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High Connectivity Among *Synedrella nodiflora* Populations in Java Island Based on Intergenic Spacer *atp*B-*rbc*L

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Abstract

The genus *Synedrella* remains monotypic in spite of its wide range of distribution, in which *S. nodiflora* (L.) Gaertn has taxonomically been the only member of the genus so far. This leads to assumption of the very low genetic difference among *S. nodiflora* populations worldwide. It may also be the case in Java Island, though rapid changes in ecosystem condition occurs. Here we report our study on *S. nodiflora* population genetics in Java Island using intergenic spacer (IGS) *atp*B-*rbc*L as a molecular marker, since it has been well known as one of the most variable chloroplast genome regions in a wide range of plant species so far. As many as 58 individuals were collected randomly from ten different locations in the island. Based on IGS *atp*B–*rbc*L sequences of 860 bp length, only two haplotypes were observed. Both show only one polymorphic site (0.12%) as well as low haplotype diversity (0.0345) and low nucleotide diversity (0.000040). In addition, the very low fixation index (FST = 0.02645; p = 1.00000) proves low genetic difference, or in other words, indicates high connectivity among populations of S. nodiflora in Java Island. At the same time provides a fact of nearly no variation among the IGS *atp*B – *rbc*L sequences.

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INTRODUCTION

Synedrella nodiflora (L.) Gaertn, which is a member of Asteraceae family, is a widely distributed tropical plant species having potentials as medicinal plant (Adjei *et al*., 2014; Amoateng *et al*., 2017; Amoateng *et al*., 2012; Bhogaonkar *et al*., 2011; Dutta, *et al.*, 2012; Islam *et al.*, 2013; Rathi & Gopalakrishnan, 2006), bioinsecticide (Belmain *et al*., 2001) and detoxificant for heavy metals such as Cu and Pb (Prekeyi & Oghenekevwe, 2007). On the other hands, it is also commonly found as broad-leaf weed in several crops, particularly in legumes, causing severe impacts with respect of productivity (Hasanudin *et al*., 2012; Moenandir *et al.*, 1996; Murrinie, 2011). The reproductive ability of *S. nodiflora* is considerably high as every individual can bear up to 100 inflorescences, each of which may have 30 fertile seeds. In other words, in every individual there can be 3,000 fertile seeds ready to germinate as they are not subjected to dormancy. It can grow in a wide range of altitudes, from 0 to approximately 1,000 m above sea level (Dwiati *et al.*, 2003), while its life cycle varies from 120 to 150 days (Souza Filho & Takaki, 2011).

Although *S. nodiflora* is widely distributed in about 50 tropical countries (Chauhan & Johnson, 2009), it has taxonomically been the only species of genus *Synedrella* so far. It is slightly peculiar, since species with a wide range of distribution are usually exposed to different environmental circumstances that may cause phenotypic changes (Kollman & Banuelos, 2004). Even in an extreme situation, e.g. when species are subjected to enormous differences in the abiotic factors, serious taxonomic problem may result (Semir *et al.*, 2014). This can be either divergent or convergent development of species (Kieltyk & Mirek, 2014).

The wide range of distribution and altitudes, along with the current taxonomy status, gives rise to assumption that *S. nodiflora* in the world should have very low intra-specific genetic diversity, either within or among populations. In other words, gene flow among *S. nodiflora* populations in any tropical areas is strongly presumed to take place. It may also be the case in Java Island, Indonesia, eventhough the rapid changes in ecosystem condition occur due to its high density over years.

Here we use intergenic spacer (IGS) *atp*B – *rbc*L as a molecular marker to study the population genetics of *S. nodiflora* in Java Island. As a region in chloroplast genome that is not responsible for protein synthesis, the sequence has some parts with rapid alteration. This is why IGS *atp*B – *rbc*L has been widely used as a molecular marker in intra-specific genetic diversity studies in various plant species (Chiang & Schaal, 2000a, 2000b; Fujii *et al.*, 1997; Small *et al*., 2005; Taberlet *et al.*, 1991).

