal selections of *C. sanguineous*, obtained from Waldo Essen, Pringle Bay, South Africa (PB) and Fred Meyer, California, USA (FM) were also pollinated; *C. sanguineus* PB × FM and *C. sanguineus* FM × PB. Seeds were sown in the greenhouse in Metro Mix 200 and germinated in 35 days in 2006. After germination, seedlings were grown in pots in a greenhouse for 3 years. Fourteen and 3 seedlings were obtained from *C. elatus* × *C. sanguineus* capsule A and D, respectively, 8 seeds from *C. sanguineus* PB × FM (capsule B), and 14 seeds from *C. sanguineus* FM × PB (capsule C). Bulbs were harvested (April, 2009) and were stored dry in a paper bag for 18 months (Table 1). Bulbs were planted in October, 2010 and cultivated in a greenhouse maintained at 21–24 °C/16–18 °C, day/night. One bulb was planted per 12.5 cm pot, except *C. elatus* and *C. herrei* (Leighton) R.A. Dyer which was planted in 15 cm and 21 cm pots, respectively.

2.2. Identification of hybrid origin based on the morphological characteristics

At anthesis, leaves from putative interspecific *C. elatus* × *C. sanguineus* hybrid A 3 (putative hybrid A 3) resembling the female parent *C. elatus*, and intraspecific *C. sanguineus* PB × FM hybrid B 23 (hybrid B 23) (Fig. 1), and parental taxa were collected during the third week of April, 2011. The abaxial and adaxial surfaces between the mid-rib and leaf margin at the mid-point of mature leaves were observed using low temperature scanning electron microscopy as described by Roh et al. (2012). The distribution of stomata from both leaf surfaces was observed from the images at 100× and the number of stoma was counted from 4 rectangular 550 μm × 500 μm areas. The size of 15 cells was measured from 100× and 200× images and the appearance of the cell's surface was observed from 200× and 400× images. The number of stomata and cell size was subjected to an analysis of variance and means were compared with Tukey' test (hsd) at $P \leq 0.01$.

On August 27, 2011, the tips of mature leaves of several selected putative hybrids (A 2, A 3, A 5, D 39 and D 40) and hybrids (B 23 and C 34) and two parental taxa, *C. elatus* (CE 1) and *C. sanguineus* (CS 24), were collected and recorded either as acuminate (as seen in *C. sanguineus*), obtuse (as in *C. elatus*), or intermediate, generally a round shape. At the mid-point of the adaxial surface of the leaves, the mid-rib was observed as raised or not raised from all plants.

2.3. Identification of hybrid origins based on the RAPD markers and sequence analysis of ITS 1, 2 region

The total genomic DNA was extracted, quantified, and subjected to polymerase chain reaction (PCR) for RAPD analysis as described previously in a PTC-100 programmable thermal cycler (MJ Research, Watertown, MA, USA) (Roh et al., 2007; Suh et al.,

Fig. 1. Floral morphology of putative hybrid A 3 (*C. elatus* × *C. sanguineus*) resembling *C. elatus* and hybrid B 23 resembling *C. sanguineus*. Facing view (F) and side view (S) of flowers are shown.

2011). Random 10-oligomer primers (OPA 01, OPA 02, OPA 13, OPB 03, OPC 01, OPC 02, OPC 13, OPC 14, and OPC 15; Operon Technologies, Inc., Alameda, CA, USA) were screened with two parents *C. elatus* and *C. sanguineus*, and putative hybrids A 2 to A 13 and D 39 to D 41, and hybrids B 16 to B 23 and C 25 to C 38. Species-specific bands for *C. elatus* and *C. sanguineus* were used to identify the putative hybrid origin.

For amplification of the ITS 1, 2 region in ribosomal RNA gene, 18S rRNA and 28S rRNA gene specific primers in ITS 1, 2 flanking region, were constructed; ITS 1 (5′-TAG AGG AAG GAG AAG TCG TAA CAA GG-3′) and ITS 2 (5′-TGA TAT GCT TAA ACT CAG CGG GTG TC-3′). The PCR reaction mix contained 2 μl of template DNA (10 ng/μl), 1 μl of 10 pmoles/μl of each ITS 1 and ITS 2 primer, 5 μl of 5× Band doctor PCR buffer (SolGent Co. Ltd., Korea), and 12.5 μl of 2× multiplex Pre-Mix (SMP01–M25H, SolGent). The PCR amplification conditions of 15 min at 95 °C followed by 38 cycles of 20 s at 95 °C, 40 s at 58 °C, and 1 min at 72 °C, followed by 3 min of 72 °C. After gel electrophoresis, the PCR products were purified using the gel purification kit (SGP04-C100, SolGent) according to the manufacturer's instructions. The purified PCR products were directly sequenced with ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

