PHYLOGENY OF THE GENUS *POMATOCALPA* BREDA (ORCHIDACEAE)

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INTRODUCTION

The orchid genus *Pomatocalpa* Breda belongs to subtribe Aeridinae (subfam. Epidendroideae, tribe Vandeae). From recently account, there are 14 species distributed from Sri Lanka to Fiji, south to Northern Australia and north to Southern China (Hainan) and Taiwan (Watthana, inprep. *a*, *b*).

The Breda's name had been disregarded for many years, until Smith (1912) re-instated the genus and gave a full generic diagnosis, finding that many entities had wrongly been place elsewhere, mainly in *Cleisostoma* and *Saccolabium*.

Garay (1972) supplied a short description of the genus "Characterized by a short footless column to which the fleshy lip is immovably adnate. Rostellum bifid, hammershaped. Pollinia 2, each split into unequal halves on a slender stipe. Lip more or less bucket-shaped with a distinct tongue or valvate callus, often forked at the tip projecting from the backwall diagonally across toward the apex".

A distinct tongue or valvate callus is observed in the genus *Trichoglottis* and *Staurochilus*. In his generic key, Seidenfaden (1988) indicated that the genus *Pomatocalpa* differes from the genus *Staurochilus* and *Trichoglottis* by having the glabrous backwall tongue placed deeper in the spur. Obviously base on morphology, these to genera close ally of the genus *Pomatocalpa.*

In the recently revision, the combination of appearing of backwall tongue and two flattened vertical elongate calli at upper front thus providing a narrow longitudinal groove at the upper front of the spur is accepted for the genus's characters. According to the new revision, three *Pomatocalpa* species: *P. bambusara*, *P. bhuthanica* and *P. armigera* were excluded (Watthana, inprep).

Although a comprehensive hypothesis about phylogeny of the genus *Pomatocalpa* is not yet available. The globally phylogenetic relationship of tribe Aeridinae base on DNA (*matK* and ITS) has revealed some information (H. Topik et al., inprep). In their analysis, two sampled of *Pomatocalpa*, *P. diffusa* and *P. kunstleri*, were employed to be representative of the genus constructing phylogeny of the subtribe. Their results revealed

that the genus *Pomatocalpa* is monophyletic with having the monotypic genus *Haraella* as sister-group.

The objective of this paper are 1) to study the phylogeny of the intergeneric relationship of the genus *Pomatocalpa* and their allied genera base on morphology; 2) to study the phylogeny of the intrageneric relationship of the genus *Pomatocalpa* base on molecular, morphology and total evidence; 3) to discuss the monophyly of the genus.

MATERIALS AND METHODS

Intergeneric relationship

Morphological data

Sampling

The closed relative genera to *Pomatocalpa* which are *Acampe*, *Smitinandia*, *Ventricularia*, *Trichoglottis*, *Staurochilus*, *Cleisostoma*, *Pelathanteria*, *Micropera*, *Sarcoglyphis*, *Robiquetia*, *Haraella* were selected to compare with 5 representative species for the genus *Pomatocalpa*, which are *P. bicolor*, *P. spicata*, *P. kunstleri*, *P. diffusa*, *P. maphersonii* and *P. tonkinensis*. A monotypic genus *Haraella* was included into ingroup with respect to the recent molecular data (see below). Additionally, previous synonym and uncertain species were also include, viz *Trichoglottis lasiocarpa* (syn. *P. lasiocarpa*), *Pomatocalpa armigera*, *P. bambusara* and *P. bhutanica*. Unfortunately, there is no specimen of *Robiquetia vaupelii* (syn. *P. vaupelii*) available for this study.

Base on recently molecular phylogeny, different outgroups were selected, viz *Seidenfadenia*, *Vanda* and *Ascocentrum*. (H. Topik et al., unpublished data).

Characters

Morphological characters are usually defined as qualitative or quantitative. However, those qualitative characters are expressible by quantitative one when expored in detail (Felsenstein, 1988?; Steven, 1991?). According to Stevens (1991), character stated used in phylogenetic analysis should be only without overlap. However, some analyses of overlapping morphometric data may track phylogeny and may contain a considerable amount of phylogenetic information (Thiele, 1993; Ryding, 1998). According to Wiens (1995), his results on overlapping character can contain significant phylogenetic information but these characters are less reliable in inferring phylogeny than non-

overlapping characters. Thus, the polymorphoic character were included in this analysis, unless those give too much variable.

