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Molecular Profile of Cogongrass [*Imperata cylindrica* (L.) Raeusch] in Java Island Based on *trnT*(UGU)-*trnL*(UAA) IGS Sequences

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Abstract. *Cogongrass is one of the top ten problem-causing weeds in the world that is invasively spread over many tropical and subtropical regions, including Java Island, the Republic of Indonesia. The wide distribution is possibly related to their adaptability to almost all terrestrial habitats from various altitudes, which may lead to a high level of genetic variation. This study aimed to assess the genetic variation and phylogenetic relationship of cogongrass in Java Island using an intergenic spacer (IGS) sequence in the cpDNA genome, i.e. trnT(UGU)-trnL(UAA), as the molecular marker of high mutation rate. Plant samples were collected randomly from five different sites on the island. These were subjected to total genomic DNA extraction. The DNAs obtained were then used as PCR templates to amplify trnT(UGU)-trnL(UAA) IGS using a pair of universal primers. The PCR products were sequenced for genetic analysis. It was shown that an extremely high genetic variation with respect to the haplotype diversity of cogongrass in Java Island was observed. Nevertheless, a very low nucleotide diversity was obtained revealing a considerably close genetic relationship among the samples. Anatomical data regarding stomata number and length were found to correspond to the high level of genetic variation, but it was not the case with the stomata index.*

Keywords: *Cogongrass, genetic diversity, Java Island, trnT(UGU)-trnL(UAA)IGS*

Citation

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INTRODUCTION

Java Island belonging to the Republic of Indonesia has various ecosystems, ranging from coastal to mountain areas. These ecosystems provide habitats for many types of living organisms including numerous plant species. Accordingly, a high level of plant biodiversity exists on the island (Nurfadilah et al., 2017; Damayanti et al., 2021). Even high awareness of preserving plant diversity in Java Island was indicated in some plant reliefs of Borobudur Temple, Central Java (Metusala et al., 2020). On the other hand, several studies on plant diversity disturbances and changes in Java Island have been reported (Hakim & Soemarno, 2017; Iskandar et al., 2018).

Cogongrass (*Imperata cylindrica* (L.) Raeusch) is one of the plant species being classified as top ten problem-causing weeds in the world that is invasively distributed over many tropical and subtropical areas (Mac-

Donald, 2004). Despite its beneficial utilization, especially as traditional herbs in developing countries, the invasiveness is much more prominent than the advantages provided (Rusdy, 2020). Originating from Southeast Asia, cogongrass is also commonly found on Java Island existing in a sufficiently large population. As an invasive plant species, it grows very rapidly despite the applications of either physical, chemical, or biological control techniques (Syah & Hidayat, 2020). Variability in the efficacy of a broad-spectrum herbicide, glyphosate, seems unlikely to be applied due to existing resistance (Enloe et al., 2018). Cogongrass can modify the soil it occupies, in which it initially takes benefits for its growth from a native microbial community. Then, the alterations in that microbial community as well as soil chemical components by cogongrass stimulate its rapid lateral growth (Radunzel-Davis & Cronin, 2019).

Cogongrass is usually found dominating critical lands, which is of approximately 2.1 million ha on Java Island in 2018 (Badan Pusat Statistik, 2018). The high competitiveness against many other plant species is mainly due to the capacity of producing allelopathy substances (Kilowasid et al., 2021). This is then supported by a wind-pollinated reproduction system enabling easy seed dispersal over remote distances although asexual reproduction by means of rhizomes is more prominent in this species (Nomura et al., 2022). It was reported that one individual plant could produce 31 rhizomes in ten months resulting in a total rhizome length of 12 m (Kato-Noguchi, 2022), although much lower rhizome germination was observed in cogongrass under continuously dark period in comparison to those in a light/dark regime (Hamidavi et al., 2021). High phenotypic plasticity and adaptability to nearly all terrestrial habitats also contribute wide distribution of cogongrass (Susanto & Dwiati, 2022).