For cloning and sequencing of the PCR product, PCR was performed using high fidelity Tag DNA polymerase, *Solg*^{TM} *Pfu* DNA Polymerase (SPD16-R250, SolGent). The PCR reaction mix contained 2 μl of template DNA (10 ng/μl), 1 μl of 10 pmoles/μl of each ITS 1 and ITS 2 primer, 5 μl of 5× Band doctor PCR buffer and 0.5 μl of *Solg*^{TM} *Pfu* DNA Polymerase. The PCR amplification conditions of 2 min at 95 °C followed by 35 cycles of 20 s at 95 °C, 40 s at 58 °C, and 2 min at 72 °C, followed by 5 min of 72 °C. The PCR products were cloned into T-Blunt vector using T-Blunt^{TM} PCR Cloning Kit (SOT01-K020, SolGent) according to the manufacturer's instructions. The ligation mix contained 1 μl T-Blunt vector, 1 μl of 6× T-Blunt buffer, and 4 μl of the PCR product. The ligation mix was transformed into *Escherichia coli* DH5α. The plasmid isolated from the clone and sequence with ABI 3730XL DNA Analyzer.

3. Results

Two capsules from *C. elatus* × *C. sanguineus* produced viable seeds (14 seeds from capsule A and 3 seeds from capsule D) that germinated (Table 1). No viable seeds were obtained from *C. sanguineus* × *C. elatus*. Viable seeds from crosses of *C. sanguineus* PB × FM (capsule B) and *C. sanguineus* FM × PB (capsule C)

Table 2
Distribution of stomata and epidermal cell sizes in abaxial and adaxial side of leaves in parental species and two hybrids.

Parental species/hybrids[a]	Designation (Fig. 4)[b]	No. of stomata on leaf surface[c]		Cell size (μm)Length
		Abaxial	Adaxial	Abaxial/adaxial
C. elatus		19	5	250/180
C. sanguineus		20	28	208/248
C. elatus × *C. sanguineus*	A 3	11	17	264/287
C. sanguineus PB × FM	B 23	6	15	193/221
hsd at $P \leq 0.01$		3.7	6.2	27.8/19.5

[a] *C. sanguineus* from Fred Meyer (FM) and Pringle Bay (PM).
[b] Designation of cross pollination and lane number in Fig. 4. A 3: *C. elatus* × *C. sanguineus* and lane number as indicated in Fig. 4.
[c] The number of stomata counted from 550 μm × 500 μm area at 100×.

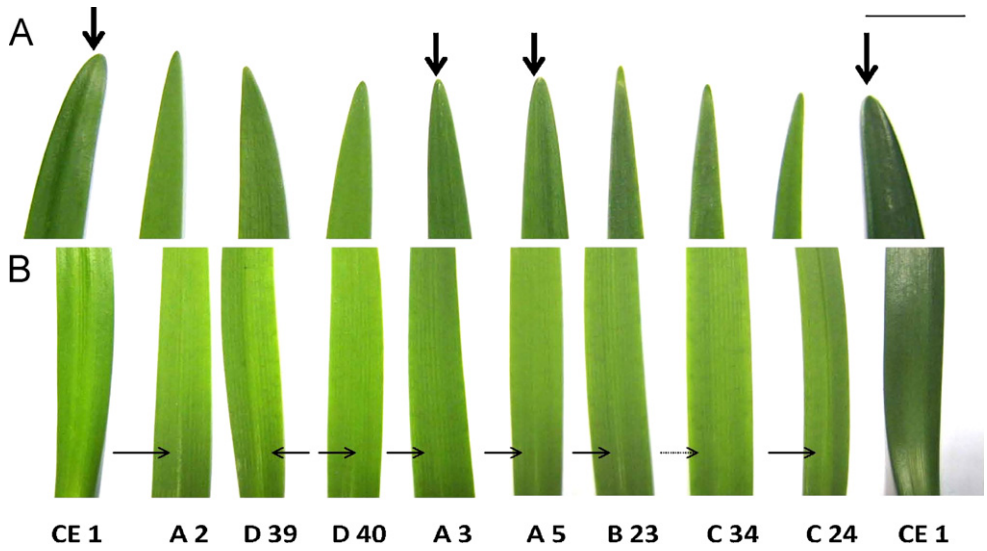

Fig. 2. Leaf morphology of *C. elatus* (CE) and *C. sanguineus* (CS) and putative hybrids of A, B, C, and D (refer to Table 1 and Fig. 4 for details). Tip of the leaves (frame A) are shown with arrow mark indicating that the tip is not too acute which resembles *C. elatus* and middle of the leaves showing the main rib which is evident in *C. sanguineus* (frame B). The numbers are sample number followed by the lane number indicated in Fig. 4. Bar = 2.5 cm. Refer to Table 1 for details.

germinated and morphological characteristics were examined and identification of hybrid origin using molecular markers have been carried out through October 2011.