The generic character in this cladistic analysis mainly considered from various key and diagnosis description of the genera (i.e. Shutiman and Vogel, 2000; Seidenfaden, 1988; Seidenfaden & Wood, 1992). Additionally, herbarium and spirit collection collection of all species of involed genera at C, as well as living collection at Copenhagen Botanical Garden, were used to be morphological comparative study.

Habit: The plant which have large stem with closed leave ex. Vanda was coded as not elongate stem. Some species of the genus *Pomatocalpa* , such as *Pomatocalpa diffusa*, are polymorphic. However, *P. spicata*, *P. macphersonii*, *P. kunstleri* have consistent short plant habit, while, *P. bicolor* has consistent rambling habit.

Inflorescence: The inflorescence type was excluded due to seeming inconsistent within the same specimen, i.e. *Pomatocalpa spicata* has usually racemous, but some specimen can produce a few inflorescence branches at peduncle nodes later.

Flower: Several characters of flower such as number, size, color, sepal and petal shape and size were excluded to this analysis due to too much variable among the taxa. The surface of labellum with having densely minutely papillose (nipple-like) and homogenous surface was separated state from pubescence, long hairs in such of *Trichoglottis*, *Staurochilos*, *Haraella*, and *Acampe* (Table 1 character2). The various ornament on midlobe, such as keel at middle midlobe, dense warts and thickened margin is treated in single state when one of this kind of ornament present, although homology of various kind of appendage are uncertain.

The presentation of calli at front wall of spur mouth seem common. However, in this study, I had observed comparatively among available spirit collection depositing at C. The present of a big callus at upper front of the spur is informative which is founded in *Pomatocalpa bambusarum*, *Smitinandia* and *Micropera*. Its shape is variable from among species such as in *Smitinandia micrantha* has globular callus but it is rather flattened in *Smitinandia helferi*.

There are several species and genera have 2 calli origin between base of front part of midlobes and spur. However, in the recently accepted *Pomatocalpa* species have 2 vertical elongate calli from basal part of the midlobe down in to the spur and thus providing a narrow longitudinal groove in between at upper front of the spur because the calli are close together. Due to this 2 characters are related, the present of a narrow

longitudinal groove at upper front of the spur is included in this analysis. It is devoice from the ratter few known information and the problematic in scoring about calli at front wall of the spur. This narrow longitudinal groove has rather short in *P. kunstleri* and *P. spicata* because a pair of callus forming a v shape (divergent). So far, the feature of front wall callus such as in the genus *Pomatocalpa* is tentatively unique for the genus.

The presentation of ligulate callus on back wall of the spur has been found in only *Trichoglottis*, *Staurochilus* and *Pomatocalpa*. The glabrous ligulate callus which is used in Seidenfaden' key to genera (1988) is found in *Trichoglottis bipunctata*. As well as, much deep attaching of the ligulate callus is also found in some species of *Trichoglottis* and *Staurochilus*.

Phylogenetic analysis

Phylogenetic analyses based on maximum parsimony were performed using PAUP* version 4.0b10 for Microsoft Window (Swofford, 2001) for a data stes of intrageneric morphological data. All characters were equally weighted, unordered. All data sets were analysed by the heuristic search method with bisection-reconnection (TBR) branch swapping and the MULTREES option on, saving all most parsimonious trees (MPTs). Evaluation of internal support of clades was conducted by the bootstrap analysis with 10,000 replicate with faststep searching.

Intrageneric relationship

Molecular data

Sampling

Seven species of *Pomatocalpa* sequences were available for this study, which were *P. acuminata, P. bicolor*, *P. diffusa*, *P. kunstleri*, *P.macphersonii*, *P. maculosa* and *P. spicata*. They were employed to be preliminary analysis together with all the available sequences of 75 genera of 103 genera of the subtribe *Aeridinae* which was done by the second author (data not show).

