ORIGINAL ARTICLE

The phylogeny of Thai *Boesenbergia* (Zingiberaceae) based on *pet*A-*psb*J spacer (chloroplast DNA)

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Abstract

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The phylogeny of Thai *Boesenbergia* (Zingiberaceae) based on *pet*A-*psb*J spacer (chloroplast DNA)
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New primers were designed to amplify cpDNA intergenic *pet*A-*psb*J sequences in *Boesenbergia* species. These primers were *pet*A-F, *psb*J-R, *psb*L-R. The aligned sequences of 18 ingroup taxa and 5 outgroup taxa resulted in 856 characters in length, including 8 parsimony informative indels. However, there were only 14 informative characters (~1.65%) in the aligned sequences. The percentage of phylogenetically informative sites was close to the other two regions in *Boesenbergia*; *mat*K and *psb*A-*trn*H. *Boesenbergia* species form a monophyletic clade with moderate support (81% bootstrap value) in parsimony and UPGMA analyses. Two subclades are recognized and supported by two different basic chromosome numbers. An unpublished new species, *Boesenbergia bambusetorum* is grouped within the clade of the other two populations of *B. longiflora* with weak support (67% bootstrap value). More study is needed to verify the status of the taxon.

Key words : *Boesenbergia*, Zingiberaceae, phylogeny, chloroplast DNA

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ประวัติวิวัฒนาการของพืชสกุลกระชายของไทยจากดีเอ็นเอชิ้นส่วน *pet*A-*psb*J ในคลอโรพลาสต์

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ไพรเมอร์ใหม่สามชนิด *pet*A-F, *psb*J-R, *psb*L-R ถูกออกแบบขึ้นมาเพื่อเพิ่มชิ้นส่วน *pet*A-*psb*J ของดีเอ็นเอในคลอโรพลาสต์ สำหรับใช้เป็นข้อมูลในการศึกษาประวัติวิวัฒนาการของพืชในสกุลกระชาย ลำดับเบสที่ได้จากการศึกษาในครั้งนี้ได้มาจากพืช 23 ตัวอย่าง โดยมีกระชาย 14 ชนิด 18 ตัวอย่างและพืชสกุลใกล้เคียงอีก 5 ชนิด ลำดับเบสที่จัดเรียงแล้วมีความยาวทั้งหมด 856 ตำแหน่ง รวม 8 ตำแหน่งจากการแทนรหัสตำแหน่งเพิ่มหรือขาดหายของชิ้นส่วนดีเอ็นเอ แต่มีเพียง 14 ตำแหน่งเท่านั้นที่มีข้อมูลเป็นประโยชน์ในการศึกษาประวัติวิวัฒนาการ (~1.65%) ซึ่งมีเปอร์เซ็นต์ใกล้เคียงกับ *mat*K และ *psb*A-*trn*H ผลการวิเคราะห์ด้วยวิธี Parsimony และ UPGMA พบว่าพืชในสกุลกระชายมีบรรพบุรุษร่วมกันมาหนึ่งสาย (monophyletic) และถูกแบ่งเป็นสองกลุ่มย่อยซึ่งเป็นไปตามจำนวนโครโมโซมพื้นฐานที่ต่างกัน ชนิดที่คาดว่าเป็นชนิดใหม่คือ *Boesenbergia bambusetorum* ถูกจัดกลุ่มร่วมกับ *B. longiflora* อีกสองตัวอย่าง เสนอแนะว่าต้องมีการศึกษาเพิ่มเติมถึงสถานภาพของพืชชนิดนี้

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Boesenbergia belongs to a ginger family, Zingiberaceae in order Zingiberales. *B. rotunda* (L.) Mansf. (กระชาย) is widely known in the genus and used in culinary in SE Asia. *Boesenbergia* species have also medicinal properties e.g. anti-inflammatory (Tuchinda *et al*., 2002) and HIV-1 protease inhibitory activity (Tewtrakul *et al*., 2003). There are about 80 species in the genus (Saensouk and Larsen, 2001), ranging from India to SE Asia in distributions. Thailand, as one of the two distribution centers apart from Borneo, is estimated to have 25 species (Larsen, 2003).

