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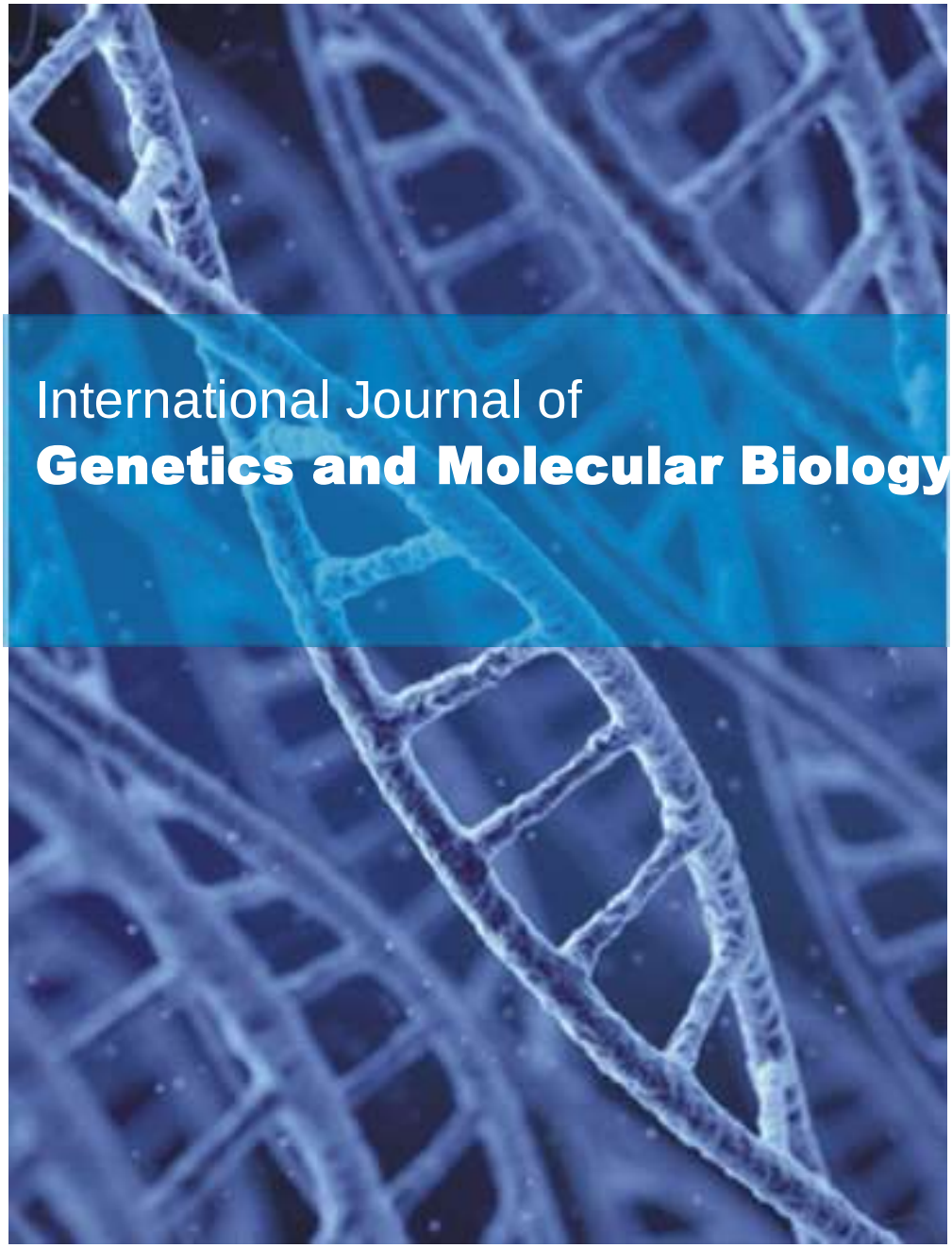

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Full Length Research Paper

The characterization of *Citrus* sp. from Parang Island Karimunjawa based on morphological, DNA barcoding and nutritional analysis

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The *Citrus* sp. from Parang Island Karimunjawa is a wild type of *Citrus* plant that grow on salt area and exhibits a high level of vitamin C. Due to its ability, morphological, molecular and nutritional characterization needs to be carried out to improve its potential. The study was carried out at the Biotechnology Laboratory and the Integrated Laboratory of Diponegoro University from 2016 to 2017. The characterization was based on the morphplogical appearence of the tree, its fuits, and leaves. Its DNA barcoding consists of 18S ribosomal RNA and the ITS region on the plants is dispersed along the coastal ends and the centre of the island. Furthermore, the nutritional characterization consists of an edible fruit part, a high vitamin C level and protein content. The research showed that the *Citrus* sp. on the coastal ends and at the center of Parang Island Karimunjawa exhibits a close relation with the *C. hystrix* and members of Papeda clade. However, the morphology of the *Citrus* is quite different from the common *C. hystrix* in Indonesia. The nutritional content also shows that the Vitamin C content is higher than that of the *C. hystrix* by almost four times. The features of the *Citrus* sp. in Parang Island Karimunjawa have potential and showed possibility to improve its superiority in industrial applications and breeding programs.

Key words: Karimunjawa, Parang, *Citrus*, 18S rRNA, ITS, nutrition.

INTRODUCTION

Citrus is one of the most popular world fruits. It also contains important nutritional elements for health. Citrus is a good source of vitamin C (ascorbic acid), phenolic compound, flavonoid, folic acid, potassium, pectin and antioxidant properties (Chiba et al., 2003; Abirami et al., 2014; Gosslau et al., 2014; Rafiq et al., 2016; Samraj and

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Figure 1. Collection sites of native *Citrus* species from Parang Island in Karimunjawa Islands (Forestry Department, 2004).

