CURRICULUM VITAE

Full Legal Name	:	Elga Renjana
Nationality	:	Indonesia
Age	:	33 years old
Email	:	elgarenjana@gmail.com
Mobile Number	:	+62 857 3040 7678
Job Position	:	Researcher
Organization	:	Research Center for Plant Conservation, Botanic Gardens and Forestry – National Research and Innovation Agency

Short Background :

I earned my M.Sc. degree from Biology Department, Airlangga University in 2017. My research interests are conservation of plants (Pteridophyte in particular) and seeds. I am also interested in modelling for plant conservation and predicting the potential of medicinal plants using docking analysis. To complete my task as plant conservation scientist, I also actively explore Indonesian forest. In addition of research activities, I am also active as an editor of Jurnal Penelitian Kehutanan Wallacea and the member of the Indonesian Researcher Union (PPI).

Relevant Skills and Experience :

I have experience in conducting plant exploration and ecological research in the forest areas of Alas Purwo National Park (East Java), Bromo Tengger Semeru National Park (East Java), and North Barito Forest (Central Kalimantan). I have basic skills in identifying plants (Pteridophyte in particular), making herbarium specimens, and collecting plants for ex-situ conservation. I also have basic skills in modelling programs to make predicted map for supporting plant conservation status.

Scientific Publications :

Assessing potential habitat suitability of parasitic plant: A case study of Rafflesia arnoldii and its host plants. (2022). Global Ecology and Conservation 34, e02063.

Morphological Characterization and Seed Germination Study of Wild Banana Musa acuminata var. flava (Ridl.) Nasution. (2022). Journal of Tropical Biodiversity and Biotechnology 7 (1), 66645.

In Silico Study of the Active Compounds of Lindera aggregata (Sims) Kosterm as Anti-coronavirus. (2021). Current Nutrition & Food Science 17 (4), 408-416.

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Assessing potential habitat suitability of parasitic plant: A case study of *Rafflesia arnoldii* and its host plants

Elga Renjana [a,*], Inggit Puji Astuti [a], Esti Munawaroh [a], Sofi Mursidawati [a], Joko Ridho Witono [a], Yuzammi [a], Izu Andry Fijridiyanto [a], Puguh Dwi Raharjo [b], Saniyatun Mar'atus Solihah [d], Iyan Robiansyah [a], Wendell P. Cropper Jr. [c], Angga Yudaputra [a]

[a] *Research Center for Plant Conservation and Botanic Gardens, National Research and Innovation Agency, Jalan Ir. H. Juanda 13, Bogor 16122, West Java, Indonesia*
[b] *Research Center for Geotechnology, National Research and Innovation Agency, Jalan Sangkuriang, Bandung 40135, West Java, Indonesia*
[c] *School of Forest, Fisheries and Geomatics Sciences, University of Florida, Gainesville, FL 32611-0410, USA*
[d] *Bogor Botanic Garden, Mitra Natura Raya, Jalan Ir. H. Juanda 13, Bogor 16122, West Java, Indonesia*

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ABSTRACT

Rafflesia are obligate endo-holoparasitic plants with the genus *Tetrastigma* playing an important role as their host plants. *Rafflesia arnoldii* is one of Indonesian endemic plants that grows in Sumatra island. This island is also known to have eleven species of *Tetrastigma*. Three of them are known as host plants of *R. arnoldii*, namely *Tetrastigma curtisii, T. pedunculare* and *T. leucostaphylum*. Unfortunately, it is not possible to survey the entire native distribution range of *R. arnoldii* and its host plants due to practical constraints. Species Distribution Modeling using Maximum Entropy (MaxEnt) is considered to be an alternative way to understand the potential regions that are suitable for a species. Predicting habitat suitability of a parasitic plant through predicting its host plant distribution may be a useful approach. The model prediction from MaxEnt for all host species has an AUC value more than 0.70, indicating the model adequately classified the occurrence records of *R. arnoldii* and its host plants. Suitable habitats for *R. arnoldii* were predicted to occur along the Bukit Barisan Mountain range from Lampung to Aceh, mostly in Lampung, Bengkulu, West Sumatra and Aceh. However, the suitable habitats of *R. arnoldii* estimated from host plants were predicted to occur predominately in Lampung, Bengkulu, West Sumatra, North Sumatra and Aceh. The important environmental variables affecting the occurrence of *R. arnoldii* and its host plants are mean temperature, slope, elevation, soil organic carbon and soil type. *Rafflesia* species can be found in various soil types, but mostly in Humic Andosols, Humic Acrisols, Orthic Acrosols, Dystric Fluvisols, Dystric Cambisols and Eutric Fluvisols based on the actual data points and predicted habitats. The suitable habitats of *R. arnoldii* based on its host plants were predicted to be mostly outside conservation areas, but suitable habitats were predicted inside approximately 46 conservation areas. The findings of this study may be used by the Government of Republic Indonesia, c.q. Ministry of Environment and Foresty for establishing

* Corresponding author.
E-mail addresses: elga.renjana@brin.go.id, prkt-iph@brin.go.id (E. Renjana), ingg001@brin.go.id (I.P. Astuti), esti001@brin.go.id (E. Munawaroh), sofi001@brin.go.id (S. Mursidawati), joko017@brin.go.id (J.R. Witono), yuza001@brin.go.id (Yuzammi), izua002@brin.go.id (I.A. Fijridiyanto), pugu003@brin.go.id (P.D. Raharjo), sani_sms@rocketmail.com (S.M. Solihah), iyan.robiansyah@brin.go.id (I. Robiansyah), wcropper@ufl.edu (W.P. Cropper), angg022@brin.go.id (A. Yudaputra).

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protected areas and conservation-based management improvement and could also help inform the *R. arnoldii* listing status in IUCN red list category in the future.

1. Introduction

Parasites exist in all types of life forms including plants (Combes, 2001). Parasitic plants obtain water and nutrients through the haustorium, which is attached to shoots or roots of their host plant. These parasitic plants consist of the plants that are able to photosynthesize (hemiparasites) and are unable to photosynthesize (holoparasites) (Kuijt, 1969). One of the plant taxa which its members are holoparasitic plants is the Rafflesiaceae family. The vegetative parts and chlorophyll are absent in this family. Rafflesiaceae grows only in the stem and root tissue of the genus *Tetrastigma* (Miq.) Planch. as their specific host. Its habitat is in tropical rainforests of Southeast Asia. Based on molecular data, this family includes three genera, namely *Rafflesia* R.Br. ex Gray, *Rhizanthes* Dumort. and *Sapria* Griff. (Bendiksby et al., 2010). Among these genera, *Rafflesia* has a species *Rafflesia arnoldii* R. Br. that produces one of the biggest flowers in the plant kingdom. Since its discovery by Joseph Arnold in 1818, surveys have greatly increased the number of known occurrence sites. In Sumatra, the species is widely distributed in Bukit Barisan Mountain range from Southeast Lampung to Aceh (Meijer, 1997; Zuhud et al., 1998), especially in five Provinces of Sumatra, namely Aceh, West Sumatra, Riau, Bengkulu and Lampung.

Rafflesia arnoldii is one of Indonesian endemic plants which grows in Sumatra island (Asiandu, 2021). This species is an obligate endo-holoparasitc plant, and host specific. Its total dependency on a host plant for growth, water and nutrition has made distribution range of this species limited to that of its host plant *Tetrastigma* (Hidayati and Walck, 2016; Susatya, 2011). At least, three species of *Tetrastigma* have been identified as host plants of *R. arnoldii*, namely *T. leucostaphylum* (Dennst.) Alston ex Mabb., *T. curtisii* (Ridl.) Suess and *T. pedunculare* (Wallich ex. Lawson) Planch (Meijer, 1997; Pranata et al., 2020; Susatya, 2011; Zuhud et al., 1998). The disappearance of those host plants will inevitably lead to the extinction of *Rafflesia*. *Tetrastigma*, with a climbing habit, also requires other plants, usually large trees, for support and access to sunlight. There are 95 validly named *Tetrastigma* species in Malesia region (Chen et al., 2011; Wen, 2007). Study of Sumatran *Tetrastigma* revealed that 11 species were recorded (Rahayu, 2017). Eight out of 11 species known as host of *Rafflesia* (*T. diepenhorstii, T. tuberculatum, T. leucostaphylum, T. rafflesiae, T. dubium, T. glabratum, T. pedunculare* and *T curtisii*). In this case, *T. pedunculare* become the host for seven *Rafflesia* species, namely *R. arnoldii, R. hasseltii, R. keithii, R. kerrii, R. patma, R. pricei* and *R. zollingeriana* (Susatya, 2011).

Previous studies of *R. arnoldii* ecology suggested that these obligate endo-holoparasitic plants are far more susceptible to extinction compared to non-parasitic plants in the same habitat. Both the host and parasite species are affected by environmental fluctuations and disturbances in the ecosystem. Thus, habitat is a critical factor affecting their survival (Ellen et al., 2019; Pranata et al., 2019; Ramadhani et al., 2017; Susatya, 2020). Even though the *Tetrastigma* population is widespread in a habitat and known to be the host, *Rafflesia* does not necessarily grow. The determining factor of *Rafflesia*-host is physiological compatibility so it is difficult to measure which is complex or not caused by single factor. Data of climbing plant is rarely covered because the leaves and flowers grow above the canopy. When it happens to be dangling, the identification is more feasible.

