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IDENTIFICATION OF PUTATIVE HYBRIDS AND NATURAL LINEAGES IN GENUS *POTAMOGETON* REVEALED BY CHLOROPLAST AND NUCLEAR DNA MARKERS

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Abstract

Phylogenetic relations of hybrids and non-hybrid species in the genus *Potamogeton* were reconstructed based on three spacers and one intron of chloroplast sequences and nuclear sequences of 5S-NTS. By comparing the phylogenetic relationships of subgenus *Potamogeton* with floral size, we propose that the split in the two main lineages reflects an early differentiation of flower size, perhaps due to the shift from out- to in-breeding; we also presume that more complex evolutionary processes exist in subgenus *Potamogeton* based on present study. As natural hybridization plays a fundamental role in the evolution of *Potamogeton*, frequently resulting in the formation of entirely new species, compelling evidence supports the hybrid origin of species: *P. anguillanus*, *P. hubeiensis*, *P. kamogawaensis*, and the triple hybrid-*P.* sp. hybrid. Incongruence between nuclear and plastid tree indicated that introgression might had occurred from subgenus *Coleogeton* to subgenus *Potamogeton*.

Key words: Chloroplast DNA; Hybridization; Molecular phylogeny; *Potamogeton;* 5S-NTS

Introduction

Potamogeton, a genus in the family of Potamogetonaceae, comprises approximately 100 species distributed world widely [1, 2]. The taxonomy of this group is notorious largely due to its highly morphological and ecological diversity [3, 4, 5]. Based on the morphology of submerged leaf, *Potamogeton* could be classified as the "broad-leaved" group and the "linear-leaved" group [6, 7]. Based on chemotaxonomy, however, *Potamogeton* may also be divided into the heterophyllous group and the homophyllous group [8]. The former contains the floating and submerged foliage; the later contains the submerged foliage without the floating foliage. Recent studies on DNA sequences supported a monophyly of *Potamogeton* [9, 10, 11, 12]. However, it is unresolved due to complex natural lineages of evolution.

Interspecific hybridization plays a significant role in plant speciation [13, 14]. Compared with the land plants, however, hybridization in aquatic plant is rare, largely due to the rarity of pollination. Clonal offspring, such as turions, winter buds, and shoot fragments, is highly effective and economical for aquatic plant survival in the stressed environment, reducing the negative selective value of sexual reproduction [15]. So, aquatic weeds distributed world widely due to clonal reproduction character, therefore, plays an essential role in their recruitment [16]. This is also greatly beneficial to hybrids survive and disperse in new habitat if formed.

Nevertheless, numerous cases of hybrid origin of *Potamogeton* have been proposed [17, 18, 19]. During field collection, our previous findings indicated four putative interspecific hybrids within the *Potamogeton*. They are *P. angullianus*, *P. hubeiensis*, *P. kamogawaensis*, and a putative triple hybrid. However, the identity and the reticulate evolution of these hybrids remain to be further studied at molecular level.

DNA sequences are informative to deeply elucidate the origin of hybrids and reciprocal relationships with its parents [20]. However, phylogenetic groups derived merely from the chloroplast DNAs are often neither in agreement with the taxonomic units by morphological characters nor inferred by nuclear markers [21]. Thus, combination of nuclear and chloroplast DNA data together with morphological characters is a valuable approach to study evolutionary pattern and process of hybrid species [22].

In this study, we collected forty-one samples, including twenty-five species and determined the DNA sequences, including the nuclear 5S non-transcribed spacer (5S-NTS) and three chloroplast DNA spacers. Based on the DNA sequence analyses, we discussed the origin of the putative hybrids and the phylogenetic relationships of *Potamogeton* species distributed in Chinese mainland.

Materials and Methods

Plant materials

Leaf tissue collected from forty-one samples of twenty-five *Potamogeton* species was stored in silica gel (Table 1). In present study, two subgenera were adopted following the traditional classification [2, 23]. Outgroup species, *Ruppia maritima* were chosen as described in previous phylogenetic analysis study [12, 24].

