INTRODUCTION

National exploration of ethnomedicine by looking at local knowledge and medicinal plants in Indonesia was carried out in 2012, observing 209 ethnic groups in 26 provinces (project known as Ristoja). The objective of Ristoja was to provide a database of ethnomedicine comprising local knowledge of herbal formulas and medicinal plants in Indonesia. Ristoja identified 15,733 used herbal formulas and 19,738 items of medicinal plant information in these herbal formulas are used across 1,740 species. Herbal formulas were found which aimed to treat diseases covered by Ministry of Health programmes, including HIV-AIDS, tuberculosis, malaria, maternal and child health and cancer (Wahyono, 2013).

Kaempferia galanga L., known in Indonesia as kencur, is a medicinal plant used empirically by 109 ethnic groups and ranked as the 16th most used medicinal plant in traditional herbal formulas. The plant’s rhizomes and leaves are used to treat common colds, coughs, wounds, headaches, ulcers, breast cancer, asthma and as an after-childbirth treatment (Wahyono, 2013).

Kaempferia galanga L. is a species in the family of Zingiberaceae, is perennial herbaceous plant and widely used as a medicinal plant, as a spice and in perfumery. K. galanga is possibly native to India, and distributed and widely cultivated throughout Southeast Asia, including southern China, Malaysia, Indonesia, and also introduced into northern Australia (Ibrahim, 1999). The plant is traditionally used for pharmacological treatments, since it has anti-inflammatory, analgesic (Umar, Zaini Bin Asmawi, Sadikun, Altaf, & Iqbal, 2011), antioxidant and antimicrobial properties (Rao V & Kaladhar, 2014).

Rhizomes of K. galanga have been reported to have volatile oils with 50 constituents (97.19% of the oil), including ethyl cinnamate, ethyl-p-methoxycinnamate, γ-cadinene, 1,8-cineole, δ-carene, borneol, ethyl-m-methoxycinnamate, camphene, linoleoyl chloride and α-pinene (Kumar, 2014).

Currently, K. galanga has been reported to be an endangered species, even though it is valuable as a medicinal and aromatic plant (Preetha, Hemanthakumar, & Krishnan, 2016). In its natural habitat the plant exhibits poor natural rhizomatous growth and reproduction, making it susceptible to habitat loss and overharvesting. The plant is also threatened by the introduction of exotic species and the expansion of agricultural land. Therefore, conservation efforts are necessary to protect this important medicinal plant.

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* Corresponding author:
E-mail: dyah.subositi@gmail.com
propagation (Shirin, Kumar, & Mishra, 2000); in addition, deforestation and over-exploitation have further reduced the plant population. Diversity assessment is required to enable genetic resources to be exploited for plant improvement (Pandotra, Gupta, Husain, Gandhiram, & Gupta, 2013). Genetic diversity can be analysed based on agronomic, biochemical, physiological, morphological and molecular markers (Moulin, Rodrigues, Gonçalves, Sudré, & Pereira, 2012). The weakness of morphological and biochemical characteristics in this evaluation is that they are influenced by environmental factors. Molecular markers are independent of environmental effects, fairly stable and show a high level of polymorphism (Prashanth, Yugander, & Bhavani, 2015). Selection of effective molecular markers is necessary for studying genetic diversity among plant accessions.

ISSR has several advantages as a tool for assessing plant genetic diversity in comparison to other molecular marker techniques such as Random Amplified Polymorphic DNA (RAPD). ISSR is a powerful tool for use as a fingerprinting technique because of its greater reliability and reproducibility of DNA fragments, and its ability to reveal more polymorphic bands. In addition, the primer is longer than RAPD primer and anneals at higher temperatures, also prior sequence information is not required (Prashanth, Yugander, & Bhavani, 2015; Zheng et al., 2015). Furthermore, ISSR has been used successfully for studying interspecific and intraspecific genetic diversity of the Zingiberaceae family, including Curcuma alismatifolia (Taheri, Abdullah, Abdullah, & Ahmad, 2012), Kaempferia galanga (Devi et al., 2015), Zingiber officinale (Ghosh, Shylaja, & Nazeem, 2015), Alpinia galanga (Rajasekharan, Kareem, Ravish, & Mini, 2016), Curcuma sp. (Saha, Sinha, Basak, & Sinha, 2016) and Elettaria cardamomum (Anjali, Ganga, Nadiya, Shefeek, & Sabu, 2016), Zingiber sp. (Bidyaleima, Kishor, & Sharma, 2019) and Elingera elatior (Ismail, Rafii, Mahmud, Hanafi, & Miah, 2019). Genetic diversity information for K. galanga is needed to provide a medicinal plant database, particularly for further conservation. The aim of this research was to study the genetic diversity of K. galanga, which is used as a medicinal plant by selected ethnic groups in Indonesia, based on ISSR markers.