Deforestation in Sumatra has been rapid and potentially represents a serious risk to native species. Eyes on the Forest (EoF, 2018) reported that Sumatra had 25 million hectares of natural forest in 1985; after over 31 years of deforestation only 11 million remained in 2016. With an average annual forest loss rate of 0.46 million hectares per year it is likely a significant threat to the future of *R. arnoldii*. It has not been listed in the International Union for Conservation of Nature (IUCN) Red List Categories as more comprehensive studies are still required. Time and financial constraints have prevented a comprehensive population survey in Sumatra. To date, a more practical method to assess population status and risks in large areas is available through habitat suitability model prediction. This method supports continuing inventory research through identifying possible new subpopulations in unsurveyed regions of Sumatra. In order to understand the prediction of habitat suitability, Species Distribution Models (SDM) are commonly used by combining species occurrence data and environmental variables (Anderson and Martínez-Meyer, 2004; Elith and Leathwick, 2009; Franklin and Miller, 2009; Raxworthy et al., 2003; Thorn et al., 2009). Among many species distribution model methods, Maximum Entropy (MaxEnt) is powerful and outperformed other tools when predicting rare species with narrow habitat ranges (Elith et al., 2006; Pearson et al., 2007; Phillips et al., 2006; Wisz et al., 2008).

This study aims to predict the most suitable habitats of *R. arnoldii* by considering the potential habitats of its host plants and identify the protected areas in Sumatra that have high suitability for *R. arnoldii*. The findings of this study may be used by the Government of Republic Indonesia for establishing protected areas and conservation-based for *R. arnoldii* and would also provide information relevant to the listing status in the IUCN red list category.

2. Materials and methods

2.1. Research sites

The occurrence records of *R. arnoldii* and three host plant species, namely *T. curtisii*, *T. pedunculare* and *T. leucostaphylum* were obtained from direct field surveys, plant inventory records, plant expedition reports, the GBIF database and reliable scientific journals. There were 28 occurrence records of *R. arnoldii* (Ellen et al., 2019; GBIF.org, 2022a; Latifah et al., 2012; Meijer, 1997; Munawaroh et al., 2013; Pranata et al., 2019, 2020; Ramadhani et al., 2017; Susatya, 2020; Zuhud et al., 1998), 3 occurrence records of *T. curtisii*

(GBIF.org, 2022b), 24 occurrence records of *T. pedunculare* (GBIF.org, 2022c; Rahayu, 2017; Susatya et al., 2017), and 19 occurrence records of *T. leucostaphylum* (GBIF.org, 2022d; Pranata et al., 2019; Rahayu, 2017; Rahma et al., 2017; Ramadhani et al., 2017; Setyowati and Wardah, 2007). Those occurrence points were recorded on the western region of Sumatra from Lampung to Aceh along the Bukit Barisan Mountain range (Fig. 1).

2.2. Study species

Morphological characters of *R. arnoldii*, and three host plant species are as follows: *Rafflesia arnoldii* R. Br has unisexual and sessile flowers, developing outside the host. Flower buds are protruding as a corky swelling with hexagonal patches, up to 34.7 cm in diam., dark brown or sometimes white or pale brown at the top when immature and pale to strong sorrel when mature. The bracts are brown to dark brown and are usually released prior to blooming. When the flowers bloom, they emit a strong odor, similar to that of a rotting carcass, attracting pollinators. The flower is up to 81 cm in diameter, with a tube up to 15.5 cm high, perigone consisting of 5 lobes, 22–29 cm long and 23.3–33 cm wide.

Tetrasigma, the host plant of *Rafflesia*, is widely dispersed in tropical and subtropical Asia through Australia (Chen et al., 2011; Rahayu et al., 2018). Amongst Indonesian archipelago, Sumatra is recognized as major dispersal of *Tetrastigma* which 11 species found, i.e. *Tetrastigma curtisii* (Ridl.) Suess., *T. dichotomum* (Blume) Planch., *T. diepenhorstii* (Miq.) Latiff, *T. dubium* (Lawson) Planch., *T. hookeri* (Lawson) Planch., *T. leucostaphylum* (Dennst.) Alston ex Mabb., *T. papillosum* (Blume) Planch., *T. pedunculare* (Wall. ex Lawson) Planch., *T. pisicarpum* (Miq.) Planch., *T. pyriforme* Gagnep. and *T. rafflesiae* (Miq.) Planch (Rahayu, 2017; Rahayu et al., 2018). Three species are commonly host of *R. arnoldii*, i.e. *T. curtisii, T. leucostapylum, T. pedunculare.* Taxonomical confusion occurred in view of (Veldkamp, 2008) where he proposed *T. rafflesiae* is a correct name instead of *T. leucostapylum* (stated the latter misapplied in Sumatera). In the following year, he sank *T. rafflesiae* together with *T. lanceolarium* (Roxb.) Planch. and *T. leucostaphylum* as synonym of *T. coriaceum* (DC.) Gagnep. (Veldkamp, 2009). However, this dispute has now been resolved after research conducted by (Rahayu et al., 2018) which designated *T. leucostapylum* and *T. rafflesiae* as two different species and sank *T. lanceolarium* as synonym of *T. leucostaphylum*. The following Table 1 showed morphological diagnosis characters of three *Tetrastigma* species (*Tetrasigma curtisii, T. leucostaphylum* and *T. pedunculare*).

This current study is focussed on *R. arnoldii* and its host plants, since distribution and habitat data of those species is available from herbarium records, scientific publications and our experience during field works. It will be a starting point to do a further study for

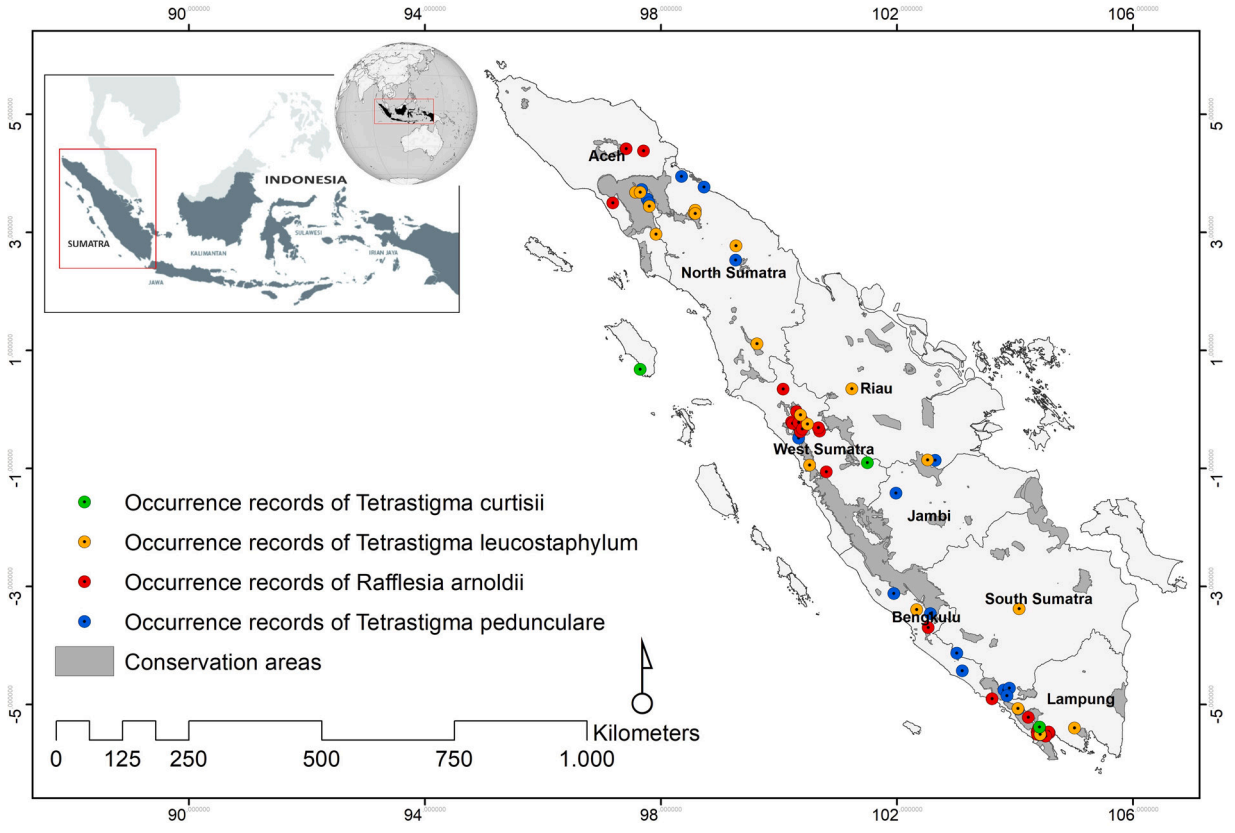


Fig. 1. The occurrence records of *Rafflesia arnoldii*, and three host plant species, namely *Tetrastigma curtisii*, *T. leucostaphylum* and *T. pedunculare* in Sumatra.

Table 1
Morphological diagnosis characters of *T. curtisii, T. leucostaphylum* and *T. pedunculare*.