Rajamurgugan, 2017). As humans lack the ability to synthesize and store ascorbic acid, their daily requirement depend on fresh fruits.and vegetables. Citrus fruit production in Indonesia reaches 2 million tons each year while its consumption in 2018 totaled almost 2.76 to 2.45 million tons. Commonly, it was planted in the highland and lowland (Direktorat Jenderal Holtikultura, 2015). Citrus plants in Indonesia that can grow in coastal areas or areas with high salt content are very rare, except Swing Orange [*Limnocitrus littoralis* (Mig) Swing] in the area of Jepara which is considered as endangered species and has the strength of disease resistance (World Conservation Monitoring Centre, 1998). We found the indigenous citrus plant that grows on salinity area on Parang Island Karimunjawa which is an archipelago located about 80 km to the north of the island of Java with a total area of about 78 km^2 and the largest island covering an area of 2700 ha (Tomascir et al., 1997). Sea salinity in the western season is 32.6 ppm and 32.2 ppm in the eastern season. Its territory consists of several islands including Parang Island with an area of 690 ha. Their geographical position is at 5°42'-6°00'S, 110°07'-110°37'E, with air temperatures reaching 23 to 32°C and altitude of about 0 to 605 mabove sea level (Forestry Department, 2004). The wild Citrus in Indonesia is generally recognized by their morphology and nutrition content as conducted on Swing orange (Nuryandani, 2012; Adelina and Adelina, 2017). Characterization of some Citrus in Indonesia had been performed by molecular methods using isozymes, RAPD and ISSR related to their high genetic diversity and environmental adaptation (Fang et al., 1998; Karsinah et al., 2002; Agisimanto et al., 2007; Novelli et al., 2006; Bayer et al., 2009; Morton, 2009; Uzun et al., 2009; Penjor et al., 2014; Shimizu et al., 2016; Uchoi et al., 2017). Identification of *Citrus* sp. from Parang Island has never been found. Molecular markers provide abundant information compared to morphological data; they are more efficient and are insensitive to environmental factors. Therefore, characterization based on a combination of them has become important in the identification of this indigenous species. Until now, phylogenetic data for oranges that grow in salt area is very limited, especially oranges from Indonesia. *Citrus* sp. from Parang Island is able to live in coastal area and it can be used as a commercial orange rootstock that is susceptible to salinity. Since the sea area of Indonesia is around 63%, salinity is a major agricultural problem in lands that decreases growth and productivity, it is also possible to develop varieties with enhanced salt tolerance that can expand citrus cultivation into the salt affected marginal lands (Singh et al., 2003; Vijayan, 2009; Syvertsen et al., 2010; Hanin et al., 2013).

MATERIALS AND METHODS

Plant material

Fresh leaves and fruits of *Citrus* sp. were collected from Parang Island *Karimunjawa*. Location, state and name of species are provided in Figure 1.

Determination of nutrition and ascorbic acid/vitamin C using iodometric titration method

The nutritional analysis and iodometric titration method was conducted according to another study with some modifications (Ciancaglini et al., 2001; AOAC, 2005; Ywassaki and Canniattii-

Brazaca, 2011; Spínola et al., 2013). *Citrus* samples was ground in a mortar and pestle followed by adding distilled water several times while grinding the sample, each time decanting off the liquid extract into a 100 mL volumetric flask. The solution was centrifuged to obtain the filtrate. About 5 ml of filtrate was placed in the erlenmeyer and a solution of starch was added as much as 1% or about 2 ml. About 20 ml of distilled water was added into the place and the solution was titrated with 0.01 N iodine solution. The endpoint of the titration was identified as the first permanent trace of a dark blue-black colour due to the starch-iodine complex.

DNA extraction

Total genomic DNA was extracted from 0.5 to 1.0 g of fresh leaf material. Genomic DNA of *Citrus* species was extracted through cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with some modification. Quantification of DNA isolation product was conducted spectrophotometrically using Nanodrop. The quality of DNA was determined by electrophoresis on 0.8% agarose gel.

DNA barcoding analysis

The DNA barcoding analysis was conducted using the 18S ribosomal RNA (Region I) and sequences of ITS1,5.8S ribosomal RNA gene, ITS2, sequence of 28S rRNA gene (Region II). Both regions are DNA barcoding commonly used for phylogenetic analysis by researchers. The 18S ribosomal RNA fragment was amplified using the primer pair of Primer F (5′-GTA GTC ATA TGC TTG TCT-3′) and Primer R (5′-GCT GGC ACC ASA CTT GCC CT-3′) (Kusumaningrum, 2008). The final PCR cocktail of 25 µL ml contained 50 ng of genomic DNA, 2.5 µL PCR buffer (KAPA), 10 mM concentration of deoxynucleoside triphosphate mix (KAPA), 2.5 pmol of each primer and 0.625 U of Taq Extra Hotstart DNA Polymerase and ddH_2O. The amplifying reactions were run for 25 cycles for 3 min of pre-denaturation at 94°C, 25 s of denaturation at 94°C, 30 s of primer annealing at 55°C, 50s of elongation at 72°C, and 1 min of final elongation at 72°C. The PCR primers ITS 4 and ITS 5 (White et al., 2016) were used to amplify the ITS region (ITS 1,5.8S, and ITS 2) utilizing same primers for sequencing. The amplification program consisted of one cycle of initial denaturation at 94°C for 4 min followed by 25 cycles of 94°C for 1 min, 55°C for 3 min and 72°C for 1 min. This was followed by a 7 min extension at 72°C to allow completion of unfinished DNA strands, which in turn links to a soak file at 4°C. The PCR mixture of ITS primer contains 50 ng of genomic DNA, 2.5 µL PCR buffer, 10 mM concentration of deoxynucleoside triphosphate mix, 2.5 pmol of forward primer, 2.5 pmol of reverse primer and 0.625 U of Taq Extra Hotstart DNA Polymerase and Nuclease Free Water until reaching volume of 50 µL. DNA amplification was performed in a thermal cycler system. Amplified PCR products were purified using QIAquick gelextraction kit.