This study aims to explore new regions of chloroplast DNA for phylogenetic information. In our labs, we have used *mat*K and *psb*A-*trn*H for phylogenetic study of the genus. We have found that *mat*K and *psb*A-*trn*H have limited information to be useful in the study of *Boesenbergia* phylogeny. The intergenic region of *pet*A-*psb*J was reported to evolve rapidly compared to other regions in the cpDNA in *Musa* (banana) cultivars and hybrids (Swangpol, 2004), a taxon in Zingiberales. The region was also used to study the populations of *Trochodendron aralioides* Siebold & Zucc. (Huang *et al*., 2004). *Pet*A is a gene encoding for photosynthetic electron transport protein, cytochrome *f*, while *psb*J is a gene encoding for a subunit in photosystem II assembly. The region is found as a single copy unit in a large single copy (LSC) region of chloroplast DNA in rice, *Oryza sativa* L. (Hiratsuka *et al*., 1989). Thus, we hope that the region would give enough information for the study of *Boesenbergia* phylogeny. Moreover, molecular evidence can also be used in the classification and identification of the plants. Morphologically *Boesenbergia* has stem and leaf form similar to other genera in Zingiberaceae, such as *Kaempferia* and *Curcuma*. There are also floral variations within the species of *Boesenbergia*. Molecular evidence may be an additional useful source for the taxonomy of the genus.

Materials and Methods

Plant Material

There are 14 species totaling in 18 populations of ingroup Thai *Boesenbergia* (Table 1). Five outgroup taxa in 4 genera, *Cornukaempferia,*

Table 1. Plant samples included in this study with locality, chromosome number and GenBank accession number.

Species name	Locality	Chromosome number	GenBank accession number
1. *Boesenbergia bambusetorum* (unpublished) (Kai Larsen, pers. comm.)	Tak	-	DQ104857
2. *B. basispicata* K.Larsen ex Sirirugsa	Nakhon Si Thammarat (Khao Luang)	2n = 20 (Angsornkitt *et al*., 2004)	DQ104864
3. *B. curtisii* (Baker) Schltr.	KB = Krabi (Than Bok Karani Waterfall)	2n = 24 (Eksomtramage *et al*., 1996)	DQ104860
4. *B. curtisii* (Baker) Schltr.	NST = Nakhon Si Thammarat (Thungsong)		DQ104861
5. *B. longiflora* (Wall.) Kuntze	T = Tak	2n = 20 (Eksomtramage *et al*., 1996)	DQ104872
6. *B. longiflora* (Wall.) Kuntze	PCB = Phetchabun		DQ104873
7. *B. longipes* (Ridl.) Schltr.	Ranong	2n = 20 (Eksomtramage *et al*., 2002)	DQ104865
8. *B. petiolata* Sirirugsa	Saraburi (Khao Sam Lun)	-	DQ104863
9. *B. plicata* (Ridl.) Holttum	Krabi (Wat Tham Sue)	2n = 20 (Eksomtramage *et al*., 2002; Angsornkitt *et al*., 2004)	DQ104858
10. *B. prainiana* (Baker) Schltr.	Not known	2n = 20 (Eksomtramage *et al*., 1996)	DQ104866
11. *B. pulcherrima* (Wall.) Kuntze	MU = Mahidol University, Kanchanaburi	-	DQ104869
12. *B. pulcherrima* (Wall.) Kuntze	TP = Tong Phabhum, Kanchanaburi		DQ104870
13. *B. pulcherrima* (Wall.) Kuntze	HY = Huay Yang Water Fall, Prachuap Khiri Khan		DQ104871
14. *B. regalis* B.Kharukanant & S.Tohdam	Narathiwat (Hala Bala)	-	DQ104862
15. *B. rotunda* (L.) Mansf.	Not known	2n = 36 (Eksomtramage & Boontum, 1995)	DQ104859
16. *B. tenuispicata* K.Larsen	Krabi	2n = 20 (Angsornkitt *et al*., 2004)	DQ104867
17. *B. thorelii* (Gagnep.) Loes.	Not known	-	DQ104868
18. *B. xiphostachya* (Gagnep.) Loes.	Mukdahan	2n = 20 (Eksomtramage *et al*., 2002)	DQ104874
19. *Cornukaempferia aurantiflora* J.Mood & K.Larsen	Phitsanulok	-	DQ104875
20. *Hedychium biflorum* Sirirugsa & K.Larsen	Not known	-	DQ104879
21. *Kaempferia parviflora* Wall.	Nakhon Ratchasima	-	DQ104877
22. *K. candida* Wall.	Not known	-	DQ104876
23. *Scaphochlamys rubescens* Jenjitt. & K.Larsen	Not known	-	DQ104878

Hedychium, Kaempferia and *Scaphochlamys* are also included (Table 1). Voucher specimens are kept either at National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok or at Walailak University herbarium, Nakhon Si Thammarat.