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from leaves deposited in silica-gel [25]. PCR primers were listed in Table 2. Four cpDNA regions were amplified in three fragments, among which the trnS-trnG spacer and trnG intron were amplified together as one fragment. The 5S-NTS region was amplified using the primers PI and PII [4]. PCR procedures were performed in a total reaction (25 μL) containing 10-20 ng of DNA template, 2.5 μL of 10 × reaction buffer, 3 mM $MgCl_2$, 100mM of each dNTP, 1.5 U of Taq (Promega), and 0.2 mM of each primer. The thermocycling program consisted of initial step at 94 ºC for 4 min, followed by 35 cycles of 30 s at 94 ºC, 1 min at 55 ºC (*trn*S-*trn*G), and 1min at 50 ºC (*trn*S-*trn*fM, *trn*D-*trn*T), 2 min at 72 ºC, with a final extension step for 10 min at 72 ºC. After purification with DNA gel extract kitTM (Axygen), PCR products were either directly sequenced or cloned into TOPO-TA (Invitrogen) for subsequent sequencing. Sequences results have been deposited in GenBank (Table 1).

Data analyses

Sequences were initially aligned using ClustalX1.83 [26] with default setting followed by manual correction. Given that the cpDNA fragments are part of the haploid chloroplast genome sharing the same evolutionary history, they were treated as a single coalescence gene for final phylogenetic analyses. Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) analyses were separately performed for the chloroplast dataset and the nuclear 5S-NTS dataset to reconstitute phylogenetic relationships of *Potamogeton*. Parsimony informative gaps were coded as binary characters using the simple gap coding method [27] in MP analyses. MP and ML analyses were conducted using PAUP* v.4.0b10 [28]. NJ analyses were implemented in MEGA* v.4.0 [29].

Table I: List of genus *Potamogeton* accessions, out-group species investigated and their geographical origin and GenBank accession numbers.

Taxon	Locality	GenBank Accession No.				
		*trn*D-*trn*T	*trn*S-*trn*G	*trn*G intron	*trn*S-*trn*fM	5S-NTS
Potamogeton L. Subgenus *Potamogeton*						
P. maackianus A. Benn.	1 Dongjiang, Jiangxi 28°16'N 116°48'E	FJ495296	FJ495422	FJ495338	FJ495380	clone 1 FJ495464
	2 Tongjiang, Heihongjiang 47°31'N 132°31'E	FJ495297	FJ495423	FJ495339	FJ495381	clone 1 FJ495465
P. wrightii Miq.	Honghu, Hubei 29°68'N 113°17'E	FJ495298	FJ495424	FJ495340	FJ495382	clone 1 FJ495467 clone 2 FJ495468 clone 3 FJ495469 clone 4 FJ495466
P. lucens L.	Honghu, Hubei 29°68'N 113°17'E	FJ495299	FJ495425	FJ495341	FJ495383	clone 1 FJ495470
P. distinctus A. Benn.	1 Erhai, Yunnan 25º36'N 100º25'E	FJ495300	FJ495426	FJ495342	FJ495384	clone 1 FJ495471
	2 Mishan, Heilongjiang 45°15'N 132°43'E	FJ495301	FJ495427	FJ495343	FJ495385	clone 1 FJ495472
P. crispus L.	1 Honghu, Hubei 29°68'N 113°17'E	FJ495302	FJ495428	FJ495344	FJ495386	clone 1 FJ495473
	2 Mishan, Heilongjiang 45°15'N 132°43'E	FJ495303	FJ495429	FJ495345	FJ495387	clone 1 FJ495474
P. perfoliatus L.	1 Wuhan, Hubei 30°34'N 114°15'E	FJ495304	FJ495430	FJ495346	FJ495388	clone 1 FJ495476 clone 2 FJ495475
	2 Ruoergai, Sichuan 33°34'N 102°28'E	FJ495305	FJ495431	FJ495347	FJ495389	clone 1 FJ495477
	3 Beian, Heilongjiang 48°44'N 127°23'E	FJ495306	FJ495432	FJ495348	FJ495390	clone 1 FJ495478
P. prealongus Wulfen	Fuyuan, Heilongjiang 48°20'N 134°25'E	FJ495307	FJ495433	FJ495349	FJ495391	clone 1 FJ495479
P. natans L.	Raohe, Heilongjiang 46°46'N 134°01'E	FJ495308	FJ495434	FJ495350	FJ495392	clone 1 FJ495480
P. gramineus L.	1 Huahu, Sichuan 33°54'N 102°49'E	FJ495309	FJ495435	FJ495351	FJ495393	clone 1 FJ495481
	2 Daocheng, Sichuan 29°04'N 100°16'E	FJ495310	FJ495436	FJ495352	FJ495394	clone 1 FJ495483 clone 2 FJ495482
	3 Tangke, Sichuan 33°24'N 103°27'E	FJ495311	FJ495437	FJ495353	FJ495395	clone 1 FJ495485 clone 2 FJ495484
	4 Ruoergai, Sichuan 33°34'N 102°28'E	FJ495312	FJ495438	FJ495354	FJ495396	clone 1 FJ495486
P. alpinus L.	Kelin, Heilongjiang 48°13'N 128°36'E	FJ495313	FJ495439	FJ495355	FJ495397	clone 1 FJ495487
P. oxyphyllus Miq.	1 Aba, Sichuan 32°54'N 101°42'E	FJ495314	FJ495440	FJ495356	FJ495398	clone 1 FJ495488
P. oxyphyllus Miq.	2 Yilan, Heilongjiang 46°208'N 129°31'E	FJ495315	FJ495441	FJ495357	FJ495399	clone 1 FJ495490 clone 2 FJ495489
P. compressus L.	1 Yilan, Heilongjiang 46°208'N 129°31'E	FJ495316	FJ495442	FJ495358	FJ495400	clone 1 FJ495491

