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Morphological and genetic variation of *Amaranthus spinosus* L.: an adaptation evidence of climate differences and gene interaction

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Abstract

Amaranthus spinosus (spiny amaranth) natively live in America, Africa, Australia, Europe and Asia. This plant can be used as medicinal plant and also as food or feed. *A. spinosus* has phenotypic variation, especially in leave and stem type. It is because of plant adaptation. Plant adaptations impact to variation on morphological and genetic. Chloroplast DNA (cpDNA) is a common molecular marker that used in the genetic variability analyses. Phenotypic variation was analyzed using morphological and molecular data. The *trnL* intron, *matK* and *rbcL* genes were amplified and sequenced. The sequence data analyses using MEGA5, Bioedit and DNAsp software's. The molecular data shown that *A. spinosus* from tropical zone was higher genetic variability then temperate zone. Plant in the tropical zone easy to be colonized and there isn't gene flow barrier. So that, *A. spinosus* that adapt to different habitat have different morphological character and have higher genetic variability.

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Introduction

Amaranthus is a core genus of Amaranth family (Amaranthaceae), and consists of 70 species and natively life in America, Africa, Australia, Asia, and Europe (Frassen *et al*, 2001). *Amaranthus spinosus* is one of seven spesies Amaranthus that natively life in Indonesia, especially in Java Island (Backer 1986). Member of these genera widely used as traditional medicinal plant, especially as antiviral, antimalarial, antidiabetic, antibacterial, antihelminthic and snake antidote (Kusumaningtyas *et al*, 2006; Vardhana, 2011; Kumar *et al*, 2010). Amaranth genera also can be used for food, feed, and as an ornamental plant (Backer, 1986; Prosea, 2012).

Amaranthus spinosus has different morphological characteristic. The morpho-logical characteristics were affected by plant adaptation and genetic variation among them (Schlichting and Pigliucci, 1998; Fatinah *et al*, 2012). *Amaranthus spinosus* can be adapted in the different ecogeographic and wide range of edaphic factor (environmental heterogeneity) (Costea *et al,* 2004). Amaranth genus has capability tointerbreed among species in the same genus. The interbreeding also causes different morphological charac-teristic of *A. spinosus* (Murray, 1940; Popa *et al*, 2010).

Chloroplast DNA is a molecular marker that widely used for taxon identification (Clegg and Zurawski, 1991). The cpDNA has an independent genome that encoded several proteins, which are protein related photosynthetic and housekeeping genes. The cpDNA encode 30-50 *tRNA* genes and 100 other protein. The gene that encode protein divided into several kinds, they are gene as splicing factors (*rpoB, rpoC1, rpoC2, rpsl6* and *matK)* and protein related photosynthetic (*rbcL, afpB, psaA* and *petB*) (Baumgartner *et al*, 1993; Sugiura, 1995; De Las Rivas *et al*, 2002).

Genetic variation in *A. spinosus* was analyzed based on PCR-sequencing cpDNA, especially analyze gene that encode tRNA (*trnL* intron), splicing factor protein (*matK*) and protein related photosynthetic (*rbcL*). The *trnL* intron is a non-coding regions, this region has higher insertion and deletions (indels) which reflect the plant evolutionary (Roy and Penny, 2007). The *matK* gene encodes maturase protein as a splicing factor and include in intron group II. The *matK* gene has high nucleotide substitution rate, insertion and deletion. Mutation in *matK* gene reflects plesiomorphic characteristics and adaptive to environmental changing (Vogel *et al*, 1999; Hao *et al*, 2010). The *rbcL* gene encodes ribulose-1.5-biphosphate carboxylase/oxygenase large subunit (Ellis, 1979). The *rbcL* gene has 1428 bp in length and has conserve primer. The *rbcL* sequence can be used for cogeneric analysis (Kress *et al*, 2005). The *rbcL* gene is a core locus in chloroplast genome (plastome) multigenes (Newmaster *et al*, 2006). The *rbcL* gene is an adaptive gene to environment heterogeneity and widely used for plant evolutionary and plant adaptation mechanism (Golmez *et al,* 2005; Sen, 2011).

The previous study using *trnL* intron indicate that *A. spinosus* has high genetic variability. The genetic variability differs among molecular marker that used. So in this paper we used the third molecular marker to analyze *A. spinosus* genetic variation to know the relationship among phenotypic variation, genetic variation and plant adaptation in the tropical and temperate zone.