3.1. Morphological characteristics

Only one interspecific *C. elatus* × *C. sanguineus* putative hybrid A 3 (putative hybrid A 3) resembling *C. elatus* (Fig. 1) flowered on July 8, 2011. Intraspecific *C. sanguineus* PB × FM hybrid B 23 (hybrid B 23) flowered on Aug. 16. The base of leaves and the neck of bulbs of putative hybrids A 3 showed the dark bronze color which is typical to *C. elatus* (data not presented). Hybrid B 23 had flowers, leaves, leaf bases and bulb neck morphologiy similar to *C. sanguineus*. All other hybrids, except parental *C. sanguineus* hybrid CS 24 and hybrid B 23, did not flower during the evaluation period in 2011 following planting of dry-stored bulbs.

The tip of the leaves of putative hybrids A 3 and A 5 showed an intermediate shape, between acuminate and obtuse, as was observed in *C. sanguineus* (i.e., hybrids C 24 and C 34) and in *C. elatus*, respectively. Other putative hybrids (A 2, D 39, and D 40) had an acuminate leaf tip similar to C. *sanguineus* (Fig. 2A). The mid-rib in the adaxial side of leaves of *C. sanguineus* and hybrids C 24 and C 34 and putative hybrids A 2 and D 39 was raised, showing the line of the mid-rib clearly (Fig. 2B).

Low temperature-scanning electron microscopy (LT-SEM) images showed that stomata were present in both the abaxial and adaxial side of epidermal cells (Fig. 3). The number of stomata in the abaxial side (Table 2) was 19 for *C. elatus* and 20 for *C. sanguineus*, which is significantly greater than the number in putative hybrid A 3 (11 stomata) and hybrid B 23 (6 stomata). The number of stomata in the adaxial side of 15 and 17 for putative hybrid A 3 and hybrid B 23, respectively, was significantly higher and fewer than the *C. elatus* (5 stomata) and *C. sanguineus* (28 stomata), respectively. The mean length of cells in the abaxial side of putative hybrid A3 (264 μm) and hybrid B 23 (193 μm) did not differ from *C. elatus* (250 μm) and *C. sanguineus* (264 μm). However, the mean length of cells in the abaxial side of putative hybrid A 3 and hybrid B 23 was either longer or shorter, respectively, than those of *C. elatus* and *C. sanguineus*.

The surface of the abaxial side of epidermis cells of *C. elatus* and *C. sanguineus* was distinct. The surface had two distinct dark outlines or undulated lines along the length of cells in the images. These were a typical prosenchymal longated shape, particularly on the abaxial and adaxial side of *C. sanguineus* and also the abaxial side of *C. elatus* as compared to cells in the adaxial side of *C. elatus* cells which had well-defined cell walls (Fig. 3). The cell shapes of putative hybrid A 3 on both sides were also well organized and undulated lines were observed in the abaxial side cells. Cells of hybrid B 23, in the abaxial and adaxial sides, had cell shapes with two prominent outlines. Observation of a close up from a 400× image of cells of putative hybrid A 3 showed no clear lines as a result of a cuticlular plication (folding) or somewhat irregular deposition of the cuticle (frame A) and lines hybrid B 23 (frame B).

3.2. Identification using molecular markers – RAPD analysis

Random primers OPA 01, OPA 13, and OPC 14 (data not presented) and primer OPC 02 produced species-specific bands; species specific bands for *C. elatus* (solid arrow, ca. 1900 bp) and for *C. sanguineus* (broken arrow, ca 360 bp) (Table 3; Fig. 4). Putative hybrids *C. elatus* × *C. sanguineus* A 3, A 4, A 5, and A 13 and D 39, D 40, and D 41 produced the species-specific band of both parental species. However, a *C. elatus* species-specific band was not produced in putative hybrids A 2, A 6–12, A 14, and A 15. All *C. sanguineus* hybrids (B 16–20, B 22, and B 23 and C 25–38) did not produce *C. elatus* specific bands. *C. herrei* which was used as out-group did not show any of these two species specific bands.