Phylogenetic analysis

The sequence characteristics of the ribosomal RNA and ITS region were calculated using MEGA version 6 (Tamura et al., 2013). For data analysis, published sequences of some members from the genus *Citrus* were downloaded from GenBank. Sequence data of the *Citrus* 18S rRNA partial sequences and ITS are listed in Table 1. Juke-Cantor method (Jukes and Cantor, 1969) was used to analyze the aligned sequence data. The phylogenetic tree was constructed using Phylip (Felsenstein, 2004). Bootstrap analysis was carried out with 999 random seed and 1000 replicates to examine the relative level of support for individual clades on the cladograms of each search. Genetic relationship was analyzed by phylogenetic tree construction.

Table 1. List of plant materials investigated in this study and their NCBI accession number.

Species	GenBank accession no.
Citrus kinokuni	AB456098.1
Citrus unshiu	JQ990161.1
Citrus medica var. *sarcodactylis*	JQ990163.1
Citrus sinensis	AB456120.1
Citrus hassaku	JQ990166.1
Citrus natsudaidai	AB456119.1
Citrus tachibana	KU535462.1
Citrus leiocarpa	JQ990180.1
Citrus tangerina	JQ990181.1
Citrus ichangensis	JQ990182.1
Citrus nippokoreana	JQ990183.1
Citrus aurantium	KU535472.1
Citrus pseudogulgul	KJ740213.1
Citrus erythrosa	JQ990187.1
Citrus platymamma	JQ990189.1
Citrusxparadisi	FJ641956.1
Citrus madurensis	KP093204.1
Citrusxtangelo	JN661211.1
Citrus clementina	XM006423861.1
Citrus nobilis	FJ641927.1
Citrus reticulata	FJ641939.1
Citrus junos	AB456113.1
Citrus japonica	JX144195.1
Citrus hindsii	JX144194.1
Citrus deliciosa	AB456093.1
Citrus maxima	JN681154.1
Citrus aurantiifolia	FJ641955.1
Citrus hystrix	FJ641961.1
Citrus macroptera	AB456052.1
Citrus montana	AB456057.1
Citrus aurantium	U38312.1
Citrus sunki	JQ990188.1
Citrus trifoliata	KJ740219.1

RESULTS

Morphological appearance of the *Citrus* sp. on Parang Island Karimunjawa

The *Citrus* sp. on Parang Island Karimunjawa is grown in the center and coastal areas of the land and based on our observation, the morphology of the plant exhibits some differences in some parts of the plant. As depicted in Figure 2, the fruit of *Citrus* plant grown on land area were big, globose, ovoid, very irregularly bumpy, glabrous with scattered glandular dots. The young green

Figure 2. Fruit of *Citrus* sp. Parang Island Karimunjawa (top= coastal, down = inland).

Figure 3. Leaf and pine of *Citrus* sp. Parang Island Karimunjawa (left= coastal, right = inland).

fruits become ripe and turns yellow with an average size of 7 to 10 cm in diameter. The peel is thick with its exterior layer of ±0.3 cm thickness and yellowish green. The inner part was white and the pulp was yellowish green. The taste is very sour and slightly bitter with a faint fragrance. The fruiting pedicel was about 0.3 to 0.5 cm long. The seeds of the *Citrus* fruit are numerous, ovoid-oblong, ridged, 1.5 to 1.7cm long, 1 to 1.1 cm wide and 0.4 cm thick.

The morphological characteristics of the Citrus fruit that grow on coastal areas were small, a little bumpy, globose, light green when ripe, feebly shining, with 5-7 cm diameter; thick peel, an exterior layer of ±0.2 cm thickness, light green, the inner part white; pulp yellowish green, very sour and slightly bitter, and releases a faint fragrance. The fruiting pedicel was about 0.3-0.5 cm long. The seeds of the *Citrus* fruit are numerous, ovoid-oblong, ridged, 1.2-1.5 cm long, 0.5-0.8 cm wide and 0.3 cm thick.

The characteristics of the *Citrus* leaf which grows on the land area as showed in Figure 3 were thin. The leaf surfaces was glabrous on both surfaces, light green adaxial, dull, light green or yellowish-green abaxial, densely pellucid dotted, fragrant when bruised, alternate, stalked unifoliate, broadly orbicular-ovate or ovate-oblong, lanceolate; base cuneate, obtuse or rounded, rarely subcordate, apex obtuse, rounded or slightly acuminate, often notched; patently serrate-crenate, coriaceous, 9.5-10 cm long and 3.5-4 cm wide.

The *Citrus* leaves that grows on the coastal area were also thin. The leaf surfaces were glabrous on both surfaces, light green adaxial, dull, light green or yellowish-green abaxial, dull, densely pellucid dotted, fragrant when bruised, alternate, stalked unifoliate, broadly orbicular-ovate or ovate-oblong, lanceolate; base cuneate, obtuse or rounded, rarely subcordate, apex obtuse, rounded or slightly acuminate, often notched; patently serrate-crenate, coriaceous, 9.5-10 cm long, and 3.5-4 cm wide. The petiole of *Citrus* leaves that grows on the coastal areas were almost the same with

Figure 4. Morphology of *Citrus* sp. of Parang Island Karimunjawa plant (left= coastal, right = inland).

the plants found in the center of the island. It is long and expands into prominent wings, the winged part are obovate or obcordate-oblong, with an acute, cuneote, obtuse or rounded base and an obtuse, truncate, rounded or slightly emarginate apex, patently crenate-exsculptate, coriaceous, 0.3-0.5 cm above the base upwards with large, foliaceous wings, light green adaxial, shining, yellowish green abaxial, the wings 1-8 cm long and 1-4.5 cm wide.

The *Citrus* trees that grows on the inland area are tall with a height of about 2-10 m (Figure 4). The trunk crooked with glabrous spiny branches, asymmetric or angular, thick, branched near the base; irregular crown, densely branched; branchlets rather thin. The tree branches were compressed-acutangular when young and become terete as they grow older. The branches are dark green, glabrous with scattered glandular dots, accomplished with axillary spines; spines long, stiff, subulate, green with hard brown or orange-coloured tips, obliquely erect, solitary, glabrous, 1.5-2 cm long.