	2 Kelin, Heilongjiang 48°42'N 128°59'E	FJ495317	FJ495443	FJ495359	FJ495401	clone 1 FJ495493 clone 2 FJ495492
	3 Beian, Heilongjiang 48°44'N 127°23'E	FJ495318	FJ495444	FJ495360	FJ495402	clone 1 FJ495494
P. octandrus Poir	1 Balan, Heilongjiang 46°21'N 129°30'E	FJ495319	FJ495445	FJ495361	FJ495403	clone 1 FJ495496 clone 2 FJ495495
	2 Beian, Heilongjiang 48°44'N 127°23'E	FJ495320	FJ495446	FJ495362	FJ495404	clone 1 FJ495497
P. obtusifolius Mertens	Balan, Heilongjiang 46°21'N 129°30'E	FJ495321	FJ495447	FJ495363	FJ495405	clone 1 FJ495498
P. pusillus L.	1 Daocheng, Sichuan 29°04'N 100°16'E	FJ495322	FJ495448	FJ495364	FJ495406	clone 1 FJ495499
	2 Ruoergai, Sichuan 33°34'N 102°28'E	FJ495323	FJ495449	FJ495365	FJ495407	clone 1 FJ495501 clone 2 FJ495500
	3 Aba, Sichuan 32°54'N 101°42'E	FJ495324	FJ495450	FJ495366	FJ495408	clone 1 FJ495502
P. cristatus Regel	Raohe, Heilongjiang 46°46'N 134°01'E	FJ495325	FJ495451	FJ495367	FJ495409	clone 1 FJ495504 clone 2 FJ495505 clone 3 FJ495503
P. hubeiensis Wang	Chongyang, Hubei 29°32'N 114°08'E	FJ495326	FJ495452	FJ495368	FJ495410	clone 1 FJ495507 clone 2 FJ495508 clone 3 FJ495506
P. anguillanus Koidz	Wuhan, Hubei 47°31'N 132°41'E	FJ495327	FJ495453	FJ495369	FJ495411	clone 1 FJ495509
P. sp. hybrid	Raohe, Heilongjiang 46°46'N 134°01'E	FJ495328	FJ495454	FJ495370	FJ495412	clone 1 FJ495511 clone 2 FJ495512 clone 3 FJ495510
P. malainoides Miki.	Chongyang, Hubei 29°32'N 114°08'E	FJ495329	FJ495455	FJ495371	FJ495413	clone 1 FJ495513
P. kamogawaensis Miki.	Kelin, Heilongjiang 48°42'N 128°59'E	FJ495330	FJ495456	FJ495372	FJ495414	clone 1 FJ495515 clone 2 FJ495516 clone 3 FJ495514
Subgenus *Coleogeton*						
P. vaginatus Turcz.	1 Caohai, Guizhou 27°38'N 106°45'E	FJ495331	FJ495457	FJ495373	FJ495415	clone 1 FJ495517
P. pectinatus L.	1 Tongjiang Heilongjiang 47°31'N 132°31'E	FJ495332	FJ495458	FJ495374	FJ495416	clone 1 FJ495518
P. filiformis Pers.	1 Daotanghe, Qinghai 36°34'N 100°44'E	FJ495333	FJ495459	FJ495375	FJ495417	clone 1 FJ495519
	2 Maduo, Qinghai 34º53'N 98º10'E	FJ495334	FJ495460	FJ495376	FJ495418	clone 1 FJ495520
P. pamiricus Baagöe	1 Gangcha, Qinghai 37º19' N 100º07'E	FJ495335	FJ495461	FJ495377	FJ495419	clone 1 FJ495521
	2 Niaodao, Qinghai 37º22'N 100º27'E	FJ495336	FJ495462	FJ495378	FJ495420	clone 1 FJ495522
Outgroup						
Ruppia maritima L.	1 Tianjin 38°44'N 117°28'E	FJ495337	FJ495463	FJ495379	FJ495421	clone 1 FJ495523