The wide distribution of cogongrass may lead to a high level of genetic variation, both within and between populations. This can be measured by using either morphological- or molecular-based analysis, where the latter is considered more representative because of the absence of environmental intervention. In addition, molecular-based analysis can be performed at any stage of plant growth and development (Tsonev et al., 2021). Molecular analysis was required to confirm some uncertain medicinal plant species used by a particular tribe in Mindanao, the Philippines (Dapar et al., 2020). Morphological data on *Cucurbita pepo* could be affected by some anthropogenic activities or other environmental factors, while the molecular data obtained had no high coverage in the complete genome. Thus, although morphological and molecular markers are not interchangeable, they are complementary methods to assess genetic diversity (Yunli et al., 2020). On the other hand, a highly significant correlation between morphological and molecular data was observed in detecting the salt tolerance of several wheat genotypes (Al-Ashkar et al., 2020).

One of the molecular markers that can be used to analyze the genetic variation of cogongrass in Java Island is *trnT*(UGU)-trnL(UAA) intergenic spacer (IGS), which is a non-coding sequence in the cpDNA genome. As a region not responsible for any protein synthesis, this sequence shows a relatively high mutation rate, so it is appropriate to be utilized as a molecular marker in the study of genetic variation in several plant species (Skuza et al., 2019). Among the three non-coding regions of cpDNA used to analyze population genetics of *Quercus cerris* in Turkey, *trnT*(UGU)-*trnL*(UAA) was found to be the most variable and parsimony informative (Tekpinar et al., 2021). It was also one of eight

highly variable markers for the identification of some *Polygonatum* species (Wang et al., 2022). By using this molecular marker, two phenotypically very similar species of Asteraceae family can be clearly distinguished (Susanto et al., 2020). Therefore, our study aimed to assess the genetic variation and phylogenetic relationship of cogongrass in Java Island employing *trnT*(UGU)-*trnL*(UAA) IGS. Some previous studies on the genetic variation of cogongrass have been reported. However, none of them has been employing *trnT*(UGU)-*trnL*(UAA) IGS as the molecular marker. Moreover, some anatomical characteristics are involved to support the molecular profiling of cogongrass in our study.

MATERIALS AND METHODS

Plant Sampling

The study was carried out using an exploratory method from April to July 2022, where plant samples were collected randomly from five different sites on Java Island. The respective altitudes and coordinates of the sites can be seen in Table 1.

All plant samples were taken entirely from the soil including their roots. They were then wrapped with wet wipes and then put into plastic bags before being planted in the greenhouse of the Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia. Subsequently, molecular analysis was carried out at the Laboratory of Genetics and Molecular Biology of the institution. A fresh and extended second leaf of each plant was used as the sample for genomic DNA extraction.

Table 1. Sampling sites of cogongrass in Java Island

Sampling Site	Site Code	Altitude (m asl)	Coordinate	
			S	E
Purwokerto, Central Java	PWT	79	7º24’33”	109º15’14”
Jetis Beach, Cilacap, Central Java	JET	6	7º43’21”	109º23’26”
Purworejo, Central Java	PWJ	21	7º51’17”	109º57’18”
Yogyakarta City, Special Territory of Yogyakarta	YOG	136	7º47’20”	110º19’21”
Ponorogo, East Java	PON	57	7º49’15”	111º32’56”

Genomic DNA extraction

Each leaf sample was cut into small pieces, washed up using tap water, sprayed with 70% ethanol, and weighed to 0.1 g for genomic DNA extraction, which was performed following CTAB protocol (Doyle & Doyle, 1990). The small pieces of leaf were put into a mortar, then ground using a pestle until smooth and added with 800 µL CTAB buffer solution previously heated in a water bath at 65°C for 30 minutes. The homogenous mixture was put into a 1.5 mL microtube and incubated at 65°C for an hour while slowly flipping the tube every 10 minutes. This was then cooled at room temperature for 2 minutes, after which 500 µL CIAA (chloroform-isoamyl alcohol) solution was added, mixed gently, and vortexed for 5 minutes. The mixture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred into a new microtube, added with 3M sodium acetate of 1/10 supernatant volume, and mixed gently. Then, isopropanol of 2/3 total volume was added and mixed gently by flipping the tube. The mixture was stored in the freezer for 24 hours prior to centrifugation at 12,000 rpm for 10 minutes. After supernatant removal, the DNA pellet was rinsed using 500 µL 70%

ethanol while gently flipping the tube. The DNA solution was centrifuged at 12,000 rpm for 5 minutes, and again the supernatant was removed while the DNA pellet was air-dried before re-dissolved in 100 µL TE buffer. This was incubated at 37°C for an hour and finally stored at 4°C prior to further analysis.