The height of the *Citrus* tree which grows on the coastal area is almost 15 m high. The trunk is also crooked with glabrous spiny branches, asymmetric or angular, thick, branched near the base; irregular crown, densely branched; branchlets are rather thin, when young compressed-acutangular, when older terete, dark green, glabrous with scattered glandular dots. The tree branch is coupled with axillary spines; very long spines, stiff, subulate, green with hard brown or orange-coloured tips, obliquely erect, solitary, glabrous, and 3.5-4 cm long. Based on the characteristics, the clear morphological differences between the coastal and land *Citrus* was the decrease of leaf thickness and greenness, folllowed by longer and bigger stem spines on the coastal plants.

Table 2. Various nutritional constituents present in fruit juices of different *Citrus* species comparing with *Citrus* sp. Parang Karimunjawa Island[37].

Citrus	Edible plant part (BDD) (%)	Vitamin C mg/100 g BDD	Protein g/100 g BDD	Ash g/100 g BDD	Water content (%)
Citrus sp Parang inland	66.67	38.35	0.25	0.14	85.50
Citrus sp Parang coastal	43.75	66.37	0.19	0.22	86.12
Indonesian *C. hystrix*	50-60	16.5 - 19.5	-	-	65.5 - 87.9
Citrus sinensis (KPI, 2009)	72	49	0.9	0.5	87.2
Citrus limon (KPI, 2009)	76	50	0.5	0.3	92.2
Citrus aurantiifolia	-	19.7	0.5	0.4	88.9

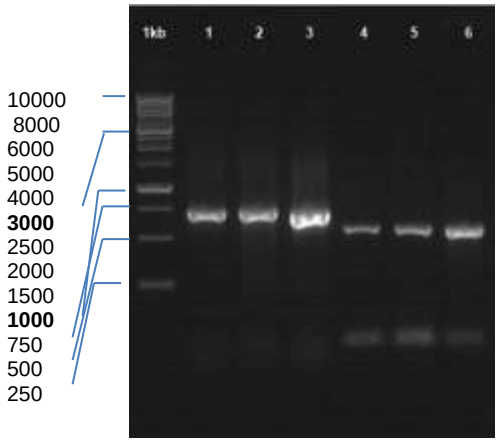

Figure 5. Electropherogram of DNA barcoding of *Citrus* sp. Parang Island Karimunjawa (10 kb=DNA ladder, 1= *Citrus* sp. inland ITS, 2= *Citrus* sp. coastal ITS, 3= ITS control positive, 4= *Citrus* sp. inland 18SrRNA , 5= *Citrus* sp. coastal 18SrRNA, 6= 18SrRNA control positive.

Nutritional analysis of *Citrus* sp. of Parang Karimunjawa Island

The *Citrus* sp. of Parang Island Karimunjawa nutritional analysis displayed in Table 2 shows a remarkable amount of Vitamin C on the *Citrus* fruit which grows on coastal region about 66.37 mg/100 g BDD. This amount was almost two times higher than *Citrus* which was grown on the center of the island. The quantity of *Citrus* nutrition showed various content in the fruit. High level of ascorbic acid in *Citrus* sp. of Parang Island will increase resistance to a lot of biotic and abiotic stresses like high salt concentration (Davey et al., 2006).

The juice contains the total amount of soluble sugar (15.43 mg/100 ml) and free amino acids (15.8 mg/100 ml) as other species of *Citrus*, whereas *C. limon* contains the least amount of the same compounds that is 4.37 and 3.6 mg/100 ml, respectively. Another researcher reported the lower (Kumar et al., 2013) and almost similar findings with the *Citrus* sp. inland of Parang Island Karimunjawa (Mahmud et al., 2009).

DNA barcoding of Karimunjawa Island *Citrus* using fragment of 18S rRNA gene, and ITS 1,5.8S ribosomal RNA gene, ITS2, fragment of 28S rRNA gene

The DNA barcoding to characterize the *Citrus* sp. on Parang Island from Karimunjawa archipelago consists of two regions, which are the 18S rRNA (region I) and fragment of 18S rRNA gene, ITS 1,5.8S ribosomal RNA gene, ITS2 and fragment of 28S rRNA gene (region II). Figure 5 shows the products of the DNA barcoding of the *Citrus* sp. in Parang Island.

The ITS of the 18S-26S nuclear ribosomal RNA separates the three gene region coding for the 18S, 5.8S, and 26S ribosomal subunits respectively. The ITS1 spacer is located between the 18S and 5.8S regions, the ITS2 spacer is between 5.8S and 26S. The annealing temperature for region I showed positive bands at 51 to 52°C and exhibited the best single DNA band. These data confirmed the primer we chose in this study was suitable for amplifying the conserved region of 18S ribosomal RNA in this Citrus. The size of the PCR product of 18S rRNA fragment was about 1000 bp. The best amplifying result of region II was achieved at the annealing temperature of 52°C. The size of the PCR product of ITS1, 5.8S rRNA gene, ITS2 region were about 750 bp.

The identity of our sequencing results using 18S rRNA is high and the value ranged from 92 to 98% compared to the existing sequence sources of existent *Citrus* species in GenBank database. This result suggested that the ITS universal primers has been successfully applied for the genus *Citrus* plants; the nrDNA ITS region could be successfully amplified using ITS universal primer sets. This result also in accordance with others (Sun et al., 2015). The sequences of region I showed homologies about 98 to 99% with 18S rRNA partial sequences of 30 *Citrus* species in the GenBank. In this study, as exhibited in Figure 6 and phylogenetic tree on Figure 7, the two *Citrus* sp. of Parang Island displayed high similarity with