For NJ analyses, datasets were performed with Complete Deletion option on, Kimura 2-Parameter nucleotide substitution model. Bootstrap test was operated with 1000 time replicates. For MP analyses, heuristic searches were performed with 1000 replicates, random stepwise addition, TBR branch swapping with the MULTREES option in effect, and all character states were equally weighted. Support for individual clades was determined by bootstrap analyses [30] of 1000 replicates using the heuristic search option for random addition sequence with TBR branch swapping. For ML analyses, evolutionary models were determined by using Akaike information criterion as implemented in Modeltest 3.07 [31]. The (TVM+I+G) model and (TIM+I+G) model was selected to best the combined chloroplast data and 5S-NTS data, respectively. Heuristic search methods using a neighbor-joining tree as starting tree with TBR branch swapping and 100 random addition sequence replicates. The confidence of branching was assessed using 500 bootstrap resamplings, each with 10 replicates using the same model and parameters above.

Results

Aligned DNA sequences

Information of the DNA sequences, including the sequence length and G+C content etc., was listed in Table 3. The aligned sequence the 5S-NTS region was 432 bp in length; the combined cpDNA region was 4466 bp. Compared with the cpDNA region, the 5S-NTS region showed a higher rate of nucleotide substitution. However, the cpDNA region provided more parsimony informative characters than those of 5S-NTS region, approximately 1.8 fold as informative as the 5S-NTS region (Table 3).

Phylogenetic analysis of the cpDNA data

We used various optimality criteria and models to analyse the cpDNA data, and all the phylogenetic trees displayed a similar topology. In one of the representative strict consensus trees, as shown in Fig. 1, a sister relationship between *Potamogeton* and *Coleogeton* was strongly supported. Furthermore, two major groups within subgenus *Potamogeton* were covered with high confidence: *P. lucens* ~ *P. crispus*.P2 (group I) and *P. pusillus*.P3 ~ *P. octandrus*.P2 (group II). ("~" signifies the inclusion of all species between the two on the trees). Group I contained the broad-leaved samples; group II included thinned linear-leaved species examined in this study. In group I, interspecific relationships within the *P. lucens* ~ *P. perfoliatus*.P3 group were ambiguous in the NJ tree but well resolved in ML and MP trees. In addition, the results supported a sister relationship between *P. alpinus* and the *P. lucens* ~ *P. perfoliatus*.P3 group. These analyses also showed *P. maackinaus* and *P. crispus* formed a monophyletic clade. In group II, two strongly supported subclades were strongly suggested, which have not been well resolved yet in previous analyses [12]. *P. pusillus* grouped with *P. oxyphyllus* with high support. *P. obtusifolius* formed sister relationship with *P. compressuss*.

Subgenus *Coleogeton* were recovered in the cpDNA analysis. Two clades clearly separated each other. Interestingly, two accessions of *P. pamiricus* clustered together with *P. filiformis*. One clade contained *P. pectinatus* and *P. vaginatus* (Fig. 1).