Amplification and Sequencing of *trn*T(UGU)-*trnL*(UAA) IGS

Both the concentration and purity of the extracted genomic DNAs were measured by using GeneQuantTM 1300. In addition, the DNAs were also visualized qualitatively in a 1% agarose gel electrophoresis using 1x TAE buffer.

Serving as PCR templates to amplify *trnT*(UGU)-*trnL*(UAA) IGS, the genomic DNAs were then diluted to 50 ng/µL. The PCR employed a pair of universal primers, i.e. B48557 (5'-CATTACAAATGCGATGCTCT-3') as the forward primer and A49291 (5'-TCTACCGATTTCGCCATATC-3') as the reverse primer (Taberlet et al., 1991). A total volume of 50 µL PCR mixture containing 10 µL template DNA, 1 µL forward primer, 1 µL reverse primer, 25 µL Kapa Taq and 13 µL nuclease-free water was run in an advanced Primus 25 thermocycler. The PCR condition used was as follows: pre-denaturation at 94°C for 5 minutes continued by 30 PCR cycles consisting of denaturation at 94°C for 1 minute, primer annealing at 50°C for 1 minute, primer extension at 72°C for 1 minute 30 seconds, and completed with a final extension at 72°C for 5 minutes.

The PCR products obtained were visualized in a 1.5% agarose gel electrophoresis using 1x TAE buffer. They were then sent to Firstbase Malaysia for sequencing. The dideoxy method of Sanger et al. (1977) was applied in an automated DNA sequencer with terminator labeling (Smith, 1989).

Measurement on Leaf Anatomical Characters

Supporting data on leaf anatomical characters were measured including stomata number, length, and index. Leaf samples were cut into some small squares with a side length of approximately ± 5 mm. These were soaked in a saturated chloral hydrate solution for a week. They were transferred then into another saturated chloral hydrate solution up to transparency, indicating that the chlorophylls have been totally removed. The leaf cut was put on an object glass for examination under the microscope connected to an Optilab Viewer 2.2. An Image Raster 3.0 Program was used to calculate stomata and epidermis cell numbers as well as to measure stomata length. The stomata index was calculated using the following formula (Wallis, 1965).

$$SI = \frac{S}{S+E} x100$$

SI : stomata index
S : stomata number
E : epidermis cell number

Data Analysis

The DNA sequences were edited using Bioedit version 7.0.4.1 (Hall, 1999) combined with manual editing. Blasting to the NCBI database was performed for validation, followed by sequence alignment using ClustalW (Thompson et al., 1994), which was also implemented in the Bioedit 7.0.4.1. Analysis of genetic variation and construction of phylogenetic trees were performed using MEGA version 11 (Tamura et al., 2021).

Supporting data on leaf anatomical characters were analyzed statistically using ANOVA. Further analysis using LSD was carried out for those showing significant differences among sampling sites.

RESULTS AND DISCUSSION

Genomic DNAs were successfully extracted from all samples resulting in sufficiently high concentrations ranging from 700 to 3,200 ng/µL, while the purity varies from 1.538 to 1.818 at 260 nm/280 nm absorbance (Table 2). When they served as PCR templates using the universal primers, amplicons of approximately 900 bp were produced (Figure 1). These were then sequenced and edited, after which DNA sequences of 850 bp long were obtained.