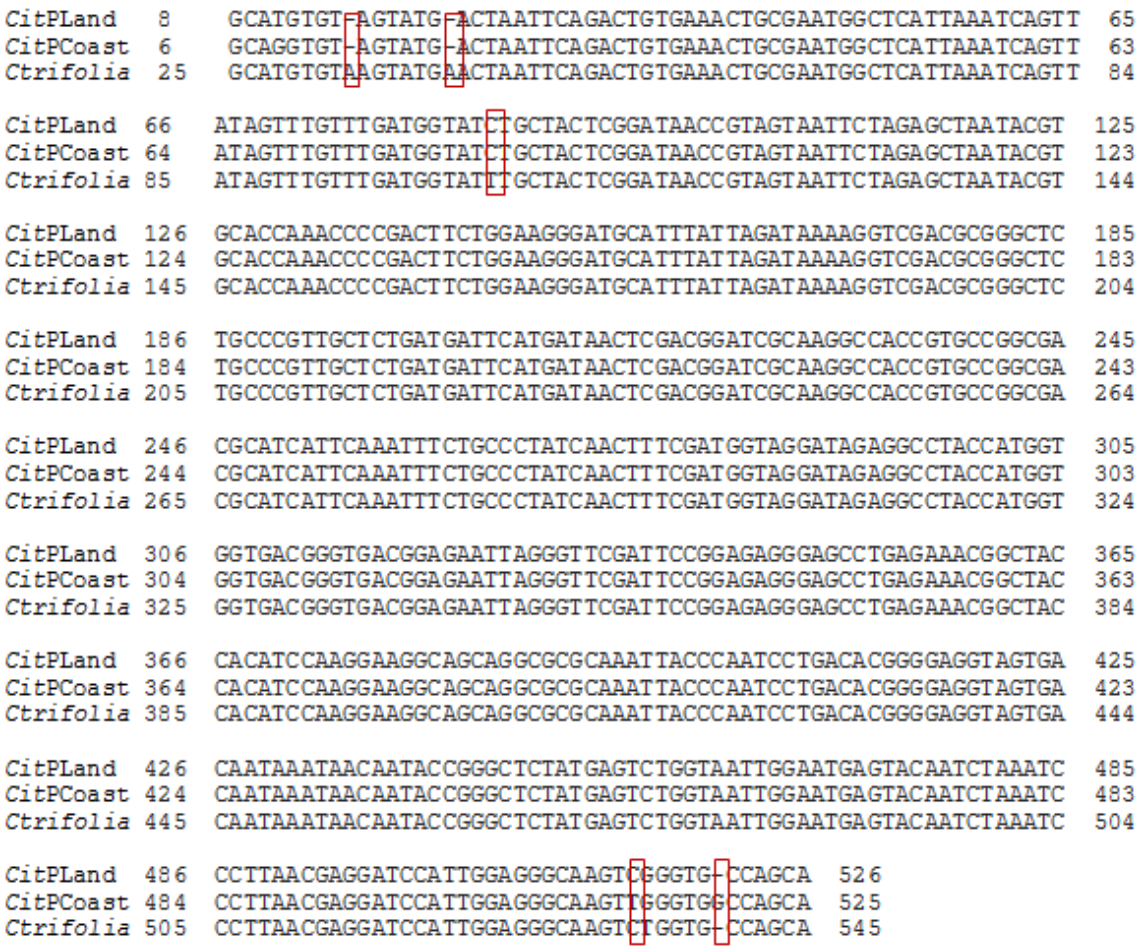

Figure 6. Close homology of 18S ribosomal RNA of *Citrus* sp Parang Island Karimunjawa inland and coastal based on 99% similarity analysis with *C. Trifoliata.*

C. trifoliata (99%) with accession number KJ740219.1. *C. tachibana*. Phylogenetic tree exhibited most of the features that is a species of *Citrus* that originated from China. A similarity analysis shows that there are 2 deletion bases and 2 subtitution bases between 18S rRNA partial sequences of the *Citrus* sp. on Parang Island with *C. trifoliata* marked with a red box.

The deletion and subtitution bases potentially indicated the nature itself for the *Citrus* sp. of Parang Island since 18S rRNA was the region with conserved sequences. The differences between the coastal and inland *Citrus* sp. of Parang Island was displayed by base substitution which exhibited a replacement of cytosine (C) on the *Citrus* sp. Parang Island land with thymine (T) on the same plant which lives on the coast. This subtitution was a turnover among the same base pairs (transitions) of pyrimidine. We also found a guanine (G) base insertion on the inland *Citrus* sp. of Parang Island comparing that of the coastal region.

Different results were reflected among the 18S rRNA partial sequences of the *Citrus* sp. on Parang Island Karimunjawa and their ITS region. These regions gained high similarity (97%) with *C. hystrix* as exhibited in Figure 8 and the phylogenetic tree in Figure 9. This result also is supported with the similarity on little bumpy morphology in their fruit skin as shown in Figure 1. The results study about high homology between *Citrus* sp. Parang Island with *C. hystrix* was not correlated with their vitamin C content. The higher vitamin C content of *Citrus* sp. Parang Island than *C. hystrix* obtained from this study exhibited the unique and specific character of this native plant. Furthermore, the sequences in the ITS1-ITS2 region also exhibited higher differences among the coastal and inland *Citrus* sp. of Parang Island Karimunjawa. In contrast with 18S rRNA partial sequences, the sequences of ITS1-ITS2 region showed lower homologies of about 89-95% with other *Citrus* from Vietnam, India, Japan, and China. This result was in agreement with the similarity analysis in showing variable sequences at the end of 18S rRNA sequences. The ITS1 sequences showed lowest differences compared to the 5.8S rRNA and ITS2. The several subtitution in the ITS2