Characters	*T. curtisii*	*T. leucostaphylum*	*T. pedunculare*
Tendrils	forked	stout	two branched
Stem	cylindrical	rough, grooved	rounded
Petiole length (cm)	3.4–8.6	3–11.6	7–15
Leaflets	3-(5)	3-(4)-5–7	(2)-3-(4)
Leaf blade	coriaceous, glabrous	sub-coriaceous, glabrous	coriaceous, glabrous above, densely covered with glaucous fine indumentum beneath
Terminal leaflet	broadly obovate to elliptic	lanceolate to oblong	broadly elliptical to obovate
Terminal leaf base	acute	acute	acute
Lateral leaflet	oblong	lanceolate to oblong, oblique	asymetrically oblong
Lateral leaflet base	obtuse	acute to rounded	oblique
Margin	serrate	serrate	serrate
Venations	easily recognized by its diffuse venation between the secondary veins, notably conspicuous adaxially	inconspicuous/smooth	cone shape adaxially and nearly rectangular abaxially

Sources: (Arshad et al., 2020; Latiff, 1984; Rahayu et al., 2018; Yeo et al., 2012) and direct observation.

other species of *Rafflesia*, not only in Sumatra but throughout the world.

2.3. Species distribution modeling (SDMs)

Habitat suitability data for species distribution modeling (SDMs) were obtained from previous research and downloaded from available websites (see below). These data include annual mean temperature, annual precipitation, elevation, soil data and land cover. Annual mean temperature and annual precipitation were extracted from WorldClim version 2.1. These variables are the average for the years 1970–2000 with the spatial resolution 30 s (1 km^2) (Fick and Hijmans, 2017) (https://www.worldclim.org/). The elevation data with resolution 30 s (1 km^2) was derived from the Shuttle Radar Topography Mission (SRTM) Digital Elevation Model Data (Farr et al., 2007; NASA SRTM, 2013). Slope was calculated from elevation data. Soil pH, soil type, soil organic carbon and Cation Exchange Capacity (CEC) were derived from SoilGrids (global gridded soil information). These data were available at a spatial resolution of 250 m (Hengl et al., 2017) (https://soilgrids.org/). Land cover data were derived from Moderate Resolution Imaging Spectroradiometer (MODIS) Land Cover Type (MCD12Q1) Version 6 (ORNL DAAC, 2018) (https://earthexplorer.usgs.gov/). The land cover data were from 2018 and represents our best estimates of the current land cover.

Habitat suitability was modeled using Maxent software version 3.4.4 for *R. arnoldii* and its host plants by implementing a machine learning technique called Maximum Entropy (MaxEnt) (Phillips et al., 2021). We used default setting on MaxEnt java program. However, for model assessment with few localities, a jackknife (or 'leave-one-out') procedure was applied. A model built using the remaining n-1 localities. Hence, for a species with n observed localities, n separate models were built for testing (Pearson et al., 2007). For the MaxEnt setting: random test percentage 1, regularization multiplier 1, number of background points 10^4, replicate run type (cross validation), maximum iteration 500, convergence threshold 10^{-5} and default prevalence value of 0.5.

The predicted suitable habitats of *R. arnoldii* and three species of *Tetrastigma* were intersected between each other to create three intersection areas (*R. arnoldii* and *T. curtisii*), (*R. arnoldii* and *T. pedunculare*) and (*R. arnoldii* and *T. leucostaphylum*). The intersection areas of those species were then overlaid with conservation areas to identify the high suitable habitats inside conservation areas. Four classes of potential habitats were grouped as follows: unsuitable ($\leq$ 0.10), low potential (0.11–0.30), moderate potential (0.31–0.70) and high potential ($\geq$ 0.71) (Choudhury et al., 2016; Qin et al., 2017; Yang et al., 2013).

3. Results

The predicted Maximum Entropy habitat models of *Rafflesia arnoldii* and three host plant species, namely *Tetrastigma curtisii*, *T. leucostaphylum* and *T. pedunculare* can be evaluated using Area Under Curve (AUC) values, representing the predictive skill of the model on a 0–1 basis. The model prediction of *R. arnoldii* has AUC value of 0.855, *T. curtisii* has 0.853, *T. pedunculare* has 0.749 and *T. leucostaphylum* has 0.725 Model outputs were used to map habitat suitability throughout Sumatra (Fig. 2).

The predicted highly suitable habitats of *R. arnoldii* covers areas an area of 3,248,189 ha throughout the Sumatra. The predicted highly suitable habitats of *T. leucostaphylum* and *T. pedunculare* cover relatively large areas throughout Sumatra compared to *T. curtisii*. The high suitable habitats of *T. curtisii*, *T. pedunculare* and *T. leucostaphylum* are respectively of 1,934,865 ha, 3,378,917 ha and 9,180,691 ha. In order to better predict the highly suitable habitats of *R. arnoldii* in conjunction with its host plants the intersection areas between *R. arnoldii* and the host plants are determined. The intersection areas of highly suitable habitat between *R. arnoldii* and *T. curtisii* covers a 307,910 ha area, *R. arnoldi* and *T. pedunculare* covers 894,630 ha and *R. arnoldii* and *T. leucostaphylum* covers 1,896,781 ha (Fig. 3).

The predicted highly suitable habitat based on the intersection areas of those species is mostly located outside of conservation

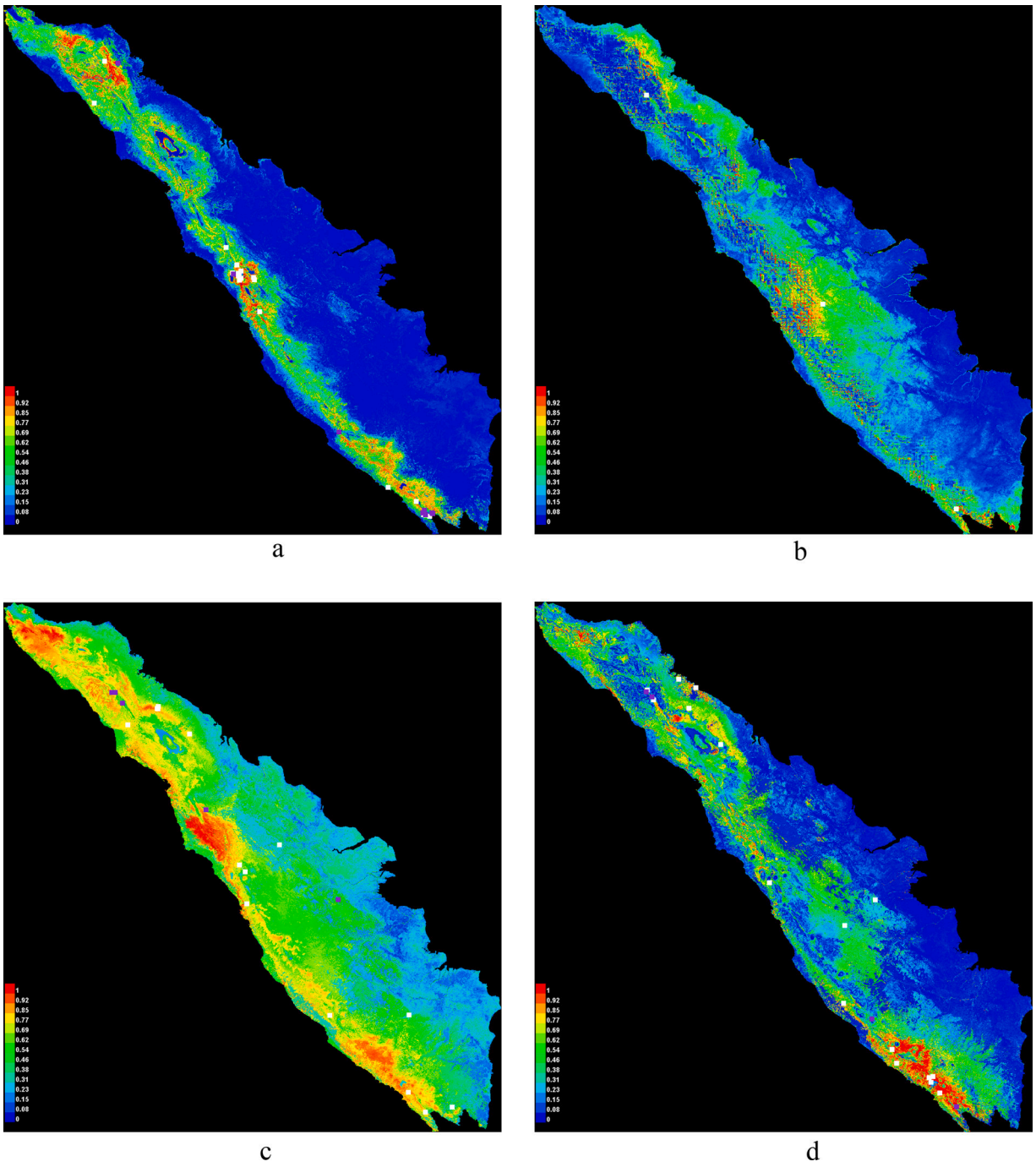
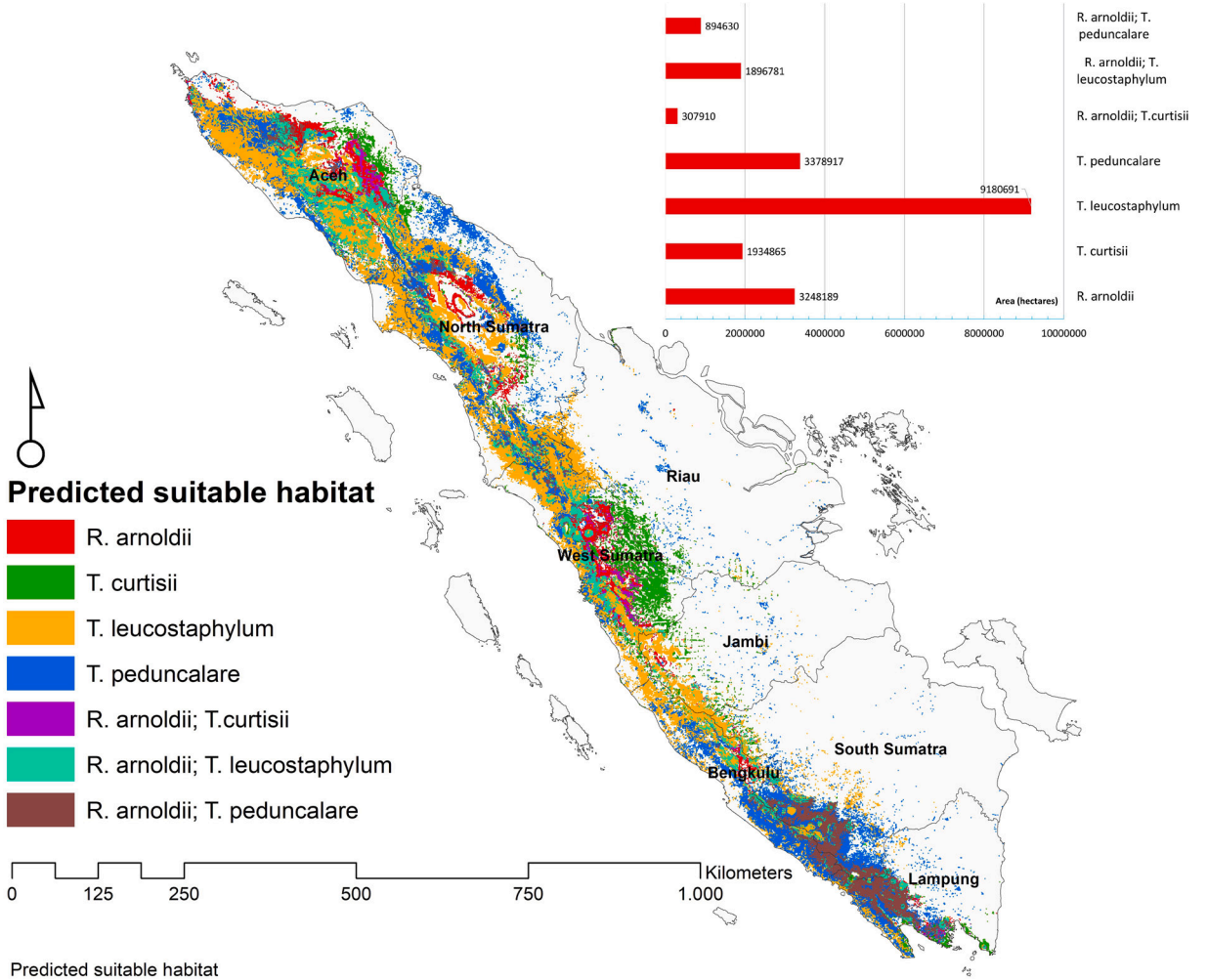