Table 2. Concentration and purity of genomic DNAs of cogongrass samples

Sampling Site	Site Code	Concentration (ng/µL)	Purity (A260 nm/A280 nm)
Purwokerto, Central Java	PWT	2,000	1.818
Jetis Beach, Cilacap, Central Java	JET	3,200	1.800
Purworejo, Central Java	PWJ	1,500	1.667
Yogyakarta City, Special Territory of Yogyakarta	YOG	700	1.750
Ponorogo, East Java	PON	2,000	1.538

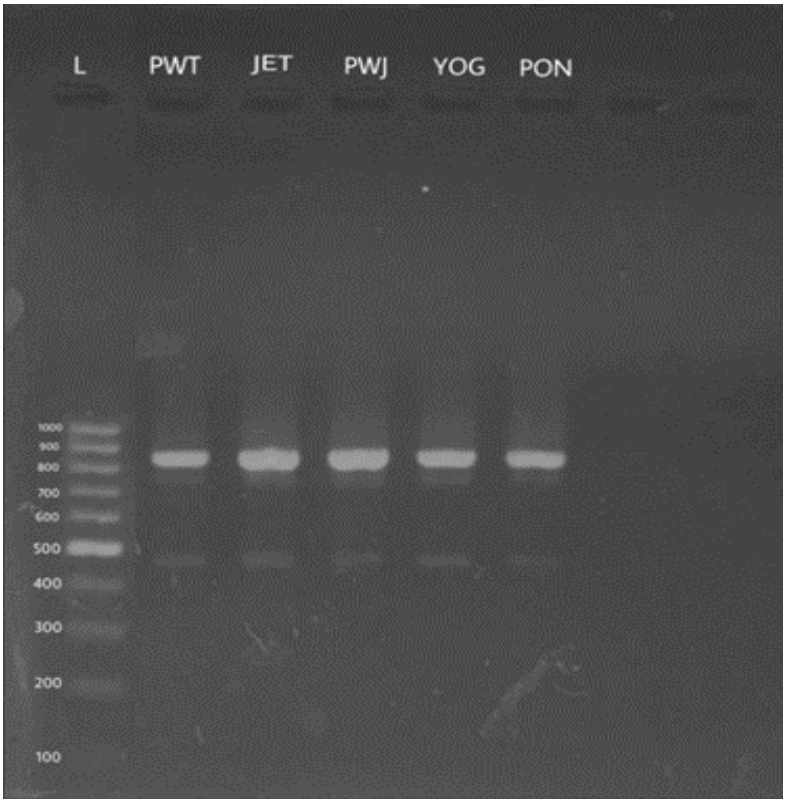

Figure 1. PCR products using a pair of universal primers to amplify *trnT*(UGU)-*trnL*(UAA) IGS (L = 1 kb ladder; PWT = Purwokerto; JET = Jetis; PWJ = Purworejo; YOG = Yogyakarta; PON = Ponorogo)

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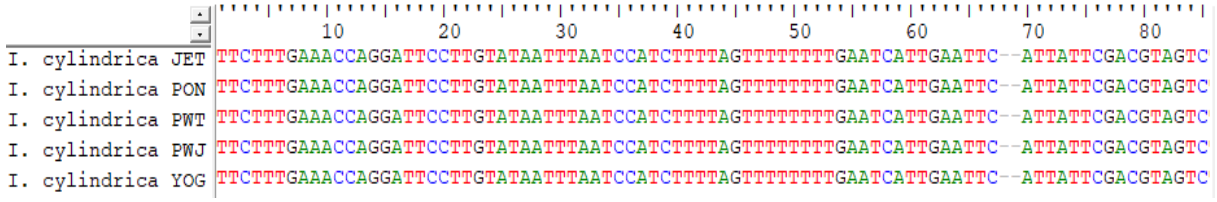
Almost similar to our results, PCR products of approximately 880 bp were obtained in *Synedrella nodiflora* and *Eleutheranthera rudealis* when amplified using a pair of universal primers for *trnT*(UGU)-*trnL*(UAA) IGS. Both species are members of Asteraceae family, which phenotypically show very identical characteristics. After manual editing, the *trnT*(UGU)-*trnL*(UAA) IGS sequence of *E. ruderalis* that slightly longer than *S. nodiflora's* was found. Despite no direct correlation between the genetic and phenotypic profiles, coincidence appeared likely to occur providing a molecular indication that they are two different species (Susanto et al., 2020). On the other hand, much shorter *trnT*(UGU)-*trnL*(UAA) IGS sequences ranging from 367 to 556 bp were found in several species of *Cinnamomum* (Lauraceae) in Sri Lanka. In addition, only a very little variation among the sequences was observed providing less contribution to the taxonomy of the species (Abeysinghe et al., 2009). Oppositely, moderate levels of variation were demonstrated with *trnT*(UGU)-*trnL*(UAA) IGS sequences in some species of *Prunus* suggesting the utilization of the molecular marker for further analysis of phylogenetic relationships within the genus (Kim et al., 2019).