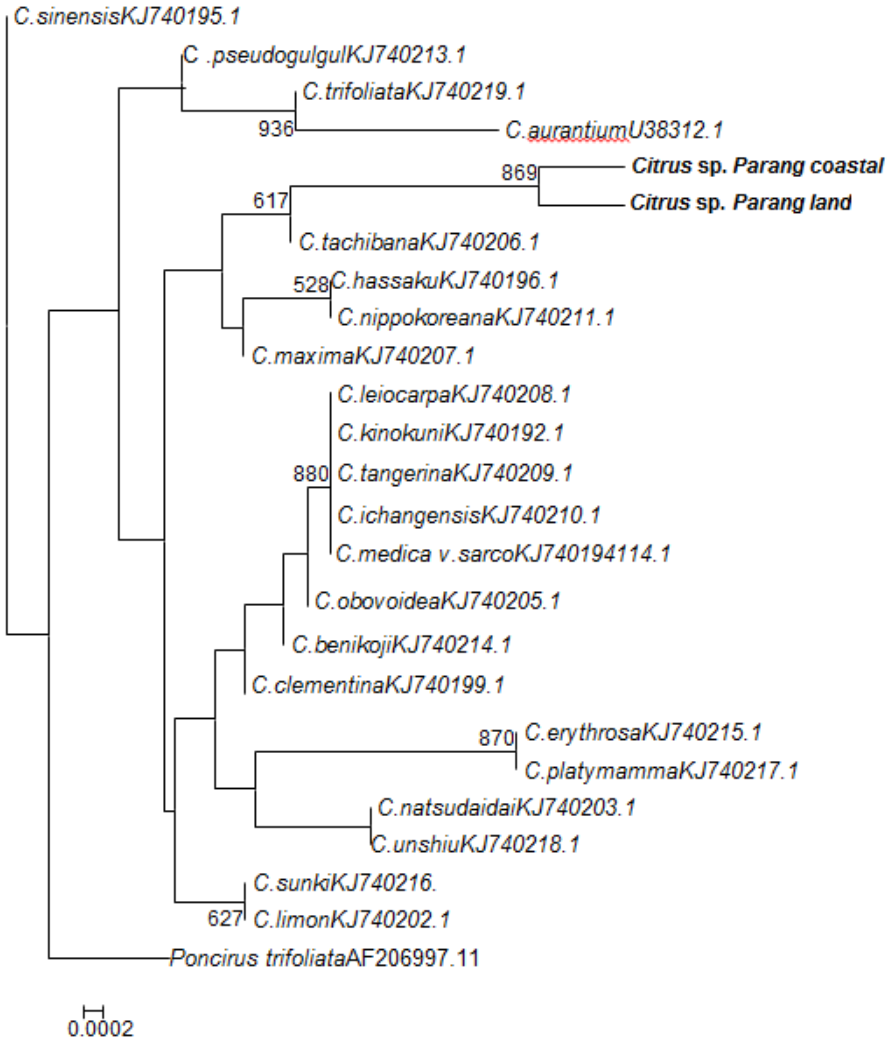

Figure 7. Phylogenetic analysis of 18S ribosomal DNA of *Citrus* sp coastal and inland Parang Island Karimunjawa.

was considered to be the specific characteristic of the *Citrus* sp. on the coastal area of Parang Island Karimunjawa because the land area *Citrus* sp. of Parang Island and *C. hystrix* did not show this subtitution in this region. The reasons for the difference in the homology obtained from phylogenetic analysis and ITS region sequencing for the both Citrus sp. from Parang Island which live inland and coastal is having the same anchestor. Nei and Kumar (2000) stated that when two DNA sequences come from the same ancestral sequence, the sequence of descendants will gradually be differentiated by nucleotide substitution. The variation in sequences and differences of ITS2 among the coastal and inland plants has been shown to be valuable in identifying both of them. This study is also in good agreement with the previous report (Alvarez and Wendel, 2003; Sun et al., 2015). Amongst the two *Citrus* sp. on Parang Island Karimunjawa investigated in this study, there were some found variations in nucleotide substitution, deletion, or insertion. Number of base substitution found between them were 8 transitions and 14 transversions.

The probability level of transversion is greater than transition which indicates that the changes in the ITS region can potentially alter the genes to ensure its position in classification at *Citrus* group. The phylogenetic and genetic distance analysis using 18S rRNA showed that *Citrus* sp. of Parang Island was in the same branch with *C. tachibana*. The ITS region showed different result in gained close genetic relationship with *C. montana, C. hystrix* and *C. macroptera*.