MATERIALS AND METHODS

The sampling locations were distributed across Sumatera, Sulawesi, Maluku, Nusa Tenggara and West Papua (Fig. 1). Sample collection was carried out in September and October 2014. This study was a continuation of the Ristoja project, collecting plant material to assess genetic diversity of K. galanga. Areas inhabited by 24 ethnic groups were selected as locations for sampling K. galanga, but the plant was found in only 12 ethnic groups.

Plant Materials

Sample selection was based on the K. galanga that was used by traditional healers in herbal formulas (Table 1). Fresh young leaves were harvested directly from the plant, preserved using silica gel and wrapped in paper bags (as used for DNA samples).

Table 1. Sample of K. galanga from 12 locations in Indonesia

| No | Local names | Ethnic group | Region/province |
|----|-------------|--------------|-----------------|
| 1. | Kencur      | Klute         | Aceh            |
| 2. | Kopuk       | Mentawai     | West Sumatera   |
| 3. | Cakua       | Minangkabau   | West Sumatera   |
| 4. | Kencur      | Sakai         | Riau            |
| 5. | Cekua       | Rejang        | Bengkulu        |
| 6. | Kencur      | Muko-muko     | Bengkulu        |
| 7. | Cekur       | Musi          | South Sumatera  |
| 8. | Tukuyo      | Buol          | Central Sulawesi|
| 9. | Gumopot     | Kaidipang     | North Sulawesi  |
| 10. | Kencur     | Alune         | Maluku          |
| 11. | Omomongere  | Togutil       | North Maluku    |
| 12. | Kencur     | Sentani       | Papua           |

DNA Extraction

The leaf samples for each accession from the field collections were scaled into 0.1 g samples and stored in a deep freezer (at -80°C) until it was used. Total genomic DNA was extracted from these samples using DNA kit isolation (Sigma GenEluteTM Plant Genomic DNA Miniprep Kit, Catalog Number G2N70). The procedure for DNA genome isolation was following the kit manual. The quality and quantity of extracted genomic DNA were determined using a spectrophotometry method using a UV-Vis spectrophotometer (UV-Vis spectrophotometer from Shimadzu, Japan) with absorbance at 260/280 nm and were also checked on electrophoresis 0.8% agarose gels at 100 V for 30 minutes (Bio-Rad, USA).
Table 2. Total number of DNA fragments and polymorphism percentage of 12 *Kaempferia galanga* accessions using 10 ISSR primers

| No | Primer sequences | Total fragments | Monomorphic fragments | Polymorphism percentage (%) | Fragment size (bp) |
|----|------------------|-----------------|------------------------|-----------------------------|-------------------|
| 1. | (AG)8T           | 11              | 0                      | 100                         | 215-2,540         |
| 2. | (ACTG)5          | 10              | 0                      | 100                         | 310-2,265         |
| 3. | (CAG)5           | 9               | 2                      | 77.8                        | 315-2,050         |
| 4. | (CA)6GT          | 10              | 2                      | 80                          | 270-1,710         |
| 5. | (AG)9C           | 9               | 1                      | 88.9                        | 210-1,130         |
| 6. | (GA)8CTT         | 16              | 0                      | 100                         | 225-1,775         |
| 7. | (AC)9G           | 11              | 0                      | 100                         | 290-1,635         |
| 8. | (TG)8C           | 9               | 0                      | 100                         | 420-1,790         |
| 9. | (AG)8YA          | 11              | 1                      | 90.90                       | 220-1,500         |
| 10.| (TC)8RG          | 10              | 0                      | 100                         | 205-2,130         |
| Total|                 | 104             | 6                      |                             |                   |
| Average |             |                 |                        |                             | 94.23             |