Material and methods

Sample collection

Plant collection from natural habitat

Eight samples of *A. spinosus* were collected during April-Mei 2012 from wild habitat in Malang, East Java, Indonesia. Leaves of each sample was packed in polyethylene bag, and immediately placed in insulated ice box until arrives in laboratory. Samples were collection with different morphological condition. The eight samples were used as tropical type of *A. spinosus*. The *A. spinosus* type from tropical region was voucher as As1, As2, As3, As4, As5, As6, As7 and As8.

Plant accessions from gene bank

The twelve sequences for *matK* gene and fifteen sequences for *rbcL* gene were used as reference sequence from temperate zone.

DNA extraction

The DNA genome was extracted from fresh young leave using Doyle and Doyle's method (1971) with modification.

Polymerase chain reaction

Polymerase chain reaction (PCR) analyses for *matK* gene were carried out with paired of primer MG1: 5`CGATCCTTTCATGCATT-3` as a forward and MG15: 5`-ATCTGGGT TGCTAACTC AATG-3` as a reverse (Hilu and Liang, 1997). The *rbcL* gene was carried out using paired of primer *rbcL*1b: 5`-ATGTCACCACAAACAGAA AC-3` and rbcL-724R: 5`-TCGCATGT ACCTGCAGTAGC-3` each primer as a forward and reverse primer respectively (CBOL, 2009). The *trnL* intron was amplified using trnL-c and d primers, that designed by Taberlet *et al* (1991).

The PCR reaction was amplified using Master Cycler Gradient Eppendorf. The PCR program for *matK* gene was started with 1 min of 95 °C incubation, followed by 35 cycles of 45 sec at 95 °C denaturing, 45 sec at 61.7 °C, 54.6 °C and 60.3 °C annealing for *trnK*, *rbcL* and *trnL* respectively, and 45 sec at 72 °C extension. The reaction was finished with 10 min at 72 °C incubation and stopped at 4 °C. PCR products were separated on 1.5 % agarose gels and detected by staining with ethidium bromide. Successful PCR amplification was result single band PCR product which has 2500 bp, 700 bp and 500 bp for trnK, *rbcL* and *trnL* genes respectively. PCR product was purified and sequence using automatic sequencer ABI 3730 XL in the Macrogen, Inc, Korea.

Data analyses

Sequences data were analyzed using MEGA5 software. MEGA5 software was used to align DNA sequences, beside that can be used to analyze Ts/Tvs ratio. Aligned DNA sequences then analyzed using DNAsp software to determine haplotype, conserved DNA region, haplotype sequence analysis and codon usage bias analysis. The haplotype networking was generated by NETWORK software. The haplotype networking can explain *A. spinosus*' phylogeography.

Results and discussions

There were 12 analyzed and divided into 34 character states. According to 34 character state, there were 4 special characters to determine *A. spinosus* variant. The special characters were stem shape (*teres* and *quadrangularis*) and leave shape(*lanceolate* and *rhomboid*) (Fig. 1). The As2, As3, As5, As6, As7 and As8 have teres stem and As1, As4 and As6 have quadrangular stem. The leave shape differ among variant from *lanceolate* (As2, AS, As6 and As8) to *rhomboid* (As1, As4, As5 and As7). The leave has different color from yellowish to dark green. The petioles have different length, with 0.5-2 cm (As3, As4, As6, As7 and As8), 2-4 cm (As2), and 4-7 cm (As1 and As5). The ratio leave length: petiole lengths are 2:1 for the *lanceolate* shape and 1:2 for the *rhomboid* shape.

Morphological differences among *A. spinosus* variant can be analyzed using molecular marker, especially using cpDNA sequences. There were three cpDNA gene that used in this research, they are the *trnL* intron, *matK* and *rbcL* genes. The *trnL* intron has 600 bp in length, the *matK* gene has 300 bp in length, and *rbcL* gene has 600 bp in length.

The *trnL* intron have two conserved region, especially in 35-98 bp and 444-551 bp with conserved sequence percentage approximately 92 %. The intron *trnL* have 476 bp monomorphic base and 43 bp polymorphic base. The polymorphic base relief the mutation events. Mutation in *trnL* intron dominated with G↔A with 12 events, and A-T (8 events), A-C and T-C (4 events), T-A (2 events) and G-C, G-T and T-G (1 event respectively). According to Fatinah *at al,* (2013), the G↔A substitution was dominate and causing the Ts/Tvs ratio high, with 1.19.