3.3. Identification using molecular markers – sequence analysis of ITS 1, 2 regions

The amplified ITS 1, 2 region from two parent species, *C. elatus* CE 1 and CE 42 (GenBank ID: JX089455) and *C. sanguineus* interspecies hybrids (B 16, B 35) were cloned, and five clones from each lines were sequenced. Sequence data showed that there was no difference between two *C. elatus* lines and only one base different compare to *C. elatus* (GQ160828). Five different ITS 1, 2 sequences were obtained from *C. sanguineus* intraspecies hybrids B 16-2 (GenBank ID: JX089456), B 16-3 (GenBank ID: JX089457), C 35-2 (GenBank ID: JX089458), C 35-4 (GenBank ID: JX089459) and C 35-5 (GenBank ID: JX089460), and aligned sequence between *C. sanguineus* (GQ160851), they showed 97–99% sequence homology (data not presented). Sequence variation between clones were presented used the International

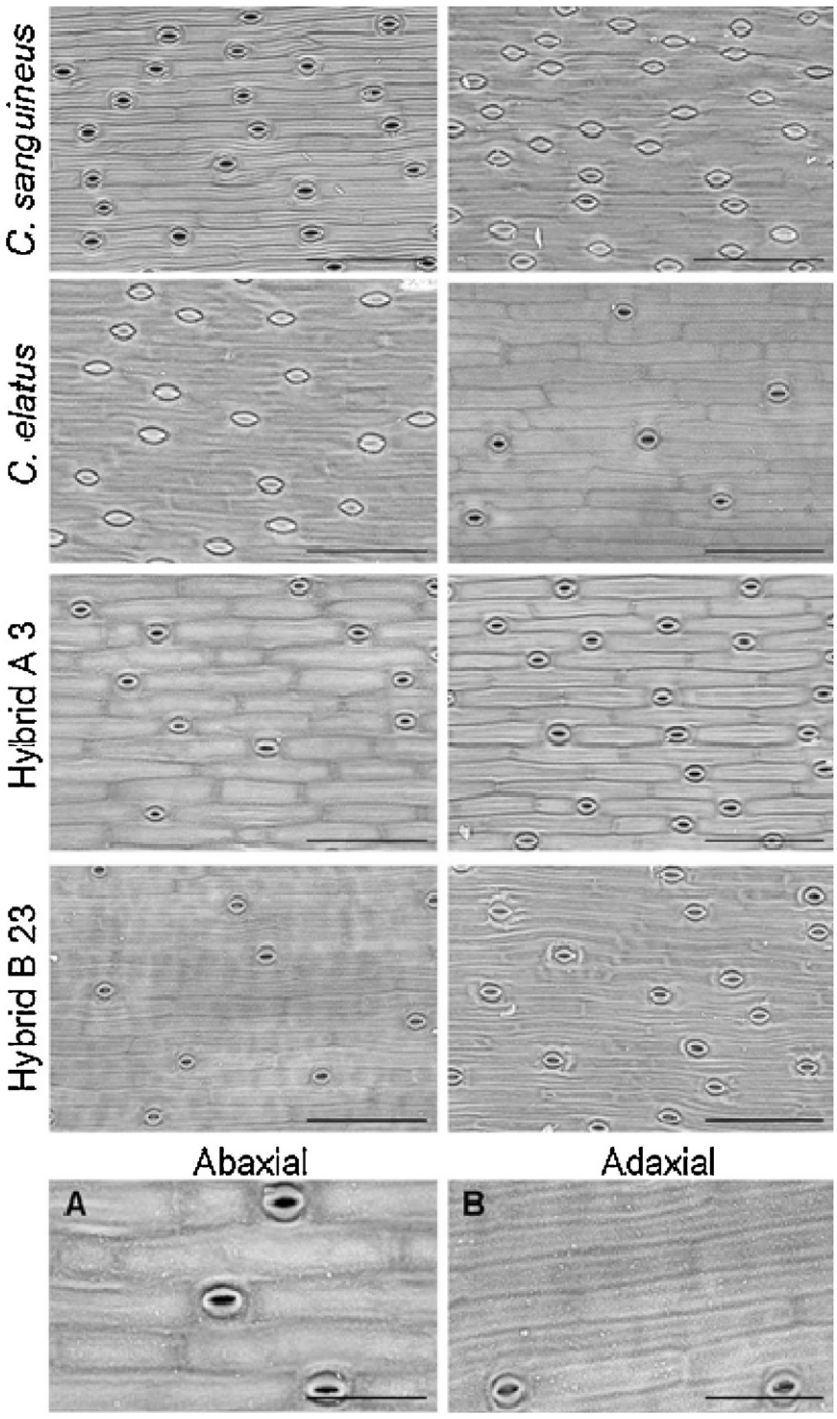

Fig. 3. Abaxial and adaxial surface low temperature scanning electron microscope pictures of *C. elatus*, *C. sanguineus*, putative hybrid A 3 resembling *C. elatus*, and hybrid B 23 resembling *C. sanguineus*. 200×, bar = 200 μm. Two types of surface images of the epidermal cells showing without (A, hybrid A 3) and with (B, hybrid 23) apparent undulating lines are presented. 400×, bar = 100 μm.