The result showed that *Pomatocalpa* is monophyletic group in matK analysis. However, in ITS data showed that *Haraella odorata* was placed among *Pomatocalpa* species. Although, the molecular analysis of the intergeneric relationship of the tribe *Aeridinae* showed that the genus *Pomatocalpa* is monophyletic with having genus *Haraella* as the sistergroup, with using only 2 taxa, which were *P. kunstleri* and *P. diffusa* (Hidayat, submit.). Indeed, this reanalysis of matK and ITS of *Pomatocalpa*, *Haraella*

and selected outgroup are shown here. The monotypic genus *Haraella* is considerably ingroup of this analysis, due to its inconsistence.

Base on preliminary phylogeny tree construction (see above), *Acampe ochracea*, *Smitinandia micrantha* and *Ventricularia tenuicaulis* were representatively employed as outgroup for matK and ITS analyses. All sequences of the outgroup and *Haraella odorata* sequences were received from the second author's project, on phylogeny of subtribe *Aeridinae* (Orchidaceae). List of voucher specimens is presented in Table xxx.

Nucleotides preparation

For the molecular analyses plant material was obtained from the living orchid collection of the botanical gardens in Tokyo, Queen Sirikit Botanic Garden and field trip from the first author. All DNA extractions and sequences were done by the second author at Department of Biological Science, The University of Tokyo, Japan.

The total DNA was extracted from fresh materials or silica-gel dried plant tissues following the instruction of QIAGEN DNeasy Mini Plant Kit. For *matK* sequences, the amplification was performed using a primer pair, OMAT1F and trnK-2R (Fig.1a). The 20-μl amplification reaction includes 2 μl 10 x of Ex-Taq buffer (Takara Bio Inc.), 1.6 μl 2.5 mM of dNTPs mix, 0.5 μl each primer (10 pmol), 0.1 micro liter 5 units/μl of Ex-Taq DNA-polymerase (Takara Bio Inc.), 2 μl of template DNAs and 13.3 μl of MilliQ water. The polymerase chain reaction (PCR) profile consists of an initial 5-min premelt at 94°C and 30 cycles of 30-s denaturation at 94°C, 30-s annealing at 53°C, and 3-min extension at 72°C, followed by a final extension of 7-min at 72°C.

Amplification of ITS region was carried out using a primer pair, AB101 and AB102 (Fig.1b). Total volume of PCR was 30 μl that includes 15 μl GC buffer I (Takara Bio Inc.), 4.8 μl 2.5 mM of dNTPs mix, 0.5 μl each primer (10 pmol), 0.21 μl 5 units/μl of LA Taq DNA-polymerase (Takara Bio Inc.), 2.4 μl of template DNAs and 6.59 μl of MilliQ water. The PCR profile consists of an initial 2-min premelt at 94°C and 30 cycles of 50-s denaturation at 94°C, 1-min annealing at 60°C, and 30-s extension at 72°C, followed by a final extension of 7-min at 72°C. To confirm the number of amplified copies for ITS regions, we performed the single-strand conformation polymorphism (SSCP) analysis based on method developed by Orita et al. (1989) with several modifications.

The PCR products were cleaned by using Montaqe PCR Centrifugal Filter Devices (Millipore Co.) and were used for auto-cycle sequencing reaction. The 10-μl auto-cycle sequencing reaction includes 3 μl of Master Mix (Beckman Coulter), 1 μl primer (1.6 pmol), and 6 μl of PCR product. The reaction was incubated with 50 cycles of 20-s at 96°C, 20-s at 50°C and 4-min at 60°C. Figure 1 also shows detailed information on primers used in auto-cycle sequencing.

Auto-cycle sequencing products were cleaned by adding STOP solution (2 μl 3M of NaOAc, 2 μl 100 mM of EDTA, and 1 μl 20 ng/μl of Glycogen) and 60 μl of 100 % ethanol; subsequently, they were centrifuged at 14000 rpm for 15-min at 4°C. The alcohol/salt mix was discarded, and the pellet was subjected to two washes with 200 μl 70% ethanol, each followed by centrifugation at 14000 rpm for 2-min at 4°C. Cleaned auto-cycle products were allowed to dry in the vacuum dry for 15-min. Both forward and reverse sequences were analyzed with CEQ8000 automated sequencer (Beckman Coulter), and electropherograms were edited and assembled with Genetyx-ATGC version 4.1 (Genetyx Corporation).