Total Genomic DNA Extraction

The CTAB method (Doyle & Doyle, 1987) was used to obtain total DNA of plant cells. Fresh leaf samples were taken and kept in dry silica gel before DNA extraction. A mixed solution of CTAB and β-mercaptoethanol (1 ml: 4 µL) 500 µL was preheated at 60°C. Leaf sample, about 1 g, was ground into powder with help of liquid nitrogen. Incubation in a water bath took place at 60°C for 30 minutes. Next, 500 µL of $CHCl_3$: Isoamyl alcohol (IAA) (24:1) was added to the tube. The tube was then centrifuged at 12000 rpm for 4 minutes. The aqueous (upper) phase was removed to a new clean tube. Cold isopropanol was added 2/3 of the extracted volume. Visible DNA was removed by a pipet tip to a new tube filled with 200 µL of 1X TBE pH 8.0. DNA precipitation was done by adding 100 µL of 7.5M ammonium acetate and 400 µL of absolute ethanol. The solution was discarded and the DNA was left to dry at room temperature. The DNA pellet was dissolved in 10-30 µL of TE and stored at -20°C until required.

Primer Design

Primers for *pet*A-*psb*J are newly designed from conserved regions which were identified by BioEdit (Hall, 2004) of 14 sequences available in GenBank. The 14 species sequences are *Amborella trichopoda* Baill. (Amborellaceae; AJ506156), *Arabidopsis thaliana* (L.) Heyn. (Brassicaceae; NC000932), *Calycanthus fertilis* Walt. (Calycanthaceae; AJ428413), *Nicotiana tabacum* L. (Olanaceae; AP006714), *Nymphaea alba* L. (Nymphaeaceae; NC006050), *Oryza nivara* Sharma et Shastry (Poaceae; NC005973), *Oryza sativa* L. (Poaceae; NC001320), *Panax ginseng* C.Meyer (Araliaceae; NC006290), *Psilotum nudum* (L.) Beauv. (Psilotaceae; AP004638), *Saccharum officinarum* L. (Poaceae; AP006714), *Spinacia oleracea* L. (Amaranthaceae; NC002202), *Triticum aestivum* L. (Poaceae; AB042240), *Trochodendron aralioides* (Trochodendraceae; AY294753), *Zea mays* L. (Poaceae; X86563)

Primer sequences designed and used in this study are (5′ to 3′), *pet*A-F = AGG TTC AAT TGT MCG AAA TG, *psb*J-R = CTG GAA GRA TTC CTC TTT GG, *psb*L-R = GTA CTT GCT GTT TTA TTT TC.

PCR Amplification and DNA Sequencing

Each PCR reaction was 50 µL in volume containing *Taq*DNA polymerase (0.02 U/µL). The PCR reaction also included buffer (1X), $MgCl_2$ (4 mM), dNTP (0.24 mM) and primers (each 0.24 µM). The heat cycles began with an initial denaturation at 94°C for 2 mins, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension of 10 mins at 72°C. Amplification of the *pet*A-*psb*J spacer was performed using either a pair of primers *pet*A-F and *psb*J-R or a pair of primers *pet*A-F and *psb*L-R. PCR products were checked on 0.8% agarose gel. Successful PCR resulted in a single band of ethidium bromide corporated-DNA viewed under ultraviolet (UV) light corresponding to approximately 1000 bp or 1200 bp. Liquid and unpurified successful PCR products were then sent to Macrogen Inc. (Seoul, Korea) (http://www.macrogen.com) for direct sequencing with primers, *pet*A-F and *psb*J-R.

Sequence Analysis

Sequence chromatograms were checked and edited manually in Chromas (Technelysium, 2004). Alignment was performed using BioEdit (Hall, 2004). Aligned sequences can be obtained by contacting the authors.

Phylogenetic Analysis

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Parsimony analyses were performed by PAUP 4.0 beta10 (Swofford, 2004). UPGMA is a distance method that produces a phenogram suggesting relationships based on overall similarities of the sequences.