Fig. 2. The predicted suitable habitats provided by MaxEnt Models. (**a**) *Rafflesia arnoldii*; (**b**) *Tetrastigma curtisii*; (**c**) *T. leucostaphylum*; (**d**) *T. pedunculare*. The regions with the grid cells that have value > 0.5 indicating more suitable, while the regions with the value of grid cells < 0.5 indicating less suitable.

areas. The total highly suitable predicted outside conservation area includes 2,239,122.161 ha (72.33%), while the total suitable habitats inside conservation areas covers around 856,625.327 ha (27.67%). The intersection areas between *R. arnoldii* and *T. curtisii* includes 86,228.956 ha (28.11%) inside conservation areas throughout Sumatra, the intersection between *R. arnoldii* and *T. pedunculare* includes 204,565.783 ha (22.87%), and the intersection between *R. arnoldii* and *T. leucostaphylum* includes 565,830.588 ha (29.86%) (Fig. 4).

There are 34 conservation areas in Sumatra that identified as high suitable habitats between *R. arnoldii* and *T. curtisii*, 26 conservation areas for *R. arnoldi* and *T. pedunculare,* and 37 conservation areas for *R. arnoldii* and *T. leucostaphylum* (Table 2).



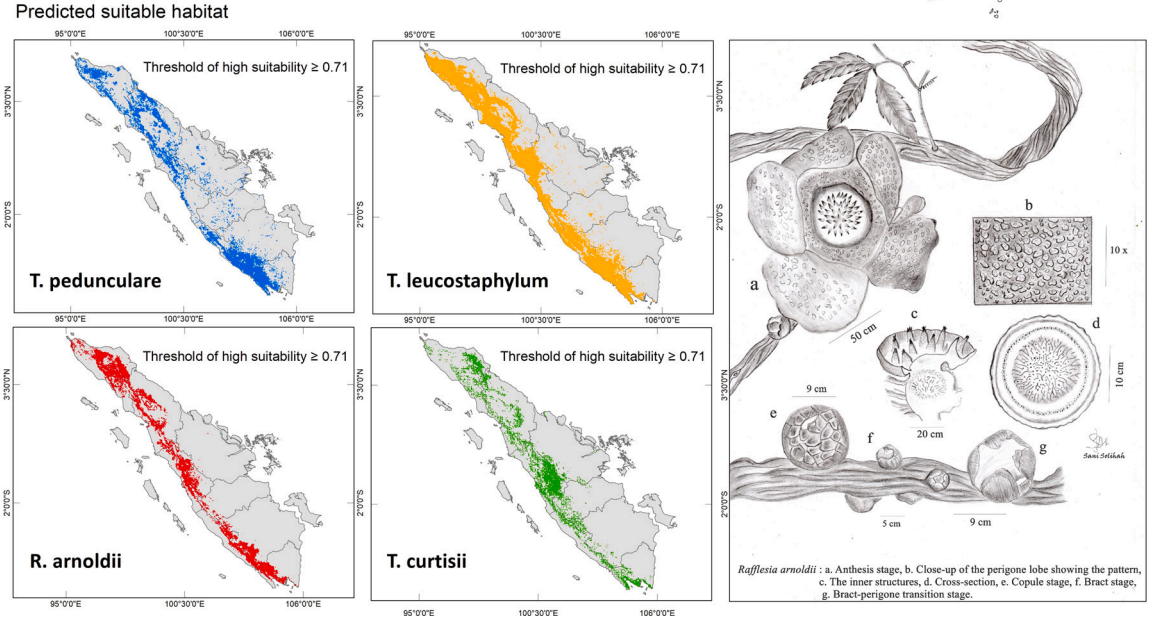


Fig. 3. The predicted intersection areas of *Rafflesia arnoldii* and three species of *Tetrastigma* as its host plants.