Phylogenetic analysis of the 5S-NTS data

We analyzed the 5S-NTS data with the MP, ML and NJ models, respectively, and the results were presented in Figure 2. In these analyses, the monophyly of subgenus *Potamogeton* and subgenus *Coleogeton* (except for *P. pusillus*.P1) was further supported. However, disagreement was also noted between the 5S-NTS tree and the cpDNA tree. For example, *P. lucens*, *P. anguillanus*, *P.* sp. hybrid, *P. wrightii*, and *P. malainoides*, which clustered together in cpDNA tree, were grouped with *P. distinctus* and *P. natans* in the MP and NJ trees.

The monophyly of *P. octandrus*P.1c.2 ~ *P. pusillus*.P1 group was supported in all three phylogenetic trees. Moreover, relationships within this clade were well resolved in MP, NJ, and ML analyses. In the NJ tree, it is showed that *P. pusillus* P1 and *P. pusillus* P2 formed a sister relationship with other species in this clade. All three model revealed well supported clade (e.g. clade of all accession of *oxyphyllus* and *P. kamogawaensis* c.1, c.3), (clade of *P. cristatus* and *P. hubeiensis* c.1, c.2), (clade of all accessions of *P. octandrus*, *P. hubeiensis*, and *P. kamogawaensis* c.2), and (clade of *P. obtusifolius* and all accessions of *P. compressuss*). Multiple sequencing of putative hybrids (*P. hubeinesis* and *P. kamogawaensis*) acquired two types of nucleotide sequences clustered with two parental lineages (Fig. 2). Two clones of *P. hubeiensis* clustered with *P. cristatus*, and the other one grouped with *P. octandrus*. Two clones of *P. kamogawaensis* formed monophyly with *P. oxyphyllus* and the third clustered with *P. octandrus*. Moderate to strong supports of *P. obtusifolius* ~ *P. compressuss* group were observed in 5S-NTS tree. From both of the genome evidence, monophyly of this group is consistent with previous studies.

5S-NTS tree showed similar results with previous studies [24]. *P. pectinatus* and *P. vaginatus* clustered together and *P. filiformis*.P1, P2 and *P. pamiricus* formed a clade with *P. pusillus*.P3.

Discussion

Our phylogenetic analyses strongly suggest a natural split of genus *Potamogeton* into two main clades, which consist with the subgenus *Potamogeton* and the subgenus *Coleogeton*. The subgenus *Potamogeton* could be further divided into two groups. One group includes the widespread species *P. perfoliatus*, *P. crispus*, and *P. macckianus*. The other group contains a few rare or narrow endemic species, such as *P. pusillus*, *P. oxyphyllus*, and *P. cristatus*. (Fig. 1 and Fig.2)

The morphological evolution of *Potamogeton* has been argued for decades. Two major split of phylogenetic trees is largely congruent with the differential pollination systems [32]. The subgenus *Coleogeton* has been subjected to different natural selection pressure possibly because of its unique characteristics in pollination system which is different from the subgenus *Potamogeton* [32]. The visibly inflorescences of subgenus *Coleogeton* is elongate flexible floating, while subgenus *Potamgeton* presents short stronger stems inflorescences. Furthermore, the lowest P/O ratios and the largest pollen volume are recorded from this group and the pollen grains in this group were of a different shape from the rest.

In subgenus *Potamogeton*, two parallel lineages derived from analyses exhibit a pattern which is correspondent with floral trait size and ploidy level [33] (Fig. 1 and Fig. 2). This correspondence suggests that variation and evolution of reproductive traits or floral isolation mechanisms have played an important role in the initial diversification and evolution of *Potamogeton*. Also, the two clades in phylogenetic trees are consistent with the submerged morphological classification, i.e., broad submerged-leaved species and linear submerged-leaved species [6]. In addition, the group of linear-leaved species, different from the group of broad-leaved species, has complex pollination systems which are anemophily, epihydrophily and/or hydroautogamy [32]. Put together, we thus argued that a strong reduction in flower size preceded the shift in reproductive strategy from out- to self-crossing. This argument could explain why hybridization is more frequent in broad-leaved species than in linear-leaved species. Moreover, it has been reported that autogamous taxa have smaller flowers than allogamous-related taxa. The ecological evolutionary advantages due to such a reproductive transition may have been facilitated colonization [34, 35, 36, 37]. However, the relationships among species of the two parallel clades in subgenus *Potamogeton*, based on molecular analyses, were discrepant from those based on the morphology of floating leaves. This discrepancy suggests that the diversification of this species may have been driven by more complex evolutionary processes in addition to the floral size isolation.