Union of Pure and Applied Chemistry (IUPAC) ambiguity codes (http://www.bioinformatics.org/sms2/iupac.html) [*C. sanguineus* (*C. s*) in Fig. 5]. The results of sequence alignment between two parent species, SNP markers for *C. elatus* and *C. sanguineus* were obtained at sixteen positions of 73, 92, 112, 142, 261, 265, 272, 446, 473, 499, 512, 610, 639, 657, 669, and 691 (Fig. 5). When the ITS 1, 2 PCR products of putative hybrids A and D were analyzed by direct sequencing, double peaks and some sequence variation were detected in the ABI sequence chromatogram. Due to this ambiguous sequence data, ITS 1, 2 PCR products from several selected putative hybrids (A 2, A 3, A 8, D 39) were cloned and sequenced.

When the sequence of ITS 1, 2 regions was analyzed following cloning, six clones of *C. elatus* × *C. sanguineus* putative hybrid A 2 had sequence data showing a *C. elatus* type (clone A 2-1, 5, 6, 7, 9, and 10) and four clones showing a *C. sanguineus* type (clone A 2-2, 3, 4, and 8). Also putative hybrid D 39 had both *C. elatus* and *C. sanguineus* type of ITS 1, 2, but A 8 had no *C. elatus* type clone (Table 3). Aligned sequence data of putative hybrid A 2-5 and A 2-8 showed that they have 22 SNPs at positions of 73, 92, 102, 104, 112, 142, 258, 261, 265, 272, 446, 473, 499, 518, 519, 512, 610, 631, 639, 657, 669, and 691 (Fig. 6A). The SNPs at the position 102, 104, 258, 518, 519, 631 were at the same position, which showed variation in

Table 3
Identification of putative interspecific hybrids of *C. elatus* × *C. sanguineus* and intraspecific hybrids of *C. sanguineus* using RAPD generated molecular markers and sequence analysis of ITS 1, 2 region.

Species	Designation[a]	Presence of RAPD band[b]		SNP analysis of ITS 1, 2 region			
				Direct sequencing[e]		Cloning[f]	
		Female	Male	Female	Male	Female	Male
C. elatus	CE	1, 42[c]		1, 42			
C. sanguineus	CS		24				
C. elatus × *C. sanguineus*	A	3, 4, 5, 13	2, 6–15		8[g]	2 (1, 5, 6, 7, 9, 10) 3 (1, 2, 5)	2 (2, 3, 4, 8)
	D	39–41				39 (1, 2, 3, 4, 5, 6, 8)	39 (7, 10)
C. sanguineus PB × FM	B		16–23 (21)[d]		16–23		16 (1, 2, 3, 4, 5)
C. sanguineus FM × PB	C		25–38		25–38		35 (1, 2, 3, 4, 5)

[a] Designation of species or inter- and intra-specific hybrids as shown in Fig. 4.
[b] RAPD bands produced with OPC 02 Operon random primer.
[c] Lane number as shown in Fig. 4.
[d] Species specific bands produced by Operon C 02 primers. Hybrid number B 21 in the parenthesis was not amplified.
[e] Sequence analysis following direct sequencing of the ITS PCR products.
[f] The ITS PCR products were cloned and sequenced. Numbers in the parenthesis were presented clone number.
[g] Presence of both parental type of ITS SNPs in A and D hybrids, except hybrid number A 8.