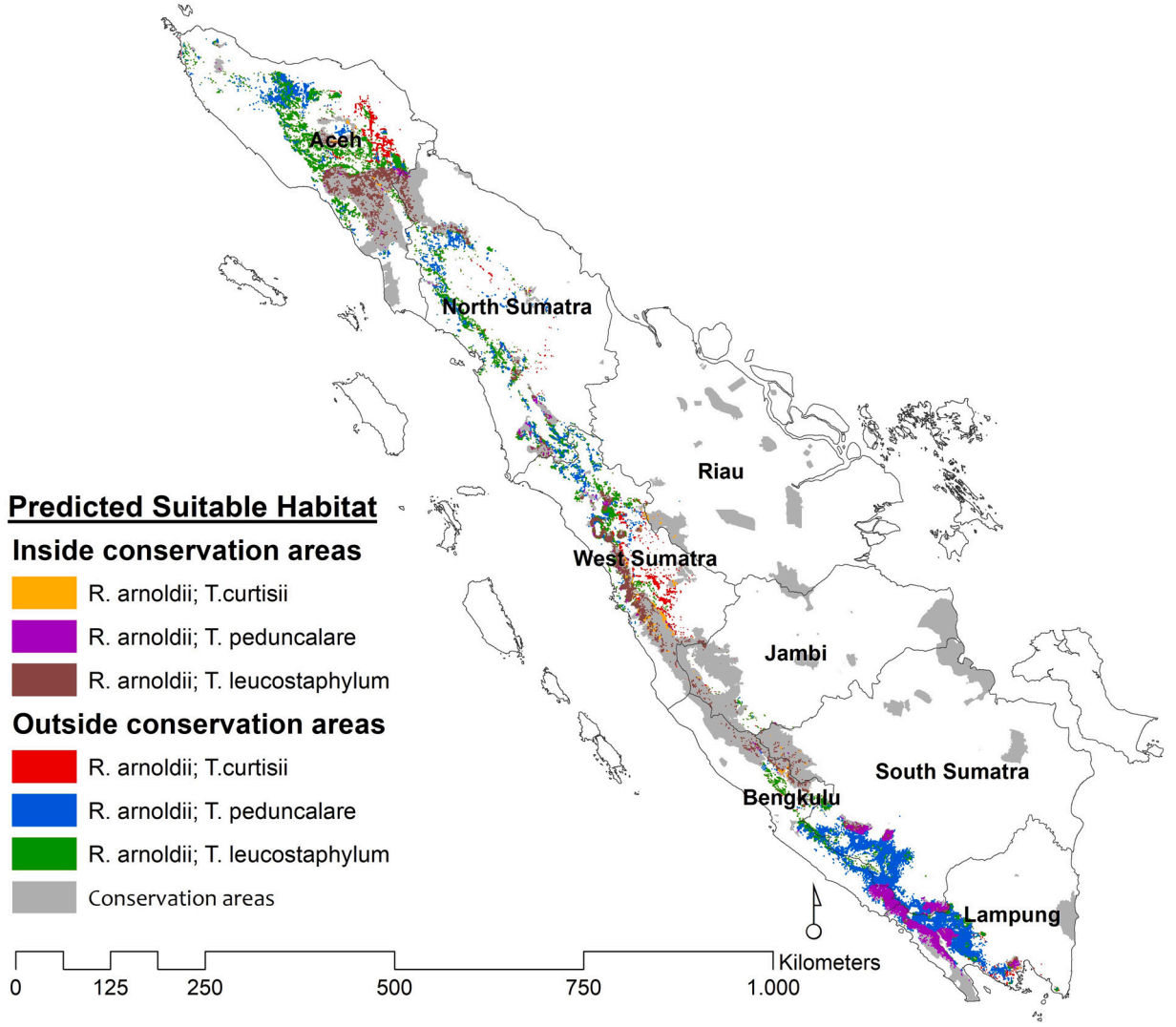


Fig. 4. The predicted intersection areas between *Rafflesia arnoldii* and three species of *Tetrastigma* that located inside and outside conservation areas.

MaxEnt modeling allows identification of the relative influence of the environmental variables on habitat suitability. The three most important variables affecting the habitat suitability of *R. arnoldii* are mean annual temperature, slope and elevation. Whereas soil organic carbon, soil type, land cover and precipitation of driest month were found to be biophysical variables moist important for predicting host plant habitat suitability (Fig. 5).

4. Discussion

According to the observed occurrence records, *R. arnoldii* is evenly distributed throughout the western side of Sumatra along the Bukit Barisan Mountain range from Lampung to Aceh. While only few occurrence records of its host plants are available due difficulty in identification in the wild. The occurrence records of *R. arnoldii* and its host plants are used to model the habitat suitability for those species. There are three criteria to choose the actual data points: 1). Using the actual data coordinate points of targeted species locations, 2) Choosing the data points with detailed location information on the targeted species, and 3). Excluding data points located in the built-up areas and human-cultivated lands. The presence data may be biased depending on accuracy of the data, sampling methods and the species detection possibility (Barbet-Massin et al., 2010).

Another inputs that are very important in the modeling process are environmental variables. Environmental variables selection process that are meaningful in determining the occurrence records of the parasitic plant and its host plants remains a challenge (Araújo et al., 2019). The choice to select the correct predictors is a crucial in determining the habitat suitability of the species (Petitpierre et al., 2017; Porfirio et al., 2014). To build this model predictions, there are four predictors (topography, soil, climate and land cover)

Table 2
Identified conservation areas that suitable habitats of the *Rafflesia arnoldii* and three species of *Tetrastigma* as its host plants.

Conservation areas	Areas status	Intersection areas		
		RA and *TC*	*RA* and *TP*	*RA* and *TL*
Air Putih	Nature Recreation Park	x		
Arau Hilir dan Air Terusan	Wildlife Reserve	x	x	x
Baringin Sati	Nature Reserve	x		
Barisan	Wildlife Reserve	x	x	x
Barumun	Wildlife Reserve	x	x	x
Batang Gadis	National Park	x	x	x
Batang Palupuh	Nature Reserve	x		
Batang Pangean I	Nature Reserve	x		
Batang Pangean II	Nature Reserve	x		
Bukit Barisan	Grand Forest Park	x		x
Bukit Barisan Selatan	National Park	x	x	x
Bukit Kaba	Nature Recreation Park	x		x
Bukit Rabang - Gluguran	Grand Forest Park		x	x
Bukit Rimbang Bukit Baling	Wildlife Reserve	x		
Cut Nyak Dien	Grand Forest Park			x
D. Sicikeh-cikeh	Nature Recreation Park			x
Danau TES	Nature Recreation Park	x	x	
Dolok Sibual-buali	Nature Reserve	x		x
Dolok Sipirok	Nature Reserve			x
Dolok Surungan	Wildlife Reserve	x	x	x
Dr. Muhammad Hatta	Grand Forest Park			x
Gumai Pasemah	Wildlife Reserve	x	x	x
Gunung Leuser	National Park	x	x	x
Gunung Leuser National Park	UNESCO-MAB Biosphere Reserve	x	x	x
Gunung Marapi	Nature Recreation Park	x		x
Gunung Raya	Wildlife Reserve	x	x	x
Gunung Sago Malintang	Nature Recreation Park	x		x
Hutan Pinus/Janthoi	Nature Reserve		x	x
Isau-isau Pasemah	Wildlife Reserve	x	x	x
Janthoi	Nature Recreation Park		x	
Kerinci Seblat	National Park	x	x	x
Kuta Malaka	Nature Recreation Park		x	x
Lembah Anai	Nature Reserve	x		x
Lembah Harau	Nature Recreation Park	x		
Lingga Isaq	Hunting Park	x	x	x
Lubuk Raya	Nature Reserve	x		x
Malampah Alahan Panjang	Wildlife Reserve	x	x	x
Maninjau	Nature Reserve	x	x	x
Mega Mendung	Nature Recreation Park			x
Pocut Meurah Intan	Grand Forest Park			x
Rimbo Panti	Nature Recreation Park		x	x
Semidang Bukit Kabu	Hunting Park		x	x
Singgalang Tandikat	Nature Recreation Park	x	x	x
Siranggas	Wildlife Reserve	x	x	x
Tropical Rainforest Heritage of Sumatra	World Heritage Site (natural or mixed)	x	x	x
Wan Abdul Rahman	Grand Forest Park	x	x	x

Note: RA = *R. arnoldii*, TC = *T. curtisii*, TP = *T. pedunculare*, TL = *T. leucostaphylum*, the mark (x) indicates the suitable areas of *R. arnoldii*.

that used as inputs. We considered those variables are meaningful for determining the habitat suitability of those species. The inclusion of land cover as input of the model was considered to be important. The inclusion of land cover improved significantly the explanatory power of bioclimatic models and the most relevant variables across groups were those not explained or poorly explained by climate (Thuiller et al., 2004).

The predictive habitat suitability model for *R. arnoldii* and *T. curtisii* have the AUC value > 0.80 indicating that those models have good performance, whereas *T. pedunculare* and *T. leucostaphylum* have the AUC value > 0.70 indicating that those models have fair performance. The value of AUC represents the categories of predictive model performance, model with AUC in range 0.9–1 (excellent), 0.8–0.9 (good), 0.7–0.8 (fair), 0.6–0.7 (poor), and 0.5–0.6 (fail) (Krzanowski and Hand, 2009). In addition, the predictive model that has the value of AUC in range 0.7–0.8 is considered acceptable, and 0.8–0.9 is considered excellent (Hosmer and Lemeshow, 2000). Although only few records of the host plants of *R. arnoldii* in which three records for *T. curtisii*, the evaluation of model performance was acceptable with the AUC value > 0.80.

A jackknife (or 'leave-one-out') procedure was applied to model few presence localities of *T. curtisii*. Using the jackknife test applied to the MaxEnt model, high success rates and statistical significance were obtained with small sample size as low as five (Pearson et al., 2007). However, a model that includes less than four presence locations has a poor success rate (Pearson et al., 2007). A critical difficulty in the current study is small number of *T. curtisii* locations of from the few available studies. In order to have a more reliable