Hybrid species could be paraphyletic or polyphyletic across multiple genes or genomes. Incomplete lineage sorting might lead

Table-II: Primers used for PCR and sequencing

Region	Primers (PCR)	Primers (sequencing)	Source	Sequence (5'-3', if from this study)
*trn*D-*trn*T	*trn*DF, *trn*TR	*trn*DF, *trn*TR	Shaw et al. (2005)	
*trn*S-*trn*G	*trn*S, *trn*G	*trn*S, *trn*G	Shaw et al. (2007)	
		5'*trn*G2S, 5'*trn*G2G	Shaw et al. (2005)	
*trn*S-*trn*fM	*trn*S, *trn*fM, *trn*FfM,	*trn*S, *trn*fM	Shaw et al. (2005)	
	*trn*RS	*trn*FS (P)	This study	AGACCGGAGCTATGAACCAC
		*trn*RfM (P)	This study	GTCACGGGTTCAAATCCTGT
5S-NTS	PI, PII	PI, PII	Cox et al. 1992	

All primers denoted with "(P)" were specifically designed for the *Potamogeton* species

Table-III: Sequence information for the 5S-NTS and cpDNA regions used in this study

Data set	Aligned length (bp)	G+C content (%)	No. parsimony sites	CI	RI
5S-NTS	471 bp	48%	280	0.5339	0.8464
Combined cpDNA	4718 bp	29%	497	0.8311	0.8787

CI: consistency index; RI: retention index

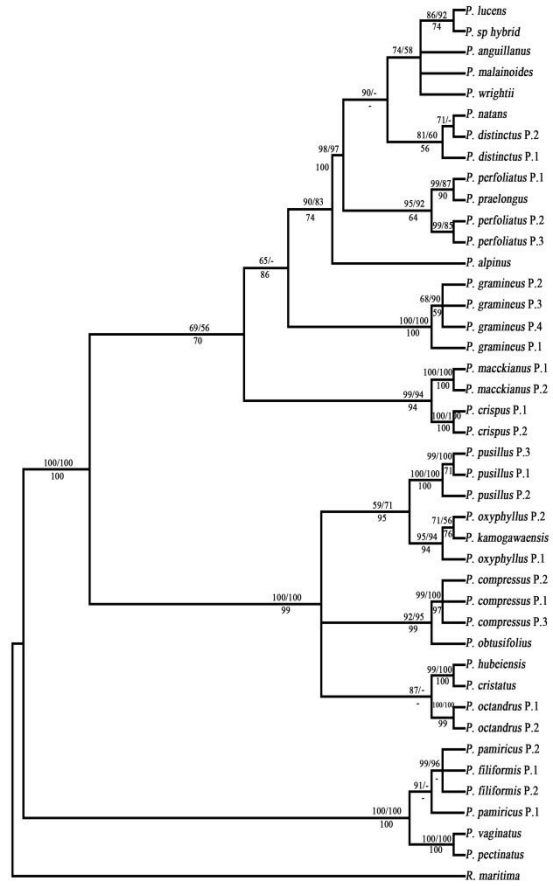

Figure 1 The strict consensus tree of combined cpDNA region. *Ruppia maritima* is served as out-group. Numbers at both "/" sides and below branch are bootstrap values above 50% by MP, ML, and NJ methods respectively. Characters after species names refer to accessions number as in Table-I