Fig. 1. DNA fragments from amplification of *K. galanga* using ISSR primer (a). (GA)8CTT, (b) (CA)6GT, (c) (CAG)5, (d) (TC)8RG. (L) Ladder 100 bp (1) Klue (Aceh), (2) Mentawai (West Sumatera), (3) Minangkabau (West Sumatera), (4) Sakai (Riau), (5) Rejang (Bengkulu), (6) Muko-muko (Bengkulu), (7) Musi (South Sumatera), (8) Buol (Central Sulawesi) (9) Kaidipang (North Sulawesi), (10) Alune (Maluku), (11) Togutil (North Maluku), (12) Sentani (Papua)
ISSR Amplification

Ten out of 25 ISSR primers for the first screening process had a high percentage of polymorphism and these were used for polymerase chain reaction (PCR) amplification (Table 2). The PCR reaction solution contained 25 ng genomic DNA (2 μl template), 1 μl primer, 12.6 μl PCR Mix (Go Taq Green Promega). The distilled water was added into these mixture until the volume reached 25 μl. The ISSR PCR reaction was conducted following the modified protocol of Heikal, Badawy, & Hafez (2008) with time and temperature modification. DNA amplification was performed using a thermal cycler (C-1000 Bio-Rad, USA) with a cycle program consisting of 95°C for 3 minutes (pre-denaturation), followed by 39 cycles of denaturation at 95°C for 1 minute, annealing at 46–52°C for 50 seconds and 72°C for 2 minutes (elongation), and at 72°C for 8 minutes (extension) as final cycle and 4°C as the holding temperature.

The amplified products were depicted using 1.8% gel agarose stained using SYBR Safe Green (Invitrogen) for electrophoresis in 1 x TBE buffer at constant 60 Volt or 80–90 minutes. Visualisation of electrophoresis gel using UV light was carried out and documented using a gel documentation system (Imaging System XR + Bio-Rad, USA).

Data Analysis

DNA fragments of the PCR product were scored for presence (1) or absence (0) of each primer used and in all accessions. Similarity indexes of all accessions were calculated based on Dice similarity (Nei & Li, 1979). The dendrogram was constructed using Unweighted Pair Group Method Using Arithmetic Mean (UPGMA) cluster analysis. This data analysis was performed by NTSYS software ver. 2.0 (Rohlf, 1998).

RESULTS AND DISCUSSION

Ten ISSR primers generated 104 DNA fragments from 12 accessions of K. galanga. The average number of fragments for each primer was 10.4. DNA fragments with size ranged from 205–2,540 bp (Table 1). These results show that the ISSR primer generated total fragments and a polymorphism percentage greater than the previous study by Devi et al. (2015), in which the genetic diversity of K. galanga from India was assessed. In that study, 11 ISSR primers produced only 72 reproducible fragments ranging from 200–1,000 bp in size, with a polymorphism average of 81.94% across eight K. galanga cultivars.

The largest percentage of polymorphic fragments (100%) was produced using primers (AG)8T, (ACTG)5, (GA)8CTT, (AC)9G, (TG)8C and (TC)8RG, and the smallest percentage (77.8%) was obtained by (CAG)5. Pandotra, Gupta, Husain, Gandhiram, & Gupta (2013) reported ISSR primers using tri-nucleotides generated lower polymorphism than ISSR primers with di-nucleotides in Zingiber officinale cultivars from the northwest Himalayan region. Furthermore, tetranucleotide ISSR primer was found more polymorphic than trinucleotide primer. The similar results were found in rice varieties genetic variability study using ISSR markers, suggesting for microsatellite marker development in rice varieties better using certain tetranucleotide motifs than trinucleotide (Blair, Panaud, & McCouch, 1999). The observed high percentages of polymorphic fragments generated using the different ISSR primers indicates genetic diversity among K. galanga accessions. According to Herison, Sutjahjo, Sulastrini, Rustikawati, & Marwiyah (2018), the level of polymorphism of DNA could be linear to the level of molecular variation among accessions, depending on accession tested and molecular markers used. Differences in DNA fragment patterns are amplified using ISSR primers through deletion/insertion or loss of primary binding sites, resulting in polymorphic fragments (Theanphong, Jenjittikul, Mingvanish, & Rungsishirunrat, 2018).