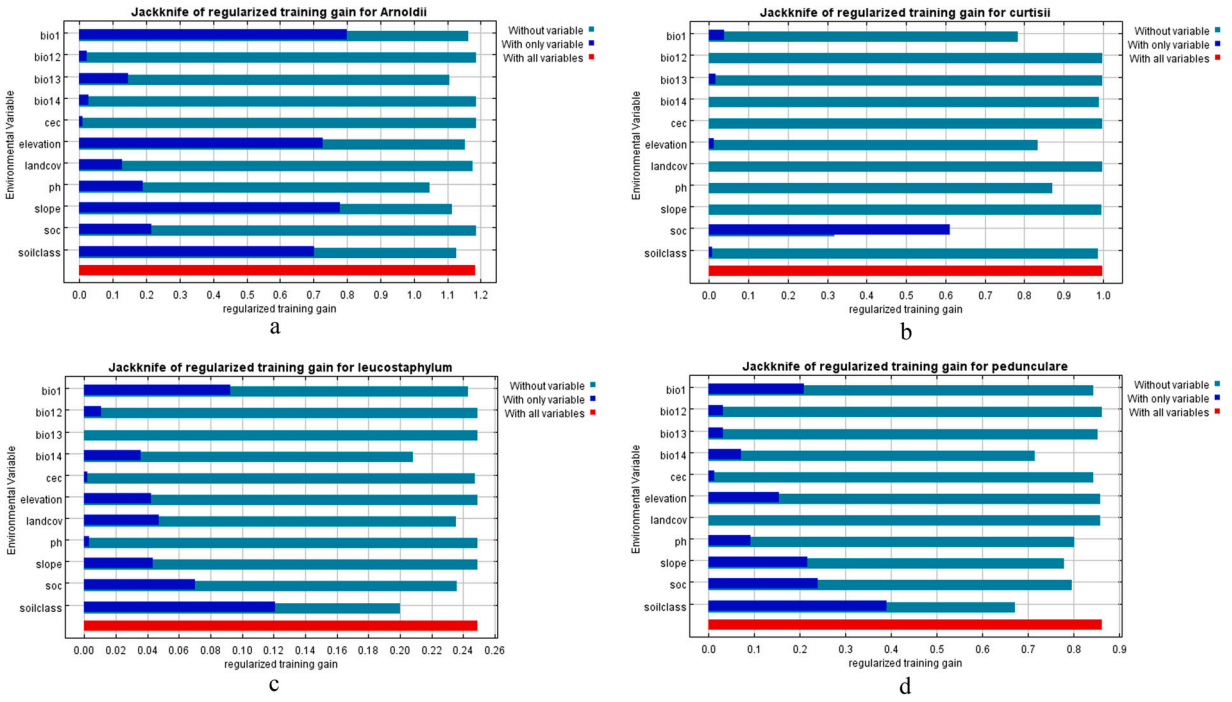


Fig. 5. The importance variables that affecting *R. arnoldii* and three species of *Tetrastigma* as its host plants. (**a**) *Rafflesia arnoldii*; (**b**) *Tetrastigma curtisii*; (**c**) *Tetrastigma leucostaphylum*; (**d**) *Tetrastigma pedunculare*.

model for *T. curtisii*, additional presence records should be obtained. Some previous studies used MaxEnt modeling to predict the potential distribution of endemic or rare species with small number of records, occurrence points of 23 (Kebede et al., 2012); occurrence points of 10 (Deb et al., 2017); occurrence points of 32 (Abdelaal et al., 2019).

The suitable habitats of the species represented by the value range from 0 to 1. The regions that have the value more than 0.5 indicating those regions are more suitable habitats of this species, while the value less than 0.5 indicating those regions are less suitable habitats of this species. The predicted suitable habitats of *R. arnoldii* are mostly in portions of southern, central and northern Sumatra. All those predicted suitable habitats are located along the Bukit Barisan Mountain range. It consists primarily of active and non-active volcanoes shrouded in dense jungle cover with higher slopes. This corresponds well with the observation records with almost all points found in western Sumatra. Eastern Sumatra is predicted as less suitable habitat represented by blue color (Fig. 2). The regions in eastern Sumatra are dominated by peat swamp forests that are not suitable for this species. The suitable habitats of *T. curtisii* are mostly predicted in West Sumatra, North Sumatra, Bengkulu and eastern Aceh. The regions in Lampung, Bengkulu, small part regions of West Sumatra, North Sumatra and some regions spread throughout Aceh are predicted as suitable habitat for *T. pedunculare*. Highly suitable habitats for *T. leucostaphylum* are predicted mostly in entire western part of Sumatra from Lampung to Aceh. The observed location data points are sometimes not predicted to be highly likely suitable habitat due to data limitations. In the evaluation of model performance there are two type of errors (commission errors and omission errors). A commission error an occurrence of absence wrongly predicted as presence (Krzanowski and Hand, 2009), while an omission error is a presence observation wrongly predicted an absence (Lobo et al., 2008). Those two type of errors may affect the accuracy of model in spatially predicting the habitat suitability of the targeted species in this study.

The most important biophysical variable contributing to the occurrence of this species is mean annual temperature, followed by slope. According to (Ramadhani et al., 2017), the habitat of *R. arnoldii* has the temperature range 25–29 °C in moderate slope hills (30–45%). This condition is likely associated with reliable water flow away from upper areas. The slope is a very complex environment for the plant community (Chiatante et al., 2002). Elevation was the third most important factor affecting the occurrence of *R. arnoldii*. This species has been found in lowland forests at an altitude from 35 to 600 m above sea level (Susatya, 2011), from 490 to 1024 m above sea level (Pranata et al., 2019) and from a 490 to 558 m above sea level (Ramadhani et al., 2017). The occurrence of *R. arnoldii* is determined by its host plants. Soil organic carbon was the most important modeled biophysical variable affecting the occurrence of the host plants of *R. arnoldii*. Those host plants tend to occupy the primary forest, secondary forest, and lowland dipterocarp-forest that have a relatively dense canopy cover (Latiff, 1984; Rahayu, 2017; Wan Zakaria et al., 2017; Yeo et al., 2012). The canopy cover of vegetation creates a warm and moist microclimate under the canopy which accelerates the decomposition of litter (Wang et al., 2010). This could affect accumulation of soil organic carbon. Soil type was also important variable that affect the presence of the host plants. According to the digital soil map of the world (Sanchez et al., 2009), the actual presence localities and predicted habitats of the host plants have various type of soils, including Humic Andosols, Humic Acrisols, Orthic Acrosols, Dystric Fluvisols, Dystric Cambisols and Eutric Fluvisols. Only few regions in eastern part of Sumatra are predicted as suitable habitats because Histosols are widely distributed

there. Histosols is a soil primarily comprised of organic materials (peaty soils). Other important prediction variables include mean annual temperature, elevation, and slope. These variables are similar to the most important contributing factors affecting the occurrence of *R. arnoldii*.

The habitat suitability map of *R. arnoldii* and its host plants resulting from this study may not precisely predict the locations where those species currently occur because it depends only on biophysical variables including topography, soils, climate and land cover. Factors such as human management and site history could also be important. Although the predicted habitat suitability of a location might be high there are many reasons why the species might not be found at that location. It is possible that the species are not able to disperse to that location. Unidentified biotic interactions could inhibit the recruitment or survival in some locations. Possibly the species was once found in the predicted habitat but has since been extirpated from the area. A failure to find *R. arnoldii* even when present might be due to dormancy. In contrast, the model may predict that a certain region is unsuitable habitat, but the species may occur in that location. Some species are able to disperse to a location and adapt physiologically to the unsuitable habitats (Peterson et al., 2011). Given the difficulty in exhaustive sampling to determine the locations of rare, but important species, MaxEnt modeling is useful to assess potential habitat suitability of *R. arnoldii* and other species of *Rafflesia* throughout the world.

The findings of this study may be used by the Government of Republic Indonesia, c.q. Ministry of Environment and Forestry for establishing protected areas and conservation-based management improvement and could also help inform the *R. arnoldii* and its host plants listing status in IUCN red list category in the future. We believe that this study is the first report modeling potential habitat suitability of a parasitic plant and its host plants. Further studies on other species of parasitic plant by incorporated biophysical variables, dispersal aspect, human activities, and site habitat history could provide an important tool for management and conservation.

5. Conclusions

The predicted best habitat suitability of *R. arnoldii* in Sumatra tends to be concentrated on the western side of Sumatra along the Bukit Barisan Mountain range. However, the suitable habitat assessed by also considering its host plants include a much smaller area. The highly suitable habitats of *R. arnoldii* and its host plants are mostly predicted to be outside conservation areas. There are 46 conservation areas that are predicted to be highly suitable habitats for *R. arnoldii* and its host plants.

CRediT authorship contribution statement

The following statements should be used “Conceptualization, E.R. and A.Y.; Methodology, A.Y., P.D.R.; Software, E.R., A.Y. and P. D.R.; Validation, J.R.W., Y. and S.M.; Formal analysis, E.R., A.Y. and P.D.R.; Investigation, E.M. and I.P.A.; Resources, E.M., I.P.A., I.A. F., I.R. and S.M.; Data curation, S.M., E.M. and S.M.S.; Writing – original draft, All authors.; Writing – review & editing, All. authors; Visualization, A.Y., P.D.R. and S.M.S.; Supervision, A.Y., S.M., J.R.W. and W.P.C.J. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research Article

Morphological Characterization and Seed Germination Study of Wild Banana *Musa acuminata* var. *flava* (Ridl.) Nasution

Trimanto[1]*, Elga Renjana[1], Dewi Ayu Lestari[1], Elok Rifqi Firdiana[1], Shofiyatul Mas'udah[1], Apriyono Rahadiantoro[1], Linda Wige Ningrum[1], Lia Hapsari[1]*

1) Purwodadi Botanic Garden – Research Center for Plant Conservation and Botanic Gardens, National Research and Innovation Agency (BRIN), Jl. Surabaya – Malang Km 65, Purwodadi, Pasuruan, East Java, 67163, Indonesia

* Corresponding author, email: triman.bios08@gmail.com; hapsari.lia@gmail.com

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Ardaning Nuriliani

ABSTRACT

Wild bananas provide important genetic materials for further banana improvement, therefore they need to be conserved and studied. This study aimed to describe morphological characteristics of plant and seed and also to study the seed germination of wild banana *M. acuminata* var. *flava* (Ridl.) Nasution. The morphological characteristics were observed descriptively by referring to the descriptor for banana. The internal and external morphology of the seeds were observed using a digital microscope. The germination testing was carried out by a completely randomized design, using fresh seeds extracted from a bunch of fruits with two ripeness levels *i.e.* fully-ripe (yellow peel) and under-ripe (green-yellow peel). The data resulted was then analyzed using an independent t-test. The results showed that *M. acuminata* var. *flava* is characterized as a perennial herb; pseudostem height ≥ 3 m; male bud like a top with prominent green-yellow bracts; fruit curved and tasted mild-sweet when ripe. The seed is angular with wrinkled surface, and dark brown-black color when ripe. The longitudinal section showed parts of the seeds comprising the seed coat, outer and inner integument, embryo, endosperm, chalazal mass, micropyle cap and channel. The seeds are classified as orthodox, with hypogeal type and gradual germination pattern. The seeds extracted from fully-ripe fruit germinated faster with higher germination percentage and growth variables (root number and plant height). Thus, it is suggested to use physiologically mature seeds (seeds from fully-ripe fruits) which should be separated from the seeds of under-ripe fruits to lower the heterogeneity.