Parsimony seeks to find the shortest tree of evolutionary changes, a cladogram, in the sequences based on shared derived characters (synapomorphies). Heuristic search that is suitable for analyzing more than 20 taxa was chosen to find the shortest tree(s). Support for individual clades was given by a bootstrap value (Felsenstein, 1985).

Results

PCR products, ~ 1,000 bp in length from a pair of primers *pet*A-F and *psb*J-R and ~ 1,200 bp in length from a pair of primers *pet*A-F and *psb*L-R, were obtained as a single band from all species in this study. The *pet*A-*psb*J spacer sequences alignment of all 23 taxa, including 18 ingroup taxa and 5 outgroup taxa, resulted in 848 bp long. Individual sequences varied from 663 to 750 bp in *Boesenbergia* and from 674 to 743 bp in outgroup species. All sequences are deposited in GenBank (see Table 1).

Intraspecific sequence variation was 0.16% in *B. curtisii*, 0.00% in *B. pulcherrima* and 0.33% in *B. longiflora* (including *B. bambusetorum*). The maximum sequence variation within ingroup was 1.57%, between *B. prainiana* and *B. rotunda*. The maximum sequence variation between ingroup and outgroup was 2.55%, between *B. tenuispicata* and *Scaphochlamys rubescens*. The percentage of parsimony informative characters of *pet*A-*psb*J, 1.65% is close to the other two regions i.e. 1.6% in *mat*K and 1.5% in *psb*A-*trn*H (unpublished data).

Analyses of the aligned sequences revealed 14 parsimony informative sites and 8 informative indels. Therefore, there were 22 informative characters (2.57%) in total for use in phylogenetic analyses. UPGMA analysis gave a phenetic tree, Figure 1, which was based on overall similarities of the sequences aligned. Parsimony analysis gave 10,044 most parsimonious trees, with length of 80, consistency index (CI) = 0.73, retention index (RI) = 0.84. The strict consensus tree, a phylogenetic tree, of 10,044 most parsimonious trees was given in Figure 2 with bootstrap values.

Discussion

Though sequences from a forward primer were all readable and posed no problems on base identification, sequences from reverse primers were not complete and even some species very short, 100-200 bp. PCR product secondary structure may be one of possible causes of the incident. Secondary structure that is formed by GC or GT rich region can inhibit DNA polymerase reaction during the sequencing process. Some modifications of the sequencing are now in trials to solve the problem. This incident was also found in *Musa* (Swangpol, 2004).

This study has shown that the *pet*A-*psb*J spacer is a limited source for phylogenetic characters in the study of *Boesenbergia* phylogeny. Due to low signal in the three regions that we have sequenced so far, search for rapidly evolving chloroplast gene in *Boesenbergia* is still ongoing. We have a plan to analyse a combined matrix of all three chloroplast DNA regions in the future as well as a combined matrix of chloroplast DNA and nuclear DNA such as the Internal Transcribed Spacer of ribosomal DNA (ITS). However, the UPGMA tree and the parsimony tree have shed some light on the evolution of *Boesenbergia*.

Both UPGMA and parsimony analyses suggest that *Boesenbergia* is monophyletic (with 81% bootstrap value). There are also some similar branching patterns in both trees. They are (1) a clade of *B. curtisii, B. regalis* and *B. rotunda* (as also found in Vanijajiva *et al*., 2003) (2) a clade of *B. basispicata, B. prainiana* and *B. tenuispicata* (as also found in Vanijajiva *et al*., 2003, 2005) (3) a clade of *B. petiolata* and *B. plicata* (4) a clade of *B. bambusetorum* and *B. longiflora*. Populations of *B. pulcherrima* and *B. curtisii* are grouped together, indicating a close relationship or a species. Two populations of *B. longiflora* and one population of *B. bambusetorum* are grouped together (67% bootstrap value). More data, both molecular (e.g DNA sequence) and morphological are needed to verify the species status of *B. bambusetorum*.