Phylogenetic analysis

DNA sequences obtained from matK and ITS were aligned with ClustalX and were then adjusted manually. Phylogenetic analyses and the evaluation of internal support of clades were performed as same as the method of the morphological intergeneric analysis (see above) for the data sets of matK and ITS.

Morphology

Sampling

Most of the morphological data used in this cladistic analysis were from investigation of spirit and herbarium material from AAU, AMES, BM, C, K, L, and P. Additionally, the following literature has also been consulted: Seidenfaden (1988, 1992), Seidenfaden and Wood (1992), and Comber (1990).

Characters

All recently accepted species of the genus *Pomatocalpa* and the monotypic genus *Haraella* were treated as ingroup. Thegenus *Acampe*, *Ventricularia* and *Smitinandia* were

employed to be outgroup. Using the generic taxon may be better than species taxon, since it would be reduced the random effect from the species of outgroup. The codding as genus taxa in stead of species taxa also presumable to represent the character state present in the ancestral species of each genus (Wiens, 2000). Thus, only the ancestral state of the outgroup taxon will be compared to ingroup.

Habit. Although it is seems to be separated habit type into 2 groups as short or fan-shape plant and rambling plant (Kerr, 1985). There are polymorphic in *Pomatocalpa diffusa*, *P. fusca* and *P. marsupialis*. All of the fan-shape plant is consistent of it lengths which is not more than 30 cm long. Leave sheath spread off or closed to the stem is too much variable and difficult to observe especially in the short stem plant due to it is depend on plant and leave size. Leave morphology, such as size, shape, apex is continue variation entirely in the genus.

Inflorescence. The orientation of inflorescence is considered at peduncle. *P. kunstleri* usually has upright peduncle with pendulous rachis, however, the pendulous inflorescence has been observed in this species. So it was scored as variable. In the common species, *Pomatocalpa spicata* often produces both unbranch or branched inflorescence and it has been observed that some inflorescence can be produced at the peduncle node later. However the relation between peduncle from base of inflorescence to the terminal rachis is comparatively informative (Fig. xxx). It was observed that *P. linearifolia* (=*P. maculosa*) has finely pubescent rachis. (Seidenfaden, 1988). From my observation, it is variable from minutely papillose to sparsely finely pubescent on rachis but it is significant on peduncle. Only *P. kunstleri* has finely pubescent peduncle.

Flower. Size of flower is representative by size of dorsal sepal. Several species of *Pomatocalpa* has obovate to obovate-oblong petal and the boundary of these two states is difficult to underline, hence they are combined into same state. Labellum of *Pomatocalpa* sometime has densely minutely papillose but it is homogenous which is different from pubescent of *Acampe Ventricularia* and *Haraellra*. Exceptionally, *P. undulata* and some plant of *P. spicata* have obtuse erect labellum-sidelobes. Almost all of the *Pomatocalpa* has labellum-sidelobes in which hind edge of the spur forming a right angle to front edge of spur. The relation of the length between hind edge and frond edge is significant (character 14). However, it can not code for *P. undulata* and some of *P. spicata* because they has erect obtuse labellum-sidelobes which is not forming a right angle between hind and frond edge. The labellum-midlobe thickeness at base is observed from spirit collection and fresh material. Back wall lamella callus outline is too much variable and

problematic to score due to it is usually keel at the middle and lay on the front mouth of the spur, thus this character is excluded (Watthana, inpre.)

Phylogenetic analysis

Phylogenetic analyses and the evaluation of internal support of clades were performed as same as morphological intergeneric relation (see above) for a morphological data set.

Total evidence

Sampling

Differences in tree topologies between the different analyses are probably due to sampling error (Huelsenbeck et al., 1996). To improve sampling, a combinaed analysis of all tree data sets was performed. *Ventricularia tenuicaulis*, *Acampe ochracea* and *Smitinandia micrantha* were used to be outgroup, because the DNA sequences were derived from single individual. The species of *Pomatocalpa* which molecular data have not been available in combine data were coded as inapplicable.