INTRODUCTION

Banana is an important cash crop worldwide; ranked next to rice, wheat, and maize. The monocotyledon Musaceae family includes three genera *i.e. Ensete*, *Musella* and *Musa* in which most edible bananas belong to the genus *Musa* (Perrier et al. 2009). It consists of approximately 70 wild species and 500 cultivars (Hakkinen & Vare 2008; Valmayor et al. 2002). In particular, in Indonesia, being part of the center of banana diversity; not less than 12 wild species and 325 cultivars have been found (Nasution & Yamada 2001). However, the evolution of wild seeded to seedless edible bananas is a complex pro-

cesses involving multiple stages and separated by time (over centuries and/or millennials and places) (Perrier et al. 2009; Langhe et al. 2009; Hapsari et al. 2018).

Most of the current banana cultivars were putatively the results of hybridization between two major wild banana species of *Musa acuminata* (A genome) and *M. balbisiana* (B genome); and few were of *M. schizocarpa* (S genome) and *M. textilis* (T genome) (Perrier et al. 2009; Langhe et al. 2009). The diversity of banana crop wild relatives (especially the putative progenitors) is important to ensure the future of modern bananas. Conservation and collection of crop wild relatives through seed is a suitable strategy to conserve germplasm in gene bank because they are the reservoir of traits and genes required to face the emerging abiotic and biotic stresses (Singh et al. 2021). Nonetheless, they have been under considerable threats due to habitat destruction, fragmentation and conversion of tropical forests, and other anthropogenic disturbances. Therefore, it is important to prioritize the collection, effective conservation, improving the availability, and providing related studies of wild bananas for use in further banana improvement (Heslop-Harrison 2011; Ford-Lloyd et al. 2011; Hapsari et al. 2020).

Two major wild species *M. acuminata* mostly grow in tropical rainforests in Southeast Asia, while *M. balbisiana* is native to monsoon climates in Southeast Asia and South Asia (Ploetz et al. 2007). In Indonesia, wild bananas can be found in Java, Borneo, Mollucas, Papua, Sulawesi and Sumatra (Sulistyaningsih et al. 2014). As an *ex-situ* conservation institution, Purwodadi Botanic Garden (PBG) which is located in Pasuruan (East Java) has been conserving various species of bananas, mostly from Eastern Indonesia. At least 103 accessions and 197 specimens, consisting of eight wild species and 95 cultivars, have been successfully collected (Hapsari 2014). Furthermore, recent publication reported that PBG has collected wild bananas comprised of three accessions of *Ensete glaucum*, three accessions of *M. balbisiana* and seven accessions of *M. acuminata* variety (Hapsari et al. 2020).

The wild species *M. acuminata* is considered to be the most important genetic material contributor for cultivated bananas (Martin et al. 2020). However, this species is thought to be a species complex; possible continuous variation and phenotypic plasticity due to environmental modifications and adaptations have made this species as a taxonomically difficult group. About fifteen varieties of *M. acuminata* have been stated by Nasution (1991) in the Memoirs of Tokyo University of Agriculture XXXII. The descriptions and key identification of *M. acuminata* varieties have been provided. However, the distinguishing characters among varieties remained confusing due to high variations, especially in the wild populations of Indonesia.

Wild banana *M. acuminata* var. *flava* is one of variety of *M. acuminata*, a status novus (STAT. NOV) or got a new rank given by Nasution (1991). It was firstly described by Ridley as *Musa flava* Ridl. (Trans. Linn. Soc. 2,3: 385-386, 1888-1894 et Ridl., F. Mal. Pen. 4: 294; Anon. Kew. Bull. 92: 249, 1894. – Type: Ridley s.n., Pulau Tijam, on Pahang River, Pahang (SING n.v.). Ac-

cording to Nasution (1991), it was generally characterized by a small to medium clump, tall and slender pseudostems, purplish brown blotching without wax. Leaf blade lanceolate, long petiole, purplish brown blotching, with erect margins. Inflorescence horizontal then pendulous, peduncle thinly pubescent, 10-12 hands per bunch, 12-21 fruits per hand. Male bud ovoid, greenish yellow or yellow in color. Fruits medium, pericarp thin, pulp yellowish. Seeds many, irregularly angular, not smooth, and black when ripe.

Related studies of wild bananas for use in further improvement of bananas are essential due to the recent global threat to cultivated bananas. Diploid wild banana produces fertile and viable seeds which are preferable as genetic material for breeding purposes because they provides more variability and any possible desired traits. Banana seeds may vary in size, shape and color, and also germination rate, depending on the species and varieties (Vineesh et al. 2015). Seed gene banks for bananas are applicable for seed-producing diploid wild *Musa* species, not for cultivar bananas. *Ex-situ* seed conservation of diploid wild banana, especially collected from wild population, brings impact on the seed collection quality (Sipen et al. 2011; Kallow et al. 2020). Furthermore ex-situ seeds conservation of wild bananas is constrained by critical knowledge gaps in their germination ecology, behavior, and also storage which need to be addressed (Kallow et al. 2020; Kallow et al. 2021).

The seed studies of some varieties of wild *M. acuminata* have been reported but still limited. One of wild bananas that has been studied its propagation method is *Musa acuminata* var. *sumatrana* (Roostika et al. 2019). Hence this study aimed to describe the plant and seed morphological characteristics of wild banana *M. acuminata* var. *flava* cultivated at PBG, and also to study the seed germination from two different fruit ripeness levels to evaluate the seed physiological maturity. A complete morphological observation on *M. acuminata* var. *flava* is important for better identification of this variety. The study of banana germination by seeds is still limited because vegetative propagation is more common in banana. Information on efficiency of seed germination is required for plant propagation and breeding programs. In addition, no previous study of seed germination particularly on *M. acuminata* var. *flava* is reported.

MATERIALS AND METHODS

Materials

Plant material used in this study is the wild banana species living collection of Purwodadi Botanic Garden *i.e. Musa acuminata* var. *flava* located at the nursery (previously located at plot XXIV.E.40-a). It was originated from wild populations in Krawak Protected Forest of Tuban, East Java (Lestarini et al. 2012). Morphological observation was conducted directly to the living collection in September 2020. A mature bunch of fruits was harvested from a single plant. The fruits were then divided in two categories *i.e.* fully-ripe ones with yellow peel and under-ripe ones with green-yellow peel (Figure 1). Later, the seeds

from both categories were extracted for further testing (September to November 2020).

A B

Figure 1. Fruit maturity categories: A. fully-ripe (yellow peel), B. under-ripe (green-yellow peel).

Methods

Morphological characterization

Plant morphological characterization was performed by referring to the banana descriptor (IPGRI-INIBAP/CIRAD 1996). All parts of the plant were documented using a digital camera, while the documentation of the seeds was carried out using a digital microscope (Dino-Lite AM3113T). External morphological characterization of seeds was conducted on quantitative characters (length, width, thickness and weight) and qualitative characters (shape, color and texture) while internal morphological characterization was conducted on seed coat, endosperm and other internal seed parts (Kallow et al. 2021).

Seed moisture content and germination testing

1. Seed extraction. Seeds were hand-extracted from the ripe fruits in the laboratory, and the seeds were washed thoroughly using tap water and sieves, until all flesh was removed. Meanwhile the under-ripe fruits were left in the laboratory at room temperature to ripen until they began to yellow and soften and then seeds were extracted following the steps described above (Kallow et al. 2021). After extraction prior to air-drying, the seeds were soaked in water for a while in which the floating seeds were discarded and only the submerged seeds were used for further testing.
2. Measurement of seed moisture content. It was started by weighing the fresh weight of 25 seeds using an analytical scale (Mettler Toledo), then they were dried using a drying oven laboratory (Finco Inc Ovin 30) at 100°C for 18 hours and their dry weight was determined. Measurements were carried out in three replications for each ripeness level. Seed moisture content was calculated using the following formula:

$$\text{Seed moisture content} = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Fresh Weight}} \text{ x } 100\%$$

Furthermore, seed storage behavior was classified as referred to (Kallow et al. 2020). Seeds with moisture content of less than 22% are predicted to have orthodox storage behavior; seeds with higher moisture content (>40%) are predicted to be recalcitrant; while seeds in-between those limits (22% - 40%) are predicted to be intermediate.