A different pair of primers were employed to amplify *trnT*(UGU)-*trnL*(UAA) IGS in *Crepidiastrum* x *muratagenii*, a putative hybrid between *C. denticulatum* and *C. lanceolatum* (Asteraceae). The marker sizes of all examined accessions were found to range from 528 to 537 bp, whereas C. denticulatum had only 528 to 530 bp long sequences due to an 8 bp deletion and a 1 bp insertion. Such indels also occurred in type I and type III hybrids, while type II hybrids did not show the mutations revealing similarity to the other parent, i.e. *C. lanceolatum*. In combination with another three cpDNA markers, i.e. *trnL*(UAA)-*trnF*(GAA) IGS, rpl16 intron, and rps16 intron, it was shown that the three hybrid types tended to be phylogenetically closer to both *C. denticulatum* and *C. lanceolatum* supporting the morphological characterization analysis (Jang et al., 2022).

The blasting result with the NCBI database proved that the sequences were undoubtedly belonging to trnT(UGU)-trnL(UAA) IGS, that indicated by the high similarity of the sequences to the cpDNA total genome of I. cylindrica (accession number MZ351433.1). The highest similarity was observed in Purworejo (99.77%), followed continuously by Jetis (99.65%), Ponorogo (99.42%), Purwokerto (99.40%) and Yogyakarta (99.31%). Then, sequence alignment of the five samples showed both base substitutions and insertions-deletions (indels) as shown in Figure 2. The polymorphic sites as well as the haplotype diversity (h) and nucleotide diversity (π) are summarized in Table 3. It is shown that three haplotypes of five cogongrass samples in Java Island were obtained resulting in an h value of 0.600.



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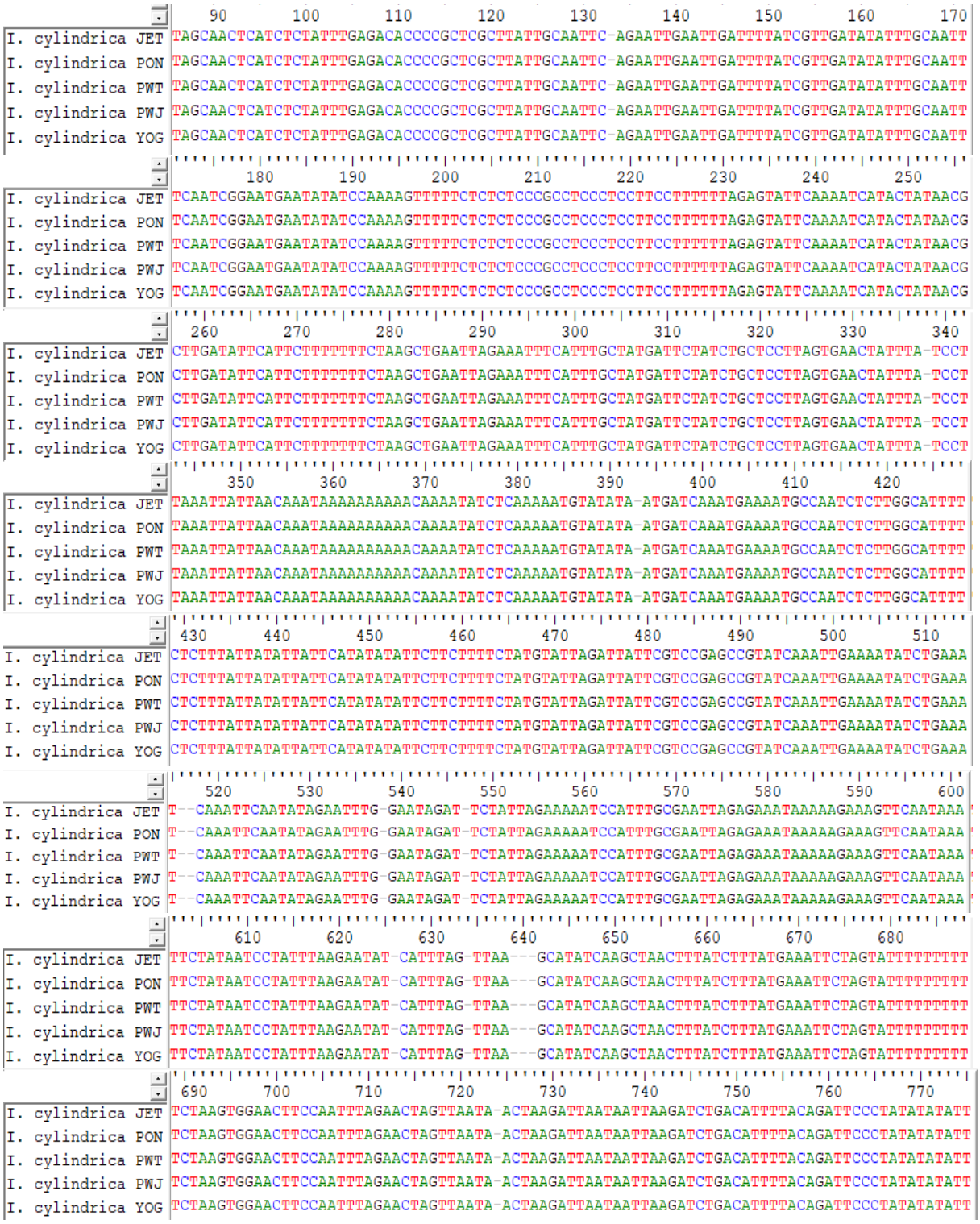