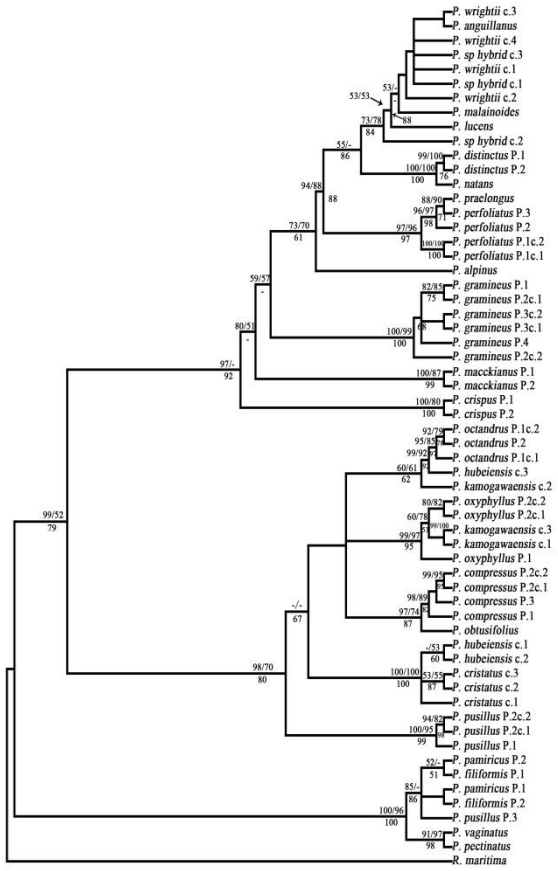

Figure 2 The 50% majority rule consensus tree of 5S-NTS region. *Ruppia maritima* is served as out-group. Numbers at both "/" sides and below branch are bootstrap values above 50% by MP, ML, and NJ methods respectively. Characters after species names refer to accessions number and its clone number as in Table-I

to monophyly of one marker while a second marker would result in unresolved lineages [38]. Hence, analyses of multiple markers for hybird are informative to clarify the mode and process of hybridization. Basically, the phylogenetic relationships derived from 5S-NTS data and cpDNA data are compatible for non-hybrid species. For the hybrid species, however, cpDNA datasets represent maternal genealogy revealed close relationships with its female parent donors.

Previous studie [24] suggest that *P.* sp. hybrid is originated from hybridization between *P. perfoliatus* and *P. wrightii*. Our phylogenetic analyses based on the cpDNA sequences indicated *P.* sp. hybrid shares the chloroplast genome with *P. lucens* (Fig. 1). By contrast, results from 5S-NTS showed that *P.* sp. hybrid clusters with *P. wrightii*, either of which might be its parents. To date, no direct evidence concluded the male parent donor of *P.* sp. hybrid. The present results reveal that *P.* sp. hybrid might be a natural triple hybrid originated from two round of hybridization. A former hybrid between *P. perfoliatus* and *P. wrightii* partial fertile could cross with *P. lucens* which is as maternal parent. Although *P. perfoliatus*, *P. wrightii*, and *P. lucens* are often self-pollinated, they are known as protogynous that give opportunity to cross-pollination occasionally. Therefore closely related species might give opportunity to bring newly fertile hybrid [2]. And some study confirmed that triple hybrid in *Potamogeton* does exist naturally [39].

P. anguillanus has been treated as the synonym of *P. intortusifolius* which is crossed from *P. perfoliatus* and *P. wrightii* [2]. In 5S-NTS tree, we note one clone of *P. anguillanus* cluster with *P. wrightii*. Based on cpDNA tree, *P. anguillanus* formed polyphyletic lineage with putative parent *P. wrightii*. In taxa of hybrid origin, multiple copies might either be retained [40, 41], or be subject to various evolutionary forces such as directional concerted evolution [38]. For this reason, single copy gene (*Adh*1) of *P. anguillanus* has shown two types of nucleotide sequences corresponding to two putative parents respectively [11]. In present study, 5S-NTS sequences of *P. anguillanus* are identical, suggesting that concerted evolution in nuclear ribosome DNA could eliminate heterogeneity sequences from parental species [42]. Furthermore, rapidly concerted evolution suggests that this hybrid has been shown to precede meiosis behavior. In combination of the leaf morphological differences between *P. anguillanus* and *P. wrightii*, we support the hybrid origin of *P. anguillanus* and its one parent genome donor is *P. wrightii*.