The purpose of this study is to know genetic diversity and the level of connectivity among *S. nodiflora* populations in Java Island based on IGS *atp*B – *rbc*L. It is expected from the study that molecular data can be obtained to compare with the existing taxonomical status of the species as the only member of genus *Synedrella*, which has been based merely on phenotypical characters.

METHODS

S. nodiflora samples were collected randomly from ten different locations in Java Island, i.e. Bogor (n = 5), Tasikmalaya (n = 5), Ciamis (n = 5), Banyumas (n = 10), Yogyakarta (n = 5), Mojokerto (n = 5), Probolinggo (n = 6), Malang (n = 6), Lumajang (n = 5) and Jember (n = 6) (Figure 1). The plants were taken with their roots and were put into plastic bottles previously filled with a little water prior to be planted in pots in a glass house.

Figure 1. Sampling locations of *Synedrella nodiflora* (L.) Gaertn in Java Island; 1 = Bogor; 2 = Tasikmalaya; 3 = Ciamis; 4 = Banyumas; 5 = Yogyakarta; 6 = Mojokerto; 7 = Probolinggo; 8 = Malang; 9 = Lumajang; 10 = Jember

Genomic DNAs were isolated from uppermost leaves following CTAB method (Doyle & Doyle, 1990). The quality and quantity of the isolated DNAs were measured using genequant. Amplification of IGS *atp*B – *rbc*L was performed using universal primers, i.e. 5'-ACA TCK ART ACK GGA CCA ATA A-3' as forward primer and 5' – AAC ACC AGC TTT RAA TCC AA-3' as reverse primer (Chiang *et al.*, 1998). A total volume of 11.5 µl PCR mixture consisting of 2 µl template DNA, 5 µl KapaTaq DNA polymerase, 4.25 µl nuclease free water (NFW) and 0.125 µl of individual primer was subjected to PCR condition as follows: pre-denaturation of 94°C 4 mins, proceeded by 40 cycles of touch down PCR (comprising 10 cycles of 94°C 45 secs, 49°C 45 secs, 72°C 2 mins; 10 cycles of 94°C 45 secs, 48°C 45 secs, 72°C 2 mins; 15 cycles of 94°C 45 secs,

47°C 45 secs, 72°C 2 mins; and 5 cycles of 94°C 45 secs, 46°C 45 secs, 72°C 2 mins), terminated by final elongation of 72°C 10 mins. The PCR products were visualized in a 1.5% agarose gel using TBE buffer. These were then sent to Firstbase Malaysia for sequencing after Sanger *et al.* (1977) automated with terminator labelling.

Data of sequences were edited using Bioedit version 7.0.4.1 (Hall, 1999) and were checked manually. Sequence alignment was carried out with ClustalW (Thompson *et al.*, 1994), which was also implemented in Bioedit version 7.0.4.1 (Hall, 1999). Arlequin 2.0 (Schneider *et al.*, 2000) was employed to calculate haplotype diversity *h* (Nei, 1987) and nucleotide diversity π (Nei & Jin, 1989). Analysis of Molecular Variance or AMOVA (Excoffier *et al.*, 1992) was used to see whether the population is subdivided into subpopulations or not.

RESULTS AND DISCUSSION

An IGS *atp*B – *rbc*L partial sequence of 860bp length was obtained from 58 *S. nodiflora* individuals collected from ten different sites across Java Island showing only two haplotypes. The sequences of both haplotypes are now available at the NCBI databases with accession numbers of KX096801.1 and KX096802.1 respectively.

Haplotype 1 consists of most individuals (i.e. 57) covering those from ten locations, while haplotype 2 consists only one individual from Lumajang. The dominant haplotype is normally assumed as the original haplotype, although sometimes this is not the case. For instance, Liao *et al.*, 2007) note that the most common haplotype of a mangrove plant species, *Ceriops tagal*, found in Borneo (haplotype 2) is not the ancestor, but instead it derives from another one (haplotype 1) found in Malay Peninsula, which is less in number.