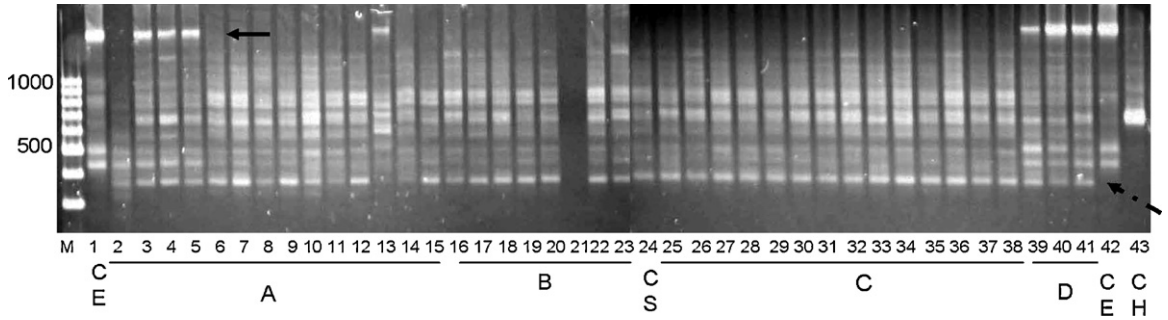

Fig. 4. Randomly amplification of polymorphic DNA gel photos using random primer OPC 02. Species specific band for *C. elatus* (solid arrow) and *C. sanguineus* (dotted arrow) is indicated. *Cyrtanthus elatus* (CE), *C. elatus* × *C. cyrtanthus* (A and D), *C. sanguineus* PB × FM (B), *C. sanguineus* (CS), *C. sanguineus* FM × PB (C), and *C. herrii* (CH) are indicated. M; DNA ladder marker.

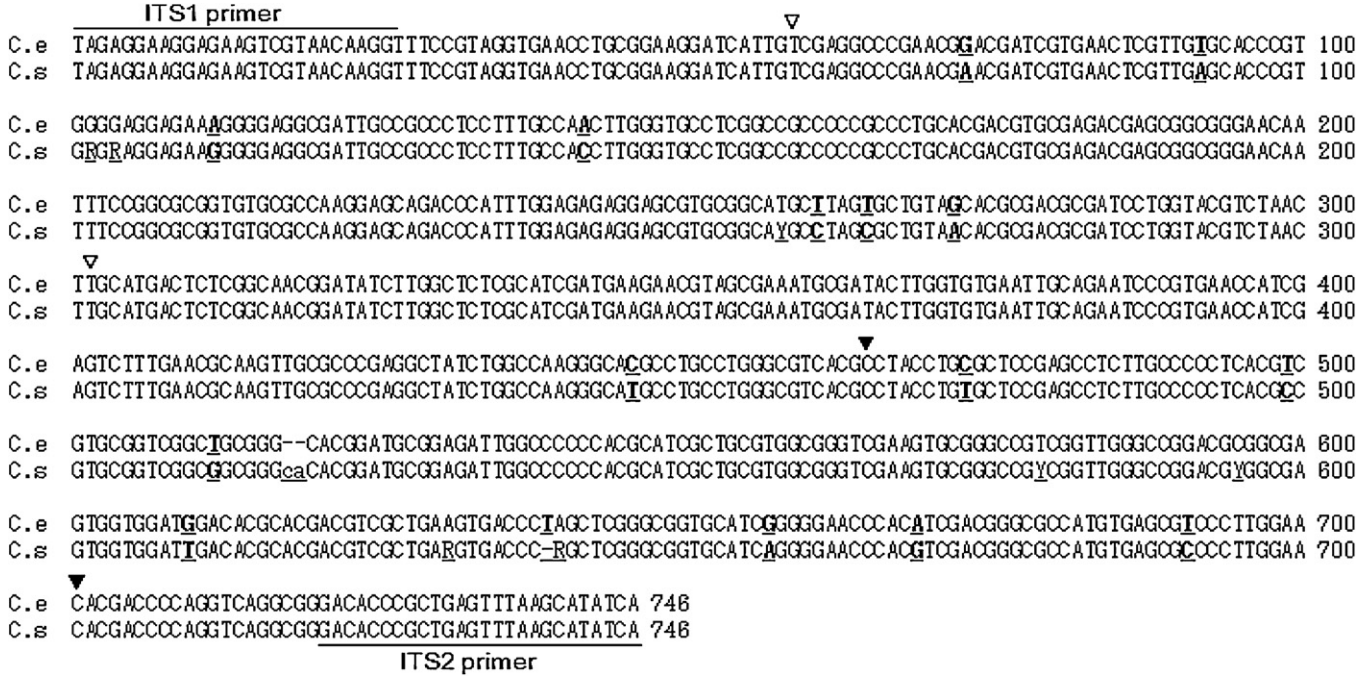

Fig. 5. Aligned sequencing data from two parent species, *C. elatus* (CE 1, CE 42) (C. e: GenBank ID: JX089455) and *C. sanguineus* (*C. s*) interspecific hybrids (B 16, B 35), B 16-2 (GenBank ID: JX089456), B 16-3 (GenBank ID: JX089457), C 35-2 (GenBank ID: JX089458), C 35-4 (GenBank ID: JX089459) and C 35-5 (GenBank ID: JX089460). SNPs between *C. elatus* and *C. sanguineus* were presented underlined and bold character. Lower case character and (–) presented insertion/deletion sequences, R presented A/G, Y presented C/T in variable sequence. Open triangle arrowhead represents the internal transcribed spacer 1 (ITS 1) between 18S rRNA and 5.8S rRNA gene, and black triangle arrowheads represent the internal transcribed spacer 1 (ITS 2) between 5.8S rRNA and 28S rRNA gene.