RESULTS

Intergeneric relationships

Morphological data

Of the 18 character scored, 14 were informative. The MP analyse yielded 3704 most parsimonious trees (length = 33; CI = 0.58; RI = 0.73). The concensus topology is shown in Fig xxx. The genus *Pomatocalpa* seems to be monophyletic group. Unfortunately, there is no bootstrap support any branches except very low supported (55%) clade of *Micropera* and *Pomatocalpa bamburarum*.

Intrageneric relationships

Molecular

The matK alignment has a total number of 1835 sites, of which 110 variable and 28 were phylogenetically informative. The MP analysis yielded 9 most parsimonious tree (length=167; CI=0.89; RI=0.65). The topology of this tree and the corresponding branch supports are shown in Fig 1. Resolution of the matK tree is very low. However, the clad of the genus *Pomatocalpa* was strongly support (91%). The clad of *Haraella* and

Pomatocalpa was moderately support (79%). While, there was rather weakly support to *Acampe* and *Ventricularia* (58%)

The ITS alignment has a total number of 668 sites, of which 69 variable and 24 were phylogenetically informative. The MP analysis yielded 6 most parsimonious tree (length=116; CI=0.85; RI=0.64). The ITS tree has more resolution more than of matK tree, but all clad were weakly supported. There is not supported for the genus *Pomatocalpa*. The topology of this tree and the corresponding branch support are shown in Fig 2.

Morphological data

Of the 32 character scored, 4 were uninformative and the remaining 28 were informative. The MP analyses yielded 2,178 most parsimonious trees (length = 64; CI = 0.63; RI = 0.69). The consensus topology with corresponding branch supports are shown in Fig.xxx. Resolution of the morphological bootstrap consensus is low. Only tree clad receive weak support: the clad of the *Pomatocalpa* (63%), the cald of *P. simalurensis*, *P. bicolor* and *P. floresceana* (69%), and the clad *P. kunstleri* and *P. tonkinensis* (66%).

Total evidence analysis

The data matrix of the combine molecular and morphological analyses contains 2534 sites, of which 187 variable and 82 phylogenetically informative. The MP analyses yielded 252 most parsimonious trees (length = 365; CI = 0.81; RI = 0.61). The consensus topology with corresponding branch supports are shown in Fig. xxx. Resolution of the total evidence analyses is a bit still lower than individual matK data set but better than the individual morphological data set. However, *Pomatocalpa* clad was supported lower than individual matK data set (78%). *Pomatocalpa* and *Haraella* clad was supported higher than individual ITS data set but lower than individual matK data set (80%).

DISCUSSION

Intergeneric relationship

There are too many equally MP trees due to many coding of missing data and polymorphism states (c.f. Wilkinson, 1995). However from the consensus tree showed that the genus *Pomatocalpa* is monophyletic group, although there is no boostrap supporting. The character of the front callus is unique character for the genus

Pomatocalpa. The resolution among intergeneric phylogeny is very low. It is not so surprised that to polytomous topology reveal in the morphological data of tribe Aeridinae, due to the high number of genera and not much unique character for each genus. There were attempts to construct phylogenetic relationship base on morphological data of this tribe but it has not success yet.

The newly preliminary of molecular study seems to be incongruent with macro-morphological data, and then it is difficult to explain the molecular topology (Hidayat, per.com.). Perhaps the new and less characters such as seed and velamen structure or chemical properties may get nearer towards a classification that appears to express a natural relationship (Seidenfaden & Wood, 1992; Rusmussen, per.com).

However, the relationship between the monotypic genus *Haraella* base on macro-morphological data showed that it is far related to the genus *Pomatocalpa*. From this analysis *Haraella* and *Robiquetia* nested with outgroup taxa.

Intrageneric relationship

The matK data provided a good resolution for the genus *Pomatocalpa* to be monophyletic group which clearly separated *Haraella* to be sister group. There is no any resolution at intrageneric level. While, ITS data showed that *Pomatocalpa* is paraphyletic group, with having *Haraella* mixed among. It is as same as the preliminary analysis of all available sequence of *Pomatocalpa* and all species of tribe Aeridinae (data not show).

Base on individually morphological data the genus *Pomatocalpa* is still monophyletic group, although it was not strongly support. The position of *Haraella* is mixed with outgroup indicated quite different in morphology.

According to the total evident of this analysis, the genus *Pomatocalpa* has a unique synapomorphy which is present of a narrow groove at the upper front of the spur and the ligulate tongue present at back wall of the labellum-spur.