As presented in Table 1 and assuming haplotype 1 as the original haplotype, only one transversion is observed, where T is replaced by G at position of 790. In other words, of the 860bp length of IGS *atp*B – *rbc*L, only one polymorphic site is observed (0.12%), indicating very low level polymorphism. This corresponds to both extremely low haplotype *(h)* and nucleotide diversity (π) values, i.e. 0.0345 ± 0.0330 and 0.000040 ± 0.000127 respectively. Both values prove that IGS *atp*B – *rbc*L of *S. nodiflora* shows considerably low level of genetic diversity. A very contrasting finding is reported among populations of *C. tagal* in Southeast Asia based on IGS *atp*B – *rbc*L (Liao *et al.*, 2007), where much higher level of genetic diversity is observed, i.e. *h* equals to 0.667 and π equals to 0.0031. As well, unlike the low level of polymorphism in *S. nodiflora* populations in Java Island, most of the variable sites in *C. tagal* include several long insertion and deletion fragments. This different result maybe because *C. tagal* as a mangrove species tends to specially adapt to environmental condition of the newly colonized regions (Mori & Kajita, 2016), while *S. nodiflora* remains genetically stable wherever its existence.

AMOVA on *S. nodiflora* (L.) Gaertn populations in Java Island is presented in Table 2, showing no significant genetic difference among populations. In other words, the populations are not subjected to spatial genetic structure (SGS). This is clearly supported by low fixation index (F_{ST} = 0.02945; p = 1.00000), which means that variation within respective population is even greater than that among populations.

The low genetic difference among populations of *S. nodiflora* (L.) Gaertn in Java Island provides also a fact that IGS *atp*B – *rbc*L does not invariably show a rapid alteration in all plant species. Similar result is obtained in safflower *(Carthamus tinctorincus)* revealing no polymorphic sequences of IGS *atp*B – *rbc*L among 76 populations (Chapman *et al.*, 2010). This is in contrast to several previous references revealing that such a marker is suitable for lower-level phylogenetic studies, especially in photosynthetic angiosperm systematics (Shaw *et al.*, 2005; Shaw *et al.*, 2014). Along with other noncoding regions, i.e. intron,

Table 1. Part of the IGS atpB – rbcL sequences of *Synedrella nodiflora* (L.) Gaertn in Java Island

Haplotype	Population sample	Number of individuals	780 to 795 position of IGS *atp*B – *rbc*L sequence (5' – 3')*)	NCBI accession number
1	Bogor, Tasikmalaya, Ciamis, Banyumas, Yogyakarta, Mojokerto, Probolinggo, Malang, Lumajang, Jember	57	tttactttatTattaattat	KX096801.1
2	Lumajang	1	tttactttatGattaattat	KX096802.1

*) The other parts of the sequences among samples are identical.

Tabel 2. AMOVA of *Synedrella nodiflora* (L.) Gaertn populations in Java Island

Source of Variation	Df	Sum of Squares	Variance Components	Percentage of Variation
Among populations	2	0.018	0.00045	2.65
Within populations	55	0.964	0.01753	102.65
Total	57			
Fixation index (F_{ST}) = 0.02645	p = 1.00000			

intergenic spacers of chloroplast genome are frequently much more variable with respect of base sequences in compare to coding regions. Perhaps this is because noncoding regions, particularly those of chloroplast genome, are not involved in the protein synthesis (Small *et al.*, 2005). Nevertheless, the circular chloroplast genomes of most seed plant species are highly conserved in terms of gene arrangement. In general, they consist of a large single copy (LSC) and a small single copy (SSC) regions, which are separated from each other by two long identical but in an opposite direction sequences known as the inverted repeats (IR) (Peredo *et al.*, 2013; Wang *et al.*, 2013). The size of chloroplast genome ranges from 120 to 220 kb, where variation in size is caused usually by the enlargement or reduction of the IR influencing LSC or SSC nearby. Otherwise, this change in size can also result from sequence alteration due to insertions or deletions (Downie & Jansen, 2015).