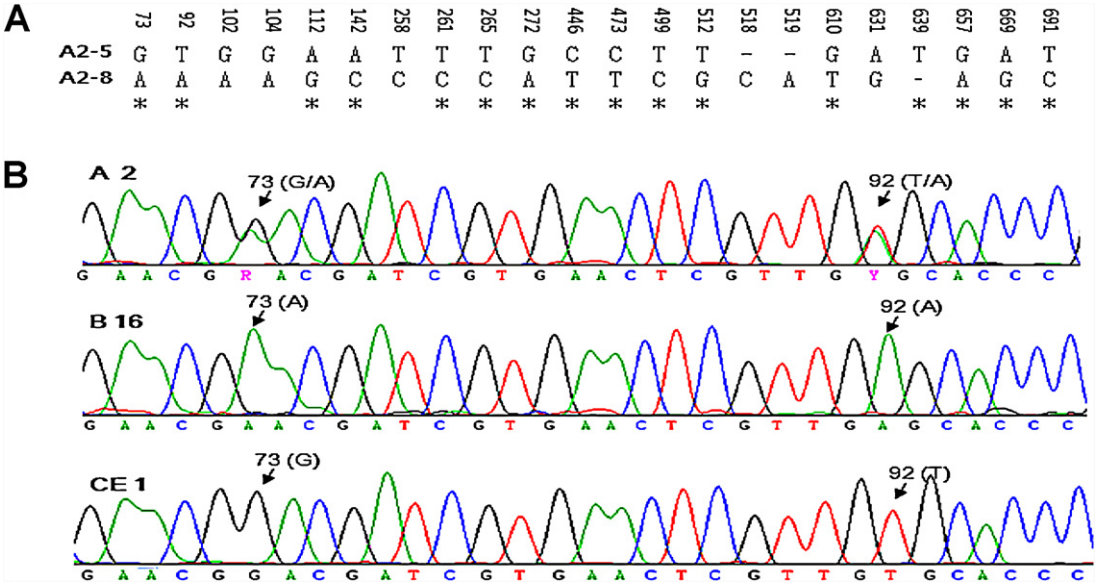

Fig. 6. (A) Aligned sequence of the putative hybrid A 2 ITS 1, 2 following cloning. A 2-1, -5, -6, -7, -9, and -10 clones had similar sequence of *C. elatus* and A 2-2, -3, -4,and -8 clones had similar sequence of *C. sanguineus*. SNPs showed twenty-two positions and asterisks presented SNPs for *C. elatus* and *C. sanguineus*. (B). Part of sequence chromatogram of putative hybrid A 2, *C. sanguineus* (*C. s*), intraspecific hybrids B 16 and *C. elatus* CE 1 following direct sequencing PCR product is presented. Arrow indicated SNP positions at the base positions 73 and 92.

C. sanguineus species (Fig. 5). When the ITS 1, 2 were sequenced by direct sequencing method, these sixteen SNPs showed double peaks in the sequencing chromatogram (Fig. 6B). The direct sequencing chromatograms of the putative hybrid A and D groups were analyzed, and all in the A and D samples showed double peaks in the SNP marker position except A 8 (data not presented). This result concluded that use of the SNPs of ITS 1, 2 is considered a more accurate tool to characterize the hybrid origin.

4. Discussion

Two parental species of *C. sanguineus* and *C. elatus* over a period of 10 years did not produce seeds following self-pollination, perhaps due to self-incompatibility or a late-acting self-incompatibility as reported in *C. breviflorus* (Vaughton et al., 2010). The lack of seed formation following hand pollination may not result from the limitation of pollen at pollination, but from the lack of pollination vectors since most of pollinators in nature are sun-birds or insects (Snijman and Meerow, 2010) that may collect pollen from different populations of a given species. Hybrids are not commonly seen because, in nature, flowers of *C. breviflorus* Harv. are pollinated primarily by bees that deposit mixtures of cross- and self-pollen (Vaughton et al., 2010). The fact that cross pollination of *C. sanguineus* PB × FM and *C. sanguineus* FM × PB produced seed in this study reflects the presence of pollen from a different population which may not involve self-compatibility.

The low flowering percentage of the interspecific and intraspecific hybrids in this study may have resulted from the adverse effect of long, dry storage. If plants were grown continuously in the greenhouse under favorable conditions that may induce flowering, more plants may have flowered. However, flowering of *C. elatus* is difficult and erratic compared to *C. sanguineus*, and this may be one of the reasons for poor flowering. Low flowering percentage in putative *C. elatus* × *C. sanguineus* hybrids except for putative hybrid A 3 and *C. sanguineus* hybrids B 23 may result from flower bud abortion during the extended dry storage after bulb harvest (Van Nes and vonk Noordegraaf, 1977).