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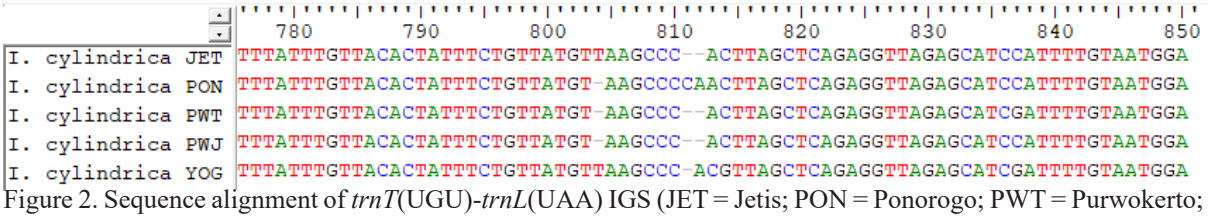

Figure 2. Sequence alignment of *trnT*(UGU)-*trnL*(UAA) IGS (JET = Jetis; PON = Ponorogo; PWT = Purwokerto; PWJ = Purworejo; YOG = Yogyakarta)

Table 3. Genetic variation of cogongrass in Java Island based on trnT(UGU)-trnL(UAA) IGS

Parameter of Variation	Number/Percentage of The Parameter	Site (s)
Number of conserved sites	844 (99.3%)	1-803, 805-810, 815-836, 838-850
Number of polymorphic sites	6 (0.7%)	804, 811, 812, 813, 814, 837
Insertion-deletions (indels)	3	804, 811, 812
Singleton two variants	2	813, 814
Parsimony informative sites two variants	1	837
Number of haplotypes	3	
Haplotype diversity (*h*)	0.600	
Nucleotide diversity (π)	0.00169	