B. curtisii, B. regalis and *B. rotunda* share a compact inflorescence, hardly elongating. Their

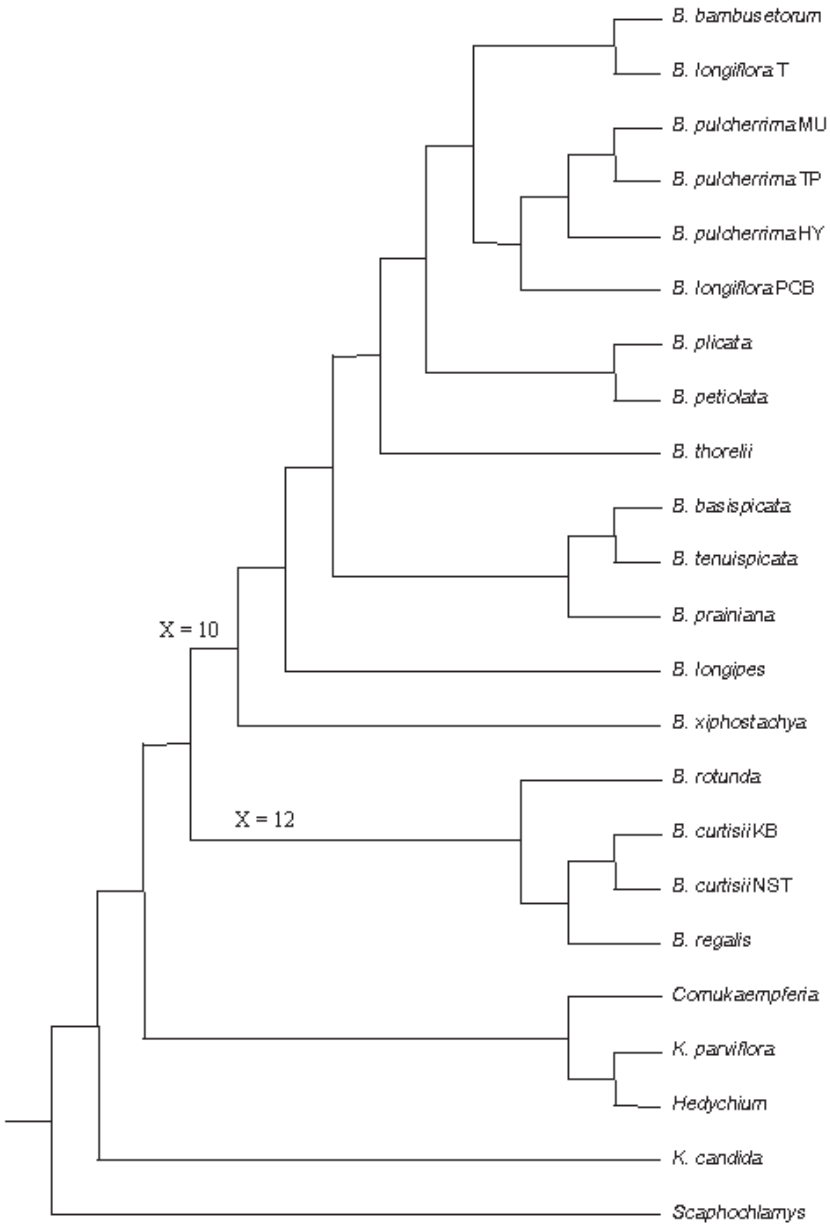

Figure 1. The UPGMA tree of *Boesenbergia* based on *pet*A-*psb*J spacer. There are two basic chromosome numbers i.e. x = 10 and x = 12 in *Boesenbergia*. The basic chromosome numbers are placed on the corresponding clades.

bracts are not or hardly projecting beyond the protecting leaf sheaths, even fully grown.

Positions of the inflorescence may well be a good character that indicates close relationships among the species. *B. basispicata, B. prainiana* and *B. tenuispicata* are grouped in a branch as also found in *B. longiflora* and *B. bambusetorum*. These species possess lateral inflorescences, while the others have terminal inflorescences. The relationships were also supported by Isozymes