Wiegleb and Kaplan [2] suggested that *P. hubeiensis* distributed in China is another ecotype of *P. octandrus*. Our previous study argued that *P. hubeiensis* was originated from hybridization between the diploid species of *P. cristatus* and *P. octandrus* [24]. This argument is further supported by the present data given that *P. hubeiensis* exhibits two types of 5S-NTS sequences, either type sharing with a form in an inferred diploid parental species. Specifically, *P. octandrus* might have provided the paternal genome based on the 5S-NTS tree, and *P. cristatus* might have served as the maternal parent based on the cpDNA tree. Based on seed and leaf morphological analyses, *P. hubeiensis* can neither be synonym of *P. cristatus* nor *P. octandrus* because of dorsal keel with hooked appendages only exist in *P. cristatus* and floating leaves of *P. hubeiensis* is extremely acuminate at apex [23].

Investigation of the reproductive biology in *P. hubeiensis* would help explain the origin and persistence of these divergent nuclear alleles. For instance, if the hybrid cannot reproduce sexually, persistence of interspecific copies would suggest that it is a F1 hybrid. Implication of phylogenetic framework based on organelle DNA and nuclear DNA is an efficient method to identify hybrid species especially in phenotype plasticity influenced aquatic plants [43].

P. kamogawaensis was described as a hybrid originated from *P. octandrus* and *P. oxyphyllus* by Wiegleb and Kaplan [2]. To our knowledge, however, no evidence is available at molecular level. In the present cpDNA tree, *P. kamogawaensis* forms one clade within *P. oxyphyllus*. In the 5S-NTS tree, by contrast, two types of nuclear sequences cluster with *P. octandrus* and *P. oxyphyllus*, respectively. These results strongly supported that *P. octandrus* is the paternal parent of *P. kamogawaensis* and maternal genome donor is *P. oxyphyllus*. These two parental species are diploid based on chromosome counting [33]. Hence, our study strongly support an allopolyploid origin of this hybrid when we consider allopolyploid/homoploid speciation involving only known diploid parents (Fig. 1 and 2). Previous results recognize that *P. octandrus* serve as maternal lineage [2], but molecular data indicate that *P. oxyphyllus* served as female progenitor. This may indicate a reciprocal hybridization that might have occurred during the hybrid formation.

It is controversial that *P. malainoides* is descendant of *P. distincts* and *P. wrightii*. Wiegleb and Kaplan [2] argued that it is a special form of *P. wrightii*, whereas conclude as hybrid based on ITS tree [24]. However, present study based on two types of genome put *P. malainoides* with *P. wrightii* together. This phenomenon may be explained that it is a shallow form of *P. wrightii* with floating leaves.

Incongruence among gene trees may be the result of processes such as reticulate evolution, lineage sorting or recombination [38]. Incongruence phylogenetic placement of *P. pusillus* P3 is observed when compared trees from organelle DNA and nuclear DNA. Morphological characterization of *P. pusillus* P3 classified this species within the section of "*Pusillu*". Our cpDNA tree further verifies that genome composition of organelle was originated from *P. pusillus* (Fig. 1 and Fig. 2). However, nuclear marker gives a different result, where *P. pusillus* P3 fall in group *P. filiformis*. The result appears consistent with previous studies using nrDNA-ITS [11]. Considering the morphological distinct from taxa in the *P. filiformis* clade, the incongruence present in our molecular data suggest a rapid reticulate evolution of the hybrid of *P. pusillus* P3. It is also possible that the incongruence is caused by incomplete lineage sorting instead of hybridization or introgression. However, the coalescence of organelle DNA is four times faster than nuclear genes, and therefore it is unlikely that the lineage sorting for nuclear had been completed before the divergence of these two genera from their common ancestor, while polymorphism of chloroplast genes were retained in that common ancestor [44].

It is rare that hybrid could come from hybridization between two subgenera because of pre-zygote barrier, such as distinct pollination pattern [2]. However, aquatic plant was easily influenced by hydrophytic circumstance, which either makes inflorescence expose itself to air or submerge in water of the inflexible peduncle species. Under this condition, pollen from *P. filifomis* species might have opportunity to fall on stigma of *P. pusillus*. Identical base chromosome number of the two species was reported [33]. Thus, factors may increase the possibility of successful hybridization. It is assumed that hybridization between species of two subgenera cannot occur because of difference in flora traits, but our results at molecular level give a successful example of hybridization arisen from two subgenera.

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