The *matK* sequences have low conserved sequenced value, with 2.3 % for tropical type and 99.8 % for temperate type. The low conserved sequence in tropical type indicated there was highly sequence variability in those samples. The *matK* sequence has sequence conserved region especially base at 59-93. The Ts/Tvs were 0.00 and 0.79 for temperate and tropical type respectively. The low Ts/Tv value ratio indicates that transversion has higher opportunity then transition. Beside that, *matK* sequence has insertion and deletions (indels) especially base at 76-78.

Table 1. Source of plant material from gene bank accession number for the 12 species *A. spinosus* were examined for *matK* gene, 15 species for *rbcL* gene sequences.

Genes	samples	Acc. No	Length (bp)	Origin
matK	*A.spinosus* voucher BioBot00664	JQ586446.1	761	Canada
	A. spinosus voucher BioBot00665	JQ586447.1	744	Canada
	A.spinosus voucher BioBot00663	JQ586445.1	749	Canada
	A.spinosus voucher Z22	JF953157.1	719	China
	*A.spinosus*_Z24	JF953155.1	713	China
	*A.spinosus*_Z26	JF953153.1	713	China
	*A.spinosus*_Z25	JF953154.1	713	China
	*A.spinosus*_Z27	JF953152.1	713	China
	*A.spinosus*_ Z29	JF953150.1	713	China
	*A.spinosus*_ NMNH.4469	GQ248076.1	791	Canada
	A.spinosus	EF590394.1	514	USA
	Amaranthus spinosus	HM850682.1	813	USA
rbcL	*Amaranthus spinosus*	HM849766.1	745	USA
	*A.spinosus*_BioBot00664	JQ590102.1	552	Canada
	*A.spinosus*_BioBot00665	JQ590103.1	552	Canada
	*A.spinosus*_BioBot00663	JQ590101.1	552	Canada
	*A.spinosus*_Z22	JF940804.1	603	China
	*A.spinosus*_Z24	JF940802.1	603	China
	*A.spinosus*_Z26	JF940800.1	603	China
	*A.spinosus*_Z28	JF940798.1	603	China
	*A.spinosus*_Z23	JF940803.1	603	China
	*A.spinosus*_Z25	JF940801.1	603	China
	*A.spinosus*_Z27	JF940799.1	603	China
	*A.spinosus*_29	JF940797.1	603	China
	A.spinosus	DQ006048.1	683	USA
	*A.spinosus*_NMNH.4469	GQ248546.1	553	USA
	*A.spinosus*_NMNH4469	EF590496.1	544	USA
trnL	*A.spinosus*	EF688743.1	943	USA

The *rbcL* sequence from tropical and temperate zone were analyzed. The *rbcL* sequence from temperate zone has higher conserved sequence value with 1.0 and 0.199 for tropical type. There were 337 bp mutation events with two conserved region especially base at 47-88 and 63-141. The Ts.Tvs values were 0.5 and 0.61 for temperate and tropical type respectively.

Phenotypic variation caused by plant morphological, functional, and developmental changing because of environmental heterogeneity to one or more genotype in one population (Radford, 1986). *Amaranthus spinosus* is one of other plant that has ability to adapt in different environmental condition (Prosea, 2012). Environmental heterogeneity adjusts plant to adapt (Coleman *et al*, 1994). Individual adaptation depends on genetic variation among them. Adaptation can increase plant vitality and survivability in face of environmental pressure. The environmental pressure causing different phenotypic expressions and this phenotypic expression majority were effected by pleotropic andepistatic genes (Wright, 1931). The gene alteration continuously will form a new phenotypic characteristic (neomorph) (Fisher, 1930).

Amaranthus spinosus from tropical zone have higher genetic variation. Genetic variation can be seen by the third molecular data was used, which have different Ts/Tvs ratio value approximately 0.5-1.19. According to Brown *et al* (1982), the Ts/Tv ratio value can be used to indicate the nucleotide substitution bias value. The DNA sequences with lower genetic distance high Ts/Tv ratio values, approximately 2-10 (Gojobori *et al*, 1982; Purvis and Bromham, 1997; Ina, 1998, Bakker *et al*, 2000). The nucleotide bias value can be informed mutation mechanism and level of DNA repair (Echol and Goodman, 1991).