Based on *trnT*(UGU)-*trnL*(UAA) IGS, we obtained three haplotypes of the five cogongrass samples in Java Island giving rise to a haplotype (h) or gene diversity of 0.600. As a comparison, the assessment using another molecular marker, i.e. amplified fragment length polymorphisms (AFLPs), detected two genetic lineages of cogongrass in the southern United States (US) resulting in gene diversity of only 0.023. One lineage was found locally restricted, while the other was widely spread over the study region. It was strongly assumed that both genetic diversity and population structure of the invasive species were influenced by differences in the historical control management applications (Lucardi et al., 2020). Meanwhile, employing digital genotyping analysis, four clonal lineages of cogongrass in the south-eastern US were identified, each of which was extremely homogeneous. Although geographical overlap existed, no hybridization among different lineages could be likely expected indicating that cogongrass has restricted evolutionary potential to adapt to novel environments. No local adaptation by means of hybridization or selection for favorable alleles is required to support cogongrass success of invasion over enormous variations of environments (Burrell et al., 2015).

To demonstrate the genetic relationship among the five sequences of *trnT*(UGU)-trnL(UAA) IGS, a phylogenetic tree was constructed involving *Cynodon dactylon* (Poaceae) as the outgroup (Figure 3). Based on the phylogenetic tree, the five samples of cogongrass showed a very close genetic relationship, confirming the considerably low value of π (0.00169). More specifically, the samples from Purwokerto and Yogyakarta were closer than those of the remaining three.

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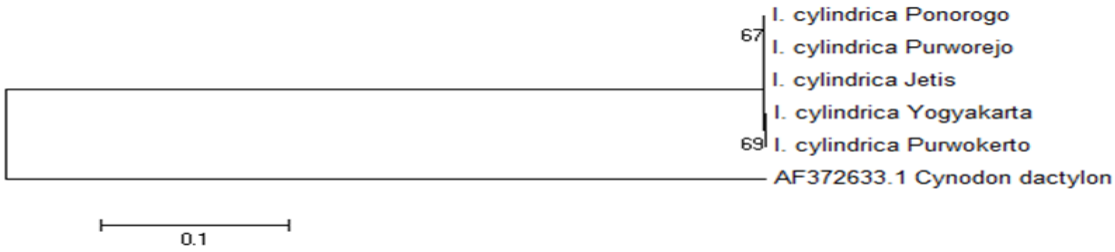

Figure 3. Phylogenetic tree of cogongrass [*Imperata cylindrica* (L.) Raeusch] in Java Island based on *trnT*(UGU)-*trnL*(UAA) IGS

Data on stomata number revealed significant differences among the five samples of cogongrass, where the sample from Jetis Beach had the smallest number of stomata, which was significantly different from those of Purwokerto, Yogyakarta, and Ponorogo (Table 4). Similarly, a significant difference with respect to stomata length was observed, in which the sample from Jetis Beach showed the shortest stomata in comparison to those of the other four (Table 5). On the other hand, no significant difference regarding stomata indices was found, although cogongrass sample from Ponorogo tended to have the densest stomata (Table 6). This means that both stomata's number and length were corresponding to the high level of genetic diversity in terms of h value, while it was not the case with the stomata index.

Table 4. Stomata number of Cogongrass in Java Island

Sample Orgin	Stomata Number
Purwokerto	8.00 ± 0.12 b
Jetis Beach	4.33 ± 0.57 a
Purworejo	7.00 ± 0.33 ab
Yogyakarta	11.97 ± 0.15 b
Ponorogo	10.33 ± 0.24 b

Note: Numbers followed by the same letter are not significantly different at LSD of 0.05

Table 5. Stomata length of Cogongrass in Java Island

Sample Orgin	Stomata Length (µm)
Purwokerto	28.12 ± 0.43 b
Jetis Beach	23.39 ± 0.85 a
Purworejo	24.62 ± 1.22 ab
Yogyakarta	24.65 ± 0.78 ab
Ponorogo	25.16 ± 1.03 ab

Note: Numbers followed by the same letter are not significantly different at LSD of 0.05

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Table 6. Stomata indices of cogongrass in Java Island

Sample Orgin	Stomata Index
Purwokerto	0.31 ± 0.04 b
Jetis Beach	0.32 ± 0.03 b
Purworejo	0.32 ± 0.04 b
Yogyakarta	0.32 ± 0.01 b
Ponorogo	0.34 ± 0.01 b

Note: Numbers followed by the same letter are not significantly different at LSD of 0.05