Inside the genus, the clad of *P. kunstleri* and *P. tonkinensis* was weak supported (57%) with having petal linearis as unique character. The clad of *P. bicolor*, *P. simalurensis*, *P. floreseana* also was weak supported (59%) without any unique character. The clad of *P. bicolor*, *P. floresana*, *P. simalurensis*, *P. maculosa*, *P. diffusa*, *P. marsupialis* has stem more than 30 cm as a unique character, however, it is showed that this character is the polymophices in *P. diffusa*, *P. fusca* and *P. marsupialis*.

Neither morphological data nor matK or ITS sequences provided sufficient resolution to study interspecific relationships within the genus *Pomatocalpa*. Variation of

matK and ITS on species level appeared to be very low, 6.0% and 10.3% respectively. Both molecular dataset seem to lack resolution due to high internal conflict among the sequences collected, as can be deduced from the relatively low RI, 0.65 and 0.64 respectively. To produce a final phylogeny of the species, data from other DNA regions should be collected.

Monophyle and generic status

The genus *Pomatocalpa* is monophyletic with strong branch support (91%) from separately *matK* data. The combined analysis of the morphological and molecular data showed that the genus *Pomatocalpa* is monophyletic but the branch support was lower than separately *matK* due to conflict between ITS and matK data set. The analysis of the morphological data also showed that the genus is monophyletic, but lower branch support,

In contrast, the ITS data showed that the genus *Pomatocalpa* is paraphyletic due to placing of the monotypic genus *Haraella*. However, chloroplast gene is more suitable for plant phylogenetic relationship among plant, since it is more stable than of the nuclear gene. **Thus, it seems to suspect the reliability of the ITS gene (reference xxx).**

Moreover the monophyletic *Pomatocalpa* required only 2 steps longer from ITS topology. It is not much when comparing to the moving, for instance, *P. acuminata* and *P. bicolor* which required 9 and 6 steps longer, respectively.

If it turns that we accept ITS data is concerned, then the genus *Pomatocalpa* is paraphyletic group. There is strictly accepted only monophyletic group in the phylogenetic classification. The paraphyletic group is in the question if it is should be accepted to the classification (xxx). Thus, there is no new combination between *Haraella* and *Pomatocalpa* in this study.

REFERENCES

Felsenstein 1988.

Garay 1972

Hidayat, et al., submit.

Huelsenbeck, J.F., Bull, J.J. & Cunningham, C.W. 1996. Combining data in phylogenetic analysis. Trends Ecol. Evol. 11: 152-158.

Kerr, A. D. 1985. The genus *Pomatocalpa*. *Amer. Orchid Soc. Bull.* 54: 312-313.

Lindley, J. 1833. Gen. et. Sp. Orchid. 227. 1833.

Ormerod, P. 1997. xxxxx. Australian Orchid Revel. 62(3):15.

Ridley, H.N. 1896. Orchidaceae and Apostasiaceae of the Malaysia Peninsula. J. Linn. Soc. (botany) 32: 213-416.

Ryding. 1998

Schuiteman and de Vogel, 2000. Orchid Genera of Thailand, Laos, Cambodia and Vietnam. Vietnamese-English ed. Nationaal Herbarium Nederland.

Seidenfaden, G. 1988. Orchid Genera in Thailand XIV. Fifty-nine Vandoid Genera. Opera Bot. 95. 1988.

Seidenfaden, G. & Wood, JJ. 1992.

Smith, J.J. 1912. Nat. Tijdssch. Ned. Ind. 72:108?. 1912.

Steven, P.F. 1991.

Thiele, 1993

Watthana, S. inprep.*a*. The Genus Pomatocalpa- Taxonomic Revision.

Watthana, S. inprep. *b.* The Pomatocalpa latifolia/maculosa complex (Orchidaceae) resolved by multivariate morphometric analysis.

Wiens, J.J. (ed.). 2000. Phylogenetic analysis of Morphological Data. Smithsonian Institution Press, Washington.

Wiens, J.J. 1995.

Wilkinson, M. 1995. Coping with Abundant Missing Entries in Phylogenetic Interference Using Parsimony. Syst. Biol. 44(4): 501-514.