The *trnL* intron has the highest Ts/Tv ratio value among all molecular marker that use with dominated by G↔A substitution. It is because of DNA repair mechanism based on *non-homologues end joining* (NHEJ). The NHEJ pathway is a common mechanism for double strand break (DSB). This pathway use short microhomology and can be repaired quickly. If DNA repair mechanism smoothly the DNA can be return to normal, but if the DSB severe damage caused deletion in these intron (Ferlow *et al*, 2011). Repairing DNA mechanism also caused nucleotide miss pairing. The G↔A substitution is a common DNA miss pairing in the DSB (Echol and Goodman, 1991). According to Bakker *et al* (2000), *trnL* intron has Ts/Tv ratio value approximately 0.8-1 in group. All angiosperm have Ts/Tv ratio value approximately 0.4-2. The Ts/Tv ratio value 0.4 indicated substitution saturated sequences and 2.0 indicated diverged sequences (Holmquist, 1983). The *matK* and *rbcL* genes have low Ts/Tv ratio value. It is caused by nonsynonymous base substitution (Li and Graur, 1991).

Table 2. Gene characterization from *matK* and *rbcL* sequences.

Genes	Origin	Ts/Tv	GC content (%)	No. of mutation	conserved sequences
rbcL	Canada	0.5[a]	42.6[a]	0	1
	China	0.5[a]	43.3[a]	0	1
	USA	0.5[a]	42.9[a]	0	1
	Indonesia	0.73[b]	54.1[b]	337	0.29
matK	Canada	0[a]	34.1[a]	1	1
	China	0.5[b]	34.2[a]	0	1
	USA	0[a]	33.6[a]	1	1
	Indonesia	0.65[b]	38.6[b]	363	0.17
trnL	Indonesia	1.14	29.6	47	0.98

According to Bakker *et al* (2000), *trnL* intron has Ts/Tv ratio value approximately 0.8-1 in group. All angiosperm have Ts/Tv ratio value approximately 0.4-2. The Ts/Tv ratio value 0.4 indicated substitution saturated sequences and 2.0 indicated diverged sequences (Holmquist, 1983). The *matK* and *rbcL* genes have low Ts/Tv ratio value. It is caused by nonsynonymous base substitution (Li and Graur, 1991).

The conserved sequence value among temperate and tropical type were different. The temperate type was more conserve then tropical type. The conserved sequence depend on phylogeography pattern, which are topography barrier, climate changing, sea increase level and volcanic activity (Gonza`lez-Rodriguez *et al,* 2004; Soltis *et al* ,2006; Jaramillo-Correa *et al*, 2009).

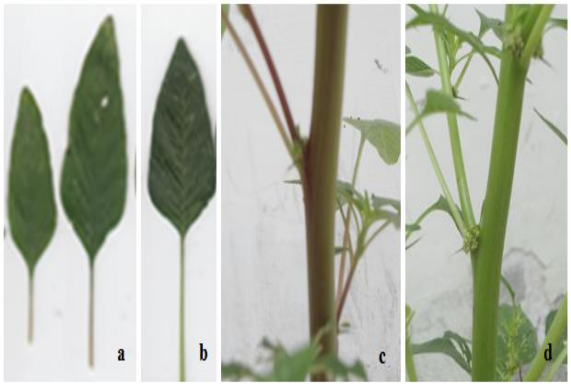

Fig. 1. Special characters to determine *A. spinosus* variants. a. *lanceolate* leave shape, b. *rhomboid* leave shape, c. teres stem shape, d. quadrangular stem shape.

Plant in the temperate zone adapted to freeze using freezing tolerant mechanism. The freezing tolerant mechanism causing genetic isolation among population and impede the genetic flow among species. Differ from temperate type; the tropical type has high genetic variability. In the tropical zone plant allow to colonize with rapid genetic flow among species. The genetic flow among species effect to high genetic variability among species but has low genetic differentiation among population (Bares *et al*, 2011). Plant adapt to spatial and temporal environmental heterogeneity. The spatial and temporal adaptations cause unique genetic variation (Levene, 1953; Haldane and Jayakar, 1963; Levins, 1968, Boza and Scheuring, 2004). Adaptations to specific environment cause plant local adaptation and contribute to plant evolutionary study and plant population size. The population size reflects plant adaptation process (Leimu and Fischer, 2008).

Conclusion

Amaranhus spinosus variant from tropical zone have high genetic variability with Ts/Ts approximately 0.5-1.19 and conserved sequenced > 70%. The high genetic variability caused by local adaptation and gene flow among species.

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