To further evaluate the potential utility of IGS *atp*B – *rbc*L as a molecular marker in lower-level phylogenetic studies, it is reasonable to apply this maker in intra-specific diversity studies in some other monospecific genera, e.g. *Cephalotus follicularis* and *Breonadia salicina*, or in genera with very few species members, e.g. *Monocharia*, *Limnocharis* and *Eichornia* (water hyacinth). The latter is well known as a very invasive aquatic plant species, both in many tropical and sub-tropical areas, often resulting in significant ecological problem. Referring to the case of *S. nodiflora*, it is also possible that IGS *atp*B – *rbc*L will show relatively little or even no variation in such plant species. In addition, evaluation on this marker usage can be performed to study the genetic diversity of another species of the family Asteraceae, i.e. *Eleutheranthera ruderalis*, which is morphologically and anatomically very similar to *S. nodiflora* (Ekeke & Mensah, 2015).

The low level of genetic difference of *S. nodiflora* populations in Java Island indicates a high connectivity among populations caused by a high gene flow, which can occur either naturally due to pollen dispersal or artificially through seed transportation. The pollens are carried in a typical structure called as cypselas (Brandel, 2007), especially central cypselas. These cypselas with their longer and lighter shapes than those of peripheral cypselas may result in farther pollen dispersal. Afterwards, cypsela germination is highly influenced by light intensity. Cypselas germinate more easily in high light intensity than they do in the dark or shaded areas. Nevertheless, other environmental factors have no significant influence on cypsela germination, so that *S. nodiflora* can grow well in a very wide range of environmental conditions (Souza Filho & Takaki, 2011). Otherwise, the pollens can also be accidentally carried in seeds of crops after harvesting if *S. nodiflora* grows in a crop field.

Gene flow among *S. nodiflora* populations in Java Island prevents the occurrence of spatial genetic structure (SGS). The absence of SGS causes no relationship between geographic and genetic distance. In this case, individuals from the same location may genetically be different from each other more than they are in compare to those from other location. Likewise, individuals from different locations may genetically be closer to each other than they are in compare to those from the same location. For instance, individuals from Lumajang number 2 and 3 are of two different haplotypes (haplotype 1 and 2 respectively), while both individuals from Lumajang number 2 and Yogyakarta number 1 belong to the same haplotype, i.e. haplotype 1.

Low fixation index also supports the absence of SGS among *S. nodiflora* (L.) Gaertn populations in Java Island. In other words, increase in random mating among populations occurs due to isolate breaking following Wahlund principle. Opposite condition, is however, reported among *Copaifera langsdorfii* Desf populations in Brazilian tropical forests undergoing fragmentation where strong SGS is observed with sufficiently high fixation index, i.e. F_{ST} = 0.152 (Sebben *et al.*, 2011). Fixation index can be used to measure the effect of isolation by distance (Slatkin, 1993).

The absence of relationship between geographic and genetic distance is also reported between *Aquilegia* populations in the southwest USA and that in Mexico (Strand *et al.*, 1996). They use

IGS from chloroplast genome, i.e. *trn*L – *trn*F of 525 bp long, obtaining that gene flow occurs between both populations.

CONCLUSIONS

In conclusion, both low level of haplotype and nucleotide diversities, as well as low fixation index, indicate that high connectivity among *S. nodiflora* (L.) Gaertn populations in Java Island is observed. It is also found that in general IGS *atp*B – *rbc*L does not show variation among the species populations, which is not in accordance with most previous studies.

To confirm this finding, the application of IGS *atp*B – *rbc*L in the study of *S. nodiflora* genetic diversity should be expanded to broader geographical areas involving more populations. For examples, it can be applied in the species population genetics studies in several adjacent islands, particularly those located in Sunda Shelf.

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