DISCUSSION

In this study, *Citrus* sp. ws characterized which grow on

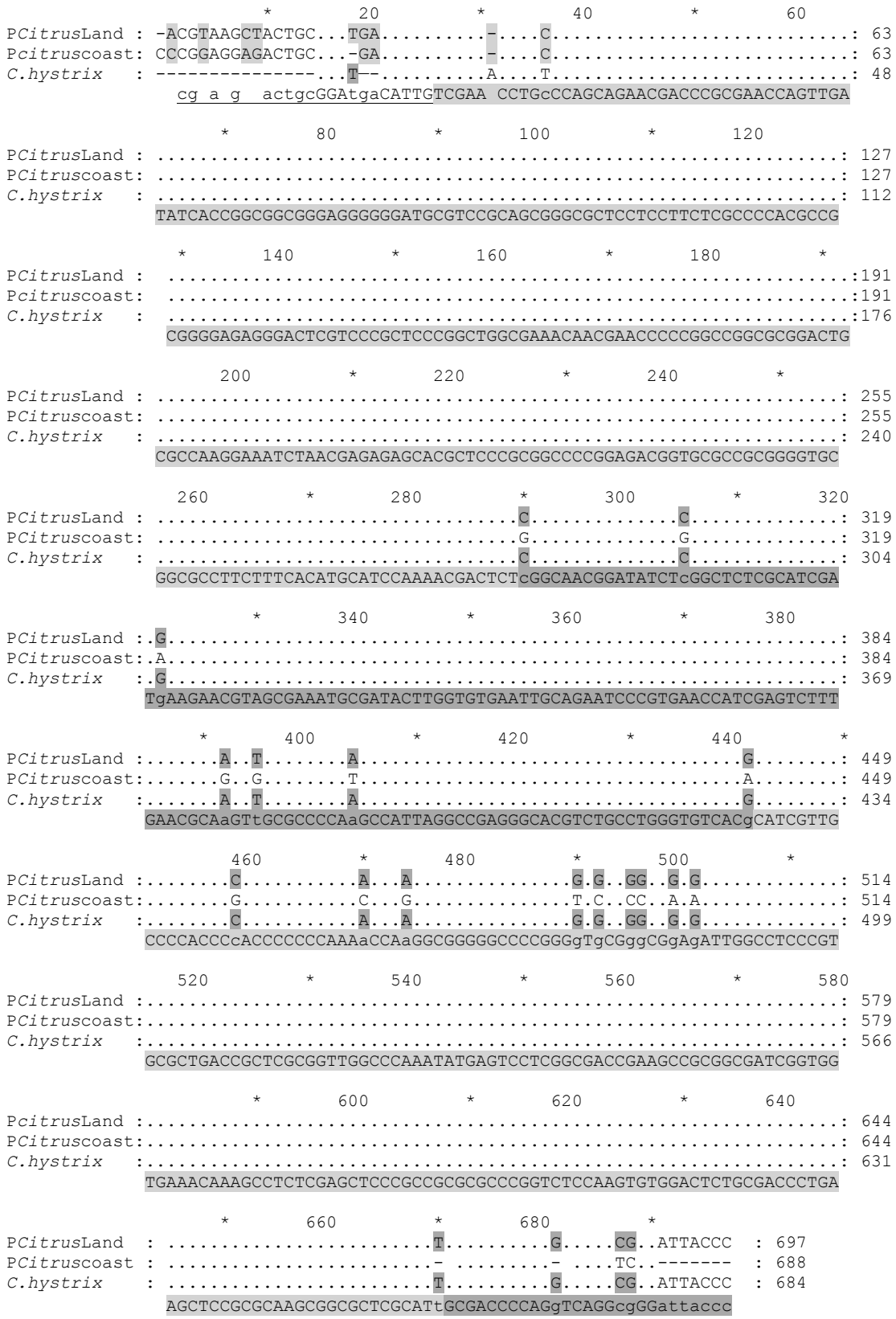

Figure 8. Closest homology of region II of *Citrus* sp Parang Island Karimunjawa coastal and inland based on 97% similarity analysis with *C. hystrix* FJ641961.1 (<1...26 = 18S rRNA, 27...281 = ITS1, 282...443 = 5.8S rRNA, 444 - 672 = ITS2, 673 - 689> = 28S rRNA).

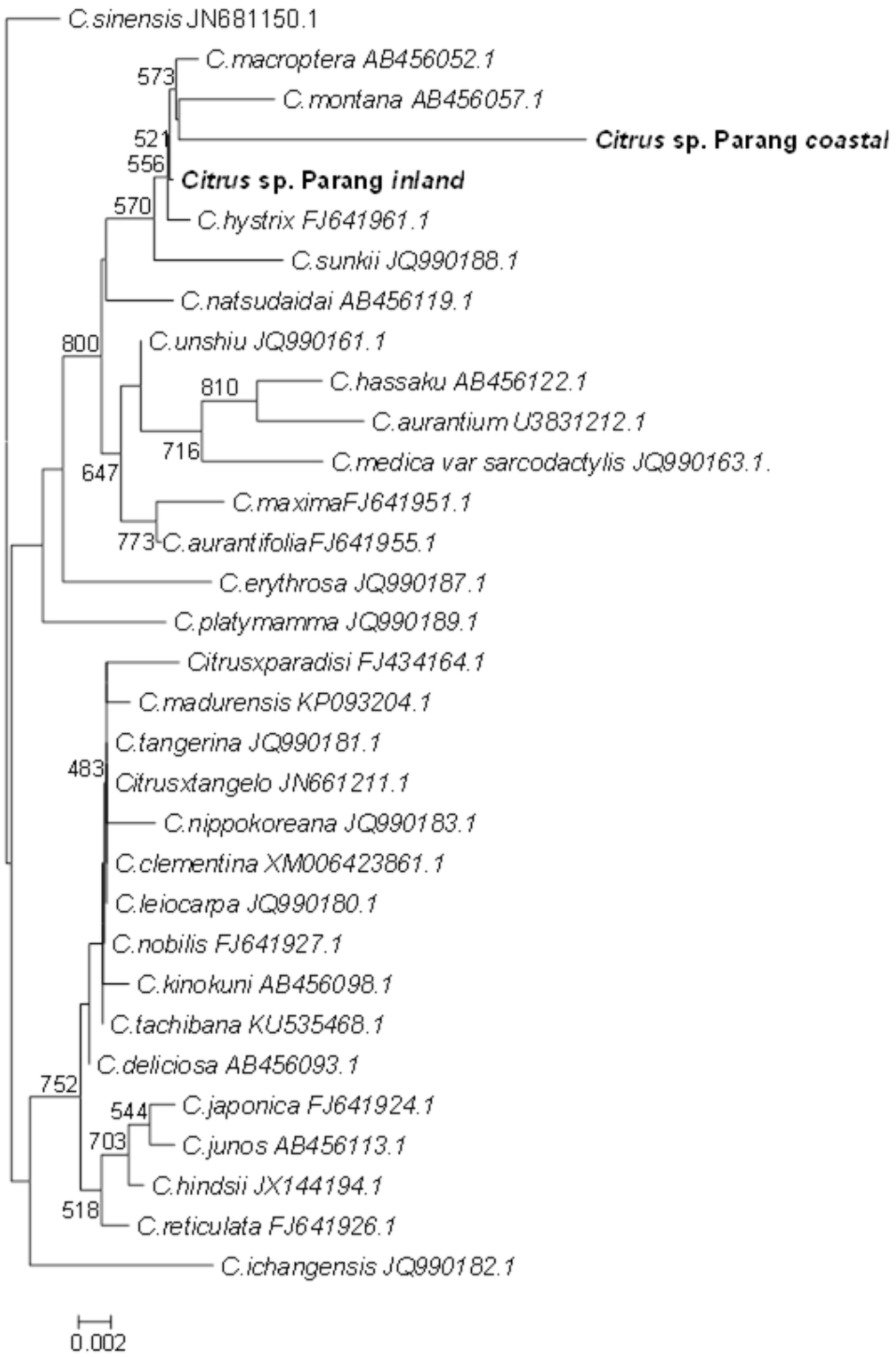

Figure 9. Phylogenetic analysis of ITS1-5.8S-ITS2 region sequence of *Citrus* sp coastal and inland Parang Island Karimunjawa.