The fact that the flower shape of putative hybrid A 3 resembled *C. elatus* and the tip of the leaves showed intermediate characteristics of its two parents suggested that interspecific hybridization may not have been successful, since hybrids do show mosaic patterns in morphology resembling to either parents or intermediate characters of two parents (Rieseberg and Ellstrand, 1993). If more plants had flowered, hybrid origin, particularly from *C. elatus* × *C. sanguineus* progeny, might have been identified based on floral morphology. However, the tip of the leaves may suggest that hybridization may have occurred; putative hybrid A 3 showed a leaf tip intermediate between the two parental species and a raised mid-rib in the middle of the leaves, resembling *C. sanguineus*. Therefore, based on the leaf morphology, putative hybrid A 3 may now be considered as an interspecific hybrid. The raised mid-rib revealed by the cross section of leaf could be used to differentiate at least some species in *Lilium*, however, it can not be verified whether it is useful characters since no hybridization has been made between *L. hansonii* and *L. tsingtauense* (Lee, 1989).

The surface image of the adaxial, not the abaxial epidermis, may be potentially useful since the cell shape of putative hybrid A 3 showed an intermediate appearance; clear cell layers similar to *C. elatus* with lines showing a valley as observed in *C. sanguineus*. The nature of two lines, which may be due to a different light reflectance in the cytoplasm showing a valley, has not been further investigated since this character may not be useful to identify the hybrid origin resulting from hybridization of these two species, as was recorded in the genus *Lilium* (Lee, 1989). Yembaturova and Korchagina (2011) stated that epidermal cell structure, especially in stems, can be of diagnostic value in wild lily species as well as in hybrids in the genus *Lilium*. However, surface images of the epidermal cells were not reported. The surface images may reflect the complex nature of the epicuticle of the plant: i.e. the cuticle proper may not be smooth, showing a valley (sunken area) along the middle lamella (Jeffree, 1996).

Therefore, these morphological characters are not useful to confirm the hybrid origin of these putative hybrids and the need of molecular markers becomes necessary. Significant morphological variations observed in *Ilex* × *wandoensis* C. F. Mill. & M. Kim has confirmed that interspecific hybridization had occurred based on the species specific bands generated following RAPD and restriction fragment length polymorphism (RFLP) analysis following

restriction enzymes digestion (Lee et al., 2006, 2010) and sequence analysis of internal transcribed spacer 2 region and restriction fragment length polymorphism in *P. tongkangensis* Y. N. Lee & T. C. Lee (Joung et al., 2011).

Based on RAPD markers, putative hybrid A 3 that flowered, and those that did not flower (A 4, A 5, A 13, D 39, D 40, and D 41) can now be considered as true interspecific hybrids based on the presence of bands from both parental taxa and supported by the SNPs analysis of ITS 1, 2. Sequence analysis of clones is a more effective tool to confirm hybrid origins as compared to the direct sequence of PCR product and RAPD analysis in investigating the hybrid origin of the putative hybrid A 2. The putative hybrid A 2 showed that two types of sequence analysis of clones indicate that there may be a limitation of RAPD markers generated by primers, not necessarily the technique of RAPD *per se* (Ramos et al., 2008), which does not produce the species specific band for *C. elatus*. A similar result was also reported in *Pulsatilla* (Joung et al., 2011). Based on the sequence analysis of ITS 1, 2 regions of putative hybrids A and D showing the *C. elatus* and *C. sanguineus* species specific SNPs, it is now certain that interspecific hybridization has occurred between *C. elatus* and *C. sanguineus*.

5. Conclusions

The use of SNPs of ITS 1, 2 is especially considered a more efficient tool to characterize the hybrid origin than morphological characteristics when putative hybrids do not exhibit intermediate morphological characteristics. The pollen fertility of these hybrids has not been examined due to a low flowering percentage. However, when interspecific hybrid A 3 and intraspecific hybrid B 23 were cross pollinated, no seeds were produced, suggesting that pollen may not be fertile. Infertility in *C. breviflorus* (Ramsey et al., 2011) is mostly likely caused by unequal ploidy, depending on the population. Doubling the chromosomes to restore fertility, as reported in *Solanum melongena* L, the egg plant (Isshiki and Taura, 2003), could be a useful way to make back crosses successful, creating hybrids with intermediate characteristics, including flower shapes, and a potentially more vigorous flowering response.

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