The very close genetic relationship among the five samples of cogongrass in Java Island indicated the presence of an adjacent common ancestor. In other words, no diverse evolutionary origin occurred as shown by the low value of nucleotide diversity (π = 0.00169). In contrast, the diverse evolutionary origin was observed in weedy rice (*Oryza sativa* f. *spontanea* Rosh.), which competed with cultivated rice causing serious problems in terms of productivity. Four to five genetically distinct weedy rice biotypes were reported in California having ancestry derived from *indica*, *aus*, and *japonica* cultivated rice and might also be from wild rice. The rapid spread of weedy rice biotypes has shown the necessity for control management of existing populations and the efforts to prevent the further introduction into the state (De Leon et al., 2019). The complicated lineage of weedy rice has confused the nomenclature so that the most appropriate name should actually be *Oryza* spp. unless the lineage was clearly known (Roma-Burgos et al., 2021).

Relatively low nucleotide diversity values were also obtained among cogongrass populations in Java Island when measured using another two molecular markers, i.e. *atpB-rbcL* IGS (π = 0.0201) and *trnL*(UAA)-*trnF*(GAA) IGS (π = 0.2277). This was related to the absence of diverse evolutionary origins, which might be due to the invasiveness of the species (Susanto & Dwiati, 2022). Invasive species are commonly subjected to a reduction in genetic diversity because of founder effects limiting their adaptability to new habitats. However, recurrent introductions, particularly in clonally reproducing invasive species, may increase genetic diversity. This was the case with Japanese knotweed (*Reynoutria japonica,* Polygonaceae), showing considerable genetic diversity without genetic structure due to local adaption (VanWallendael et al., 2021). Correspondingly, high genetic diversity was found within populations of *Sida rhombifolia* (Malvaceae) in several Indonesian ethnicities attributable to extensive genetic flow. Nevertheless, homogenous genetic characteristics among populations were observed suggesting the absence of local adaptation (Solihah et al., 2021). Regarding control management of invasive species, more developed countries had a tendency to apply more chemical approaches, while less developed countries were inclined to use non-chemical methods (Weidlich et al., 2020).

In contrast to the case with weed species, the level of genetic relationships and genetic differences that were created artificially could provide a better strategy for broadening

genetic diversity in crop species. For example, in *Brassica juncea* (Brassicaceae) several synthetic species were generated from its ancestor, *B. nigra*, by means of allopolyploidy. Some of the synthetic species showed low genetic differences from the natural *B. juncea*, while some others exhibited a distant genetic relationship to the normal *B. juncea* and revealed wide genetic diversity of the species. Hence, the latter offered a greater possibility of successful genetic improvement of *B. juncea* (Liu et al., 2021).

Phenotypical traits regarding the number and length of stomata were found corresponding to the molecular analysis, particularly with respect to haplotype diversity. However, it was not the case with the stomata index. As a comparison, no significant correlation between morphological and molecular marker systems of analysis was obtained in *Asparagus officinalis* (Asparagaceae). No consistency was found in clustering based on the two types of markers (Chen et al., 2020). Similarly, morphological and molecular markers among 64 *Cucurbita pepo* (Cucurbitaceae) were found as non-interchangeable approaches although they could be complementary to result in the comprehensiveness of data analysis (Yunli et al., 2020). Such comprehensive and accurate analysis was obtained when RAPD markers were used in a combination with some micromorphological markers to demonstrate the genetic relationship among several taxa of Asteraceae (Abdel-Hamid, 2020).

CONCLUSION

Our study concludes that cogongrass in Java Island shows a very high genetic variation pertaining to haplotype diversity when assessed using *trnT*(UGU)-*trnL*(UAA) IGS. This is appropriate with anatomical data on stomata's number and length but not with stomata's index. On the other hand, the considerably low value of nucleotide diversity indicates the absence of diverse evolutionary origin. To obtain a more comprehensive genetic profile of cogongrass in Java Island, more molecular markers should be involved.

AUTHOR CONTRIBUTION

M.D. proposed and took the responsibility of the entire project. S.A.R. collected and analyzed both molecular and morphological data. A.H.S. assisted data analysis and wrote the manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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