Parang Island Karimunjawa based on morphogical, nutritional analysis, and DNA barcoding. In some studies, morphological characterization fails to obtain the representative identity of the *Citrus*. The study using morphologic appearence proved these characteristics were independent and related to diverse pressures and evolutionary factors and require other methods to complete the assessment. Therefore, it still raises a lot of questions in attempt to further improve breeding programs (Koehler-Santos et al., 2003; Yahata et al.,

2017). Morphological characterization of the *Citrus* sp. on Parang Island Karimunjawa also showed some differences in some parts of the plant compared with other *Citrus* plants in Indonesia. These characteristics seems quite specific for the *Citrus* on Parang Island Karimunjawa. The fruit size and weight on the coastal region tends to be smaller but higher in Vitamin C content. It have greener leaf, narrower, bigger and stronger stem pine. Presumably, these character was related to salinity susceptibility and is essential for plant survival and maintained growth rate. This characteristics were also verified from other studies (Moya et al., 1999; Murkute et al., 2005; Syvertsen et al., 2010; Hussain et al., 2012; Acosta-Motos et al., 2017). Some study explain that the effects of high salt environment had induce plant adaptation and rise the level of ascorbic acid in several plants (Hernandez et al., 2000; Davey et al., 2006; Acosta-Motos et al., 2017). These studies supported the nutritional analysis of the *Citrus* sp. from Parang Island which showed that its Vitamin C content is higher than *C. hystrix* almost by four times, although they have close genetic relationship and similarity in morphology (Murkute et al., 2005). Vitamin C is one of the known forms of ascorbic acid which is a water-soluble chemical in fruits (Kumar et al., 2013). This substance amount has been shown to be valuable since vitamin C is one of important components of citrus. Another study reported that it contributes to the antioxidant activity about 56 to 77% from *Citrus* extract (Kumar et al., 2013; Abirami et al., 2014). In higher plants, ascorbic acid biosynthesis is from glucose using L-galactose pathway. Overexpression of several structural ascorbic acid-related genes from various ascorbic acid metabolic pathways is not succesfull in most species in order to enrich ascorbic acid levels (Mellidou and Kannelis, 2017). The role of two genes of Guanosin diphosphate (GDP) in the L-galactose pathway, GDP-D-mannose pyrophosphorylase (VTC1 or GMP) and GDP-D-mannose-3,5-epimerase (GME), has been correlated with ascorbic acid concentrations in fruit of some species such as kiwifruit (Bulley et al., 2009), apple (Li et al., 2010) and blueberry (Liu et al., 2015). It is also related with ascorbic acid accumulation under salt stress (Zhang et al., 2011). Several studies on genetic factors indicated that ascorbic acid accumulation showed influence of heritability (Davey et al., 2006). The high concentration of absisic acid in the *Citrus* sp. from Costal area of Parang Island offers a potency to be exploited to enhance it accumulation in fruit due to its importance as bioactive nutrients and dietary antioxidants.

The DNA barcoding approach involving the 18S rRNA region evolves relatively slowly compared to the ITS region due to their high conservation. Despite their weakness, the use of ribosomal RNA as an alignment tool and similarity analysis in plant phylogenetic studies still offers advantages because it is not influenced by environmental changes compared with other methods such as SRAP, *matK*, ISSR (Rogers and Bendich, 1987; Fang et al., 1998; Johnson et al., 1999; Uzun et al., 2009; Penjor et al., 2014). The rapidly evolving ITS spacer sequences have been used extensively in phylogenetic studies due to its application not only in lower levels, but also help resolve intra-family relationships (Rogers and Bendich, 1987, Johnson et al., 1999; Kyndt et al., 2010). This was exhibited by the inconsistent results of similarity analysis which showed that the *Citrus* sp. on Parang Island Karimunjawa was the closest to *C. aurantium* but the phylogenetic tree of 18S rRNA shows its relationship with *C. tachibana*. Interestingly, Hirai et al. (1990) and Yamaji et al. (2013) found that *C. tachibana* was known as wild species of *Citrus* with intraspecific nuclear rDNA (nrDNA) variation in ITS that was thought to be fixed into a single ribotype. The study with ITS region found that based on the number of base substitution, deletion and insertion between the coastal and inland *Citrus* sp. on Parang island, it was clustered together with *C. montana*, *C. hystrix* and *C. macroptera*. This study of ITS was supported with other in showing that *C. hystrix*, *C. macroptera*, and *C. montana* had belong to one clade Papeda respectively (Li et al., 2010, Hynniewta et al., 2014, Yamaji et al., 2013). Papeda is a common name for a group of native tropical Asian citrus. Some species of Papeda have been used as genomic sources for breeding disease-resistance (Wang et al., 2017). Based on molecular studies, papeda is one of the ancestors of many types of commercial limes (Xu et al., 2013; Wang et al., 2017; Wu et al., 2018). The morphological, nutritional and DNA barcoding analysis showed the possibility that *Citrus* sp. on Parang Island Karimunjawa was considered to have the specific characters and belongs to the Papeda clade. The ITS analysis showed that the longer branch of *Citrus* sp. which grows in coastal area suggests a possibility of a more advanced evolution. This study showed that *Citrus* sp. from Parang Island with spesific emphasis on the ones that grow on the coast has genetic potentials and a remarkable vitamin C content and can live on salinity of 32.2-32.6 ppm. This potential will increase the genetic resources of *Citrus* and increase the possibility to elevate their superiority for breeding programs and industrial applications. Previous studies have shown different levels of ascorbic acid in fruits during their growth in saline soil (Bulley et al., 2009; Li et al., 2010; Liu et al., 2015). It raised open question whether the character of *Citrus* sp. Parang Island is inherited from the parent or is the result of adaptation to the environment. Further experiment will deal with salt treatment to the Citrus on the laboratory and the examinination on the effect of the EC or TDS of water and the soil on *Citrus* sp. Parang Island vitamin C content.

Conclusion

Characterization of *Citrus* sp. Pulau Parang Karimunjawa

based on morphology, DNA barcoding and nutritional analysis shows the closest relationship with *C. hystrix* and members of the *Citrus* species in Papepa clade. However, some variations in the ITS region sequence and vitamin C content indicate the specific character of *Citrus* sp. of Parang Island Karimunjawa, especially in the coastal area.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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