Hao et al. (2006) reported that genetic diversity could be shown by the degree of polymorphism of fragments, with loss of genetic diversity of a species indicating low adaptation to climate change. However it is not clearly stated that the level of polymorphism indicates a low adaptation, other factors need to be considered (Chaudhary et al., 2012). Amplification using several ISSR primers showed presence or absence of specific fragments in some accessions (Fig. 1. b–d). The specific DNA fragment for accessions may be applied for accession discrimination or authentication in certain accessions of K. galanga. Jianming et al. (2006) reported that Populus accessions possible to identified using unique or specific DNA fragments produced from each primer.

The similarity index (SI) of K. galanga accessions was used to develop dendrograms, and shows a clear separation of all accessions. The
dendrogram divided the 12 *K. galanga* accessions into four major clusters of SI 61.12%. Index similarity among accessions ranged from 49.6–93.3%, indicating high genetic diversity. Devi et al. (2015) reported that the genetic similarity of eight *K. galanga* cultivars from India ranged from 35.9–94.9%.

Cluster I consisted of *K. galanga* from different geographical locations – Sumatera, Sulawesi, and Maluku Island – and showed them to be closely related (Fig. 2). It can be inferred that the parental lines or ancestor in *K. galanga* accessions from different origins may share a similar genetic background (Devi et al., 2015). Accessions from Musi and Rejang did not group in cluster I, which consists of *K. galanga* accessions from a location in the same island (Sumatera: Muko-muko, Mentawai, Sakai, Kluet and Minangkabau). According to Pharmawati & Candra (2015), a *Pogostemon cablin* sample from a closer location was not grouped in the same cluster. Singh, Panda, & Nayak (2012) reported that *Curcuma longa* accessions collected from ten different agroclimates in India based on RAPD and ISSR molecular markers showed no relationship between dendrogram grouping patterns with accession collection locations. Similar results were found in 57 accessions of *Etlingera elatior* from seven states in Malaysia, there was no correlation between molecular grouping based on ISSR and their geographical origin (Ismail, Rafii, Mahmud, Hanafi, & Miah, 2019).

At Cluster II – IV, each cluster had one accession of *K. galanga*. Cluster II and III consisted of *K. galanga* from Sumatera (Rejang and Musi), with *K. galanga* from Papua in cluster IV. A single cluster that consists of one *K. galanga* accession could indicate the existence of wide genetic variability. A single cluster separated from the main cluster was also found in the *Curcuma longa* accession in India based on RAPD molecular marker, indicating that there was a high gene diversity and a strong genetic structure between accessions of *C. longa* (Ashraf, Ahmad, Adnan Ali Shah, & Mujeeb, 2017).

The Kaidipang and Buol accessions were the most similar, with SI of 93.33%. These accessions are from the Northern Sulawesi region. High SI among accessions is slightly correlated to their close geographic locations (Shafie, Zain Hasan, Zain, & Shah, 2011). Plants exhibiting clonal propagation usually have low genetic variation. Low genetic diversity results in a decreased ability to adapt to the environment, as well as decreasing product quality and yield. Zheng et al. (2015) reported that people often select robust rhizomes of *Curcuma wenyujin* to generate new generations with identical genetic makeup, and these are probably more likely to accumulate deleterious mutations that reduce genotype diversity.

![Dendrogram of 12 K. galanga accessions based on ISSR molecular markers](image)

**Fig. 2.** Dendrogram of 12 *K. galanga* accessions based on ISSR molecular markers
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CONCLUSION

ISSR molecular markers were used for grouping and assessing genetic diversity of Kaempferia galanga accessions collected from 12 locations. The genetic Similarity Index of K. galanga ranged from 49.6–93.3%, indicating a high level of genetic diversity among accessions. In general, high genetic diversity in K. galanga was due to the various environmental conditions found in its wide distribution area in Indonesia. Analysis of more accessions from different locations and using another molecular marker would provide complete information of genetic diversity of K. galanga accessions in Indonesia.
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