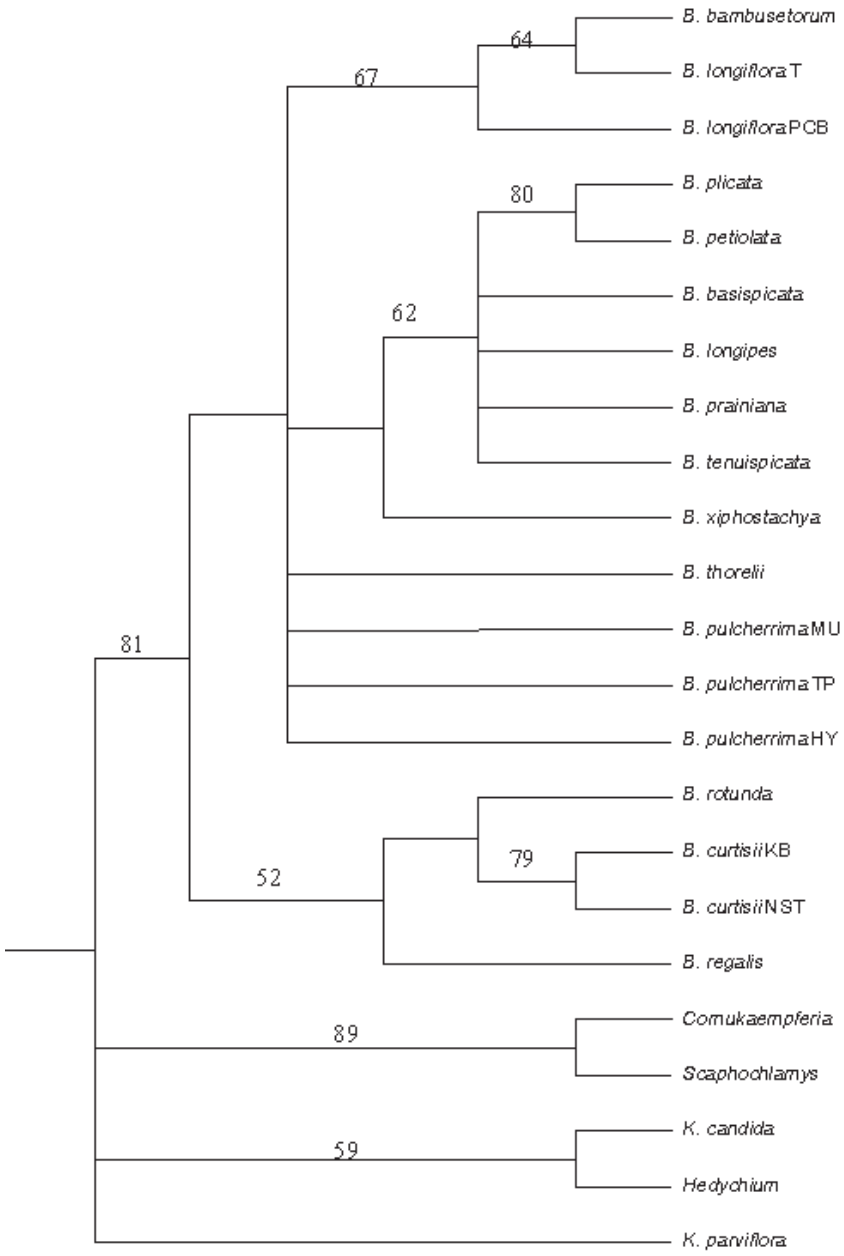

Figure 2. Strict consensus tree of the 10,044 most parsimonious trees resulting from analysis of *pet*A-*psb*J data for 23 taxa. Numbers above the branches are bootstrap values of 1000 replicates. (CI = 0.73, RI = 0.84)

and RAPD studies (Vanijajiva *et al*., 2003, 2005).

B. *bambusetorum* may be a variation of *B*. *longiflora*. *B*. *bambusetorum* possesses pale yellow floral parts, including lateral staminodes and the labellum with red stripe in the center while *B*. *longiflora* has whitish floral parts and the red stripe at the center of its labellum. The labellum of *B*. *bambusetorum* is more saccate than *B*. *longiflora*'s labellum which is rather spreading. However, red stripe in *B*. *longiflora* is divided into two different

shades, a strong red inner part and a pinkish or whitish red outer part.

Chromosome number as a character in the evolutionary history of *Boesenbergia* suggests that there are two different lines of evolution of the genus. One is that the basic chromosome number is x = 10, while the other is x = 12. The two separate clades of *Boesenbergia* was also found in Kress *et al*. (2002) and Ngamriabsakul *et al*. (2003). Confirmation on the two different lines of evolution and the monophyly of the genus require broader sampling of *Boesenbergia* species, especially in addition of species that are found in Borneo, one of the two centers of diversity of the genus. In addition, genera in the tribe Zingibereae, in which *Boesenbergia* belongs to, should be more included in the phylogeny analysis for possibly clearer patterns of its evolution.

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