3. Sowing the seeds. The seeds were sterilized before sowing by immersing them in a 10% chlorox solution (active substance NaOCl 5.25%) for 10 minutes. The experiment used a completely randomized design (CRD) with two treatments (two seed categories *i.e.* extracted from fully-ripe fruits and under-ripe fruits) and three replications for each treatment. The seeds were sown on plastic seed tray with moist straw paper media. After being sown, the seed trays were then covered with black plastic to keep them moist (regular watering was carried out when the media started to dry). Maintaining the air circulation was conducted by opening the plastic cover during observation in order to prevent the seeds from rotting.
4. Observation of germination. The seed germination variables, the number of seeds germinated, pattern and type of germination, were observed every day. Whereas to observe the growth variables *i.e.* plant height and number of roots; seven seedlings were taken randomly at 60 days after sowing (DAS). The percentage of seed germination was determined using the following formula (Sutopo 2010; Muschick et al. 2010; Darmayanti et al. 2017):

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \text{ x } 100\%$$

Data Analysis

The morphological characterization was described qualitatively, while the seed moisture content, seed germination, and growth variables data were analyzed quantitatively. Quantitative data were compiled and analyzed using Microsoft Excel 2016 to figure out the data characteristics and distribution. Later, the data normality was tested using Shapiro-Wilk ensued by the homogeneity test using Lavene. Independent t-test was conducted using SPSS 16.0 to determine the statistical significant difference between the means of the two fruit ripeness levels on the seed moisture content, seed germination and growth variables.

RESULTS AND DISCUSSION

Plant morphological characteristics of *M. acuminata* var. *flava.*

The detailed morphological characterization of both the plant and the seeds of *M. acuminata* var. *flava* from this study `are presented below (Figure 2, 3 & 4). The plant habitus of *M. acuminata* var. *flava* is perennial herbaceous plant, leaf habit intermediate, small to medium clump, number of suckers 2-5, close to parent (vertical growth). ***Pseudostem*** height ≥ 3 m, slender to normal circumference, light green, shiny (not waxy), predominant underlying watery

green with purple pigmentation, milky sap, very little or no visible sign of wax on leaf sheaths. ***Petiole*** black-purple blotches, large blotches at petiole base, length 53-70 cm, width 2.5-3 cm, green, the third petiole canal leaf wide with erect margins (not clasping), margin width ≤ 1 cm and dried. ***Leaf blade*** length 185-205 cm, width 45-50 cm, upper surface green and shiny, lower surface medium green and dull, moderately waxy, the symmetric insertion point of blades on the petiole, very corrugated, green midrib dorsal surface, light green midrib ventral surface, green cigar leaf dorsal surface, leaves of water suckers without blotches. ***Peduncle*** length ≤ 30 cm, width ≤ 6 cm, green, slightly hairy. ***Bunch*** position horizontal, spiral to asymmetric shape, compact appearance. ***Rachis*** type present, horizontal position with some neutral flowers (one to few hands only, the stalk is bare below. ***Male bud*** present, like a top, diameter 6-7 cm, length 8-9 cm; ***bract*** base shape large shoulder, length 15.7 cm, width 7.5 cm, pointed old bracts overlap at the apex of bud, green-yellow external face, green-yellow internal face, color homogenous green-yellow, without discolored lines, lanceolate shape, lifting two or more at a time, revolute behaviour before falling, very few waxes, moderate grooving; bract scars on rachis very prominent. Female flowers not observed. ***Male flowers*** small, 17-18 flowers per hand; ***compound tepal*** cream, rust colored spots, yellow lobe; ***free tepal*** tinted with yellow, oblong, corrugated (several folding under apex), apex thread-like; ***anthers*** exserted, cream to yellow, 2.1 cm; ***filament*** cream, 1 cm; ***pollen*** brown/rusty brown; ***style*** cream, without pigmentation, same level exserted, straight; ***stigma*** pale orange; ***ovary*** cream, very little or no visible sign of pigmentation, male flower cream, two arrangement of ovules. ***Fruit*** biseriate, 10-12 hands per bunch, position curved upward, ≥17 fruits per hand, length 13 - 15 cm, 1.5-2.0 cm in diameter, curved (sharp curve) shape, rounded transverse section, bottle necked apex, without any floral relicts at apex; ***pedicel*** length 11-15 mm, width 5 to 10 mm, hairless surface, deciduous at maturity (fruits fall from hand); ***peel*** light green when immature, yellow when mature, two or less thickness, cracked at maturity; ***pulp*** cream before maturity, soft flesh texture, mild to sweet taste. ***Seeds*** numerous 20-170 seeds per fruit, wrinkled surface, angular (more or less pyramidal), dark brown to black when ripe.

The habitat of *M. acuminata* var *flava* is at open places, along the rivers or roads, 300-600 m above sea level (Nasution 1991), with distribution reported in Central Borneo and the Malay Peninsula. Even though the specimen examined in this study was originated from Krawak Protected Forest of Tuban (East Java) which considered far from its original type species, but the morphological characterization results matched very well to the description of *M. acuminata* var. *flava*, particularly for the green-yellow male bud color. There were some morphological variations observed, however it may possibly due to continuous variation and phenotypic plasticity of environmental modifications. It is locally named by East Javanese as the *pisang jantung kuning* (yellow bud banana), and they utilize its leaves for food wrapping. All plant parts are also used as fodder for wild animals and cattle (Hapsari 2014).

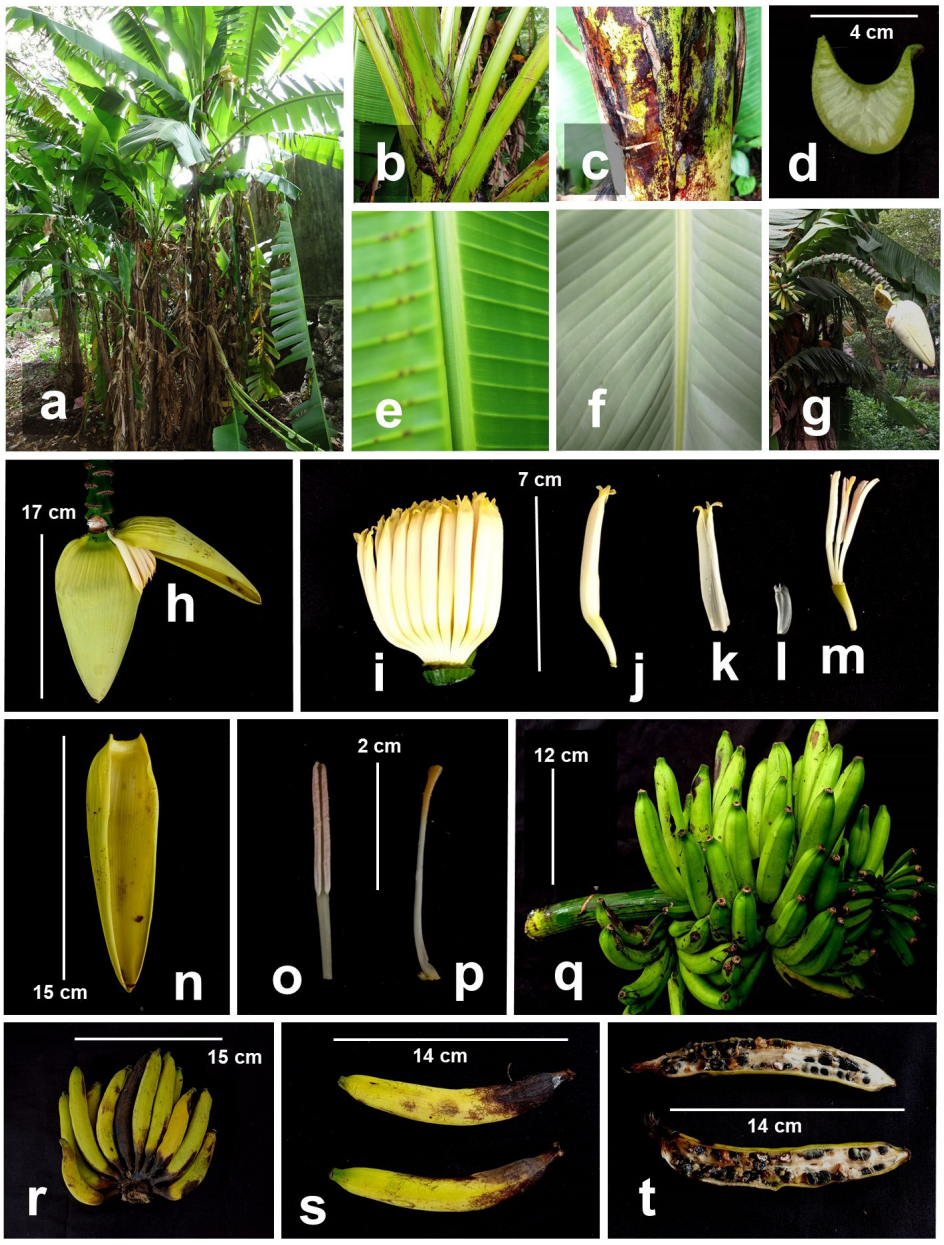

Figure 2. Plant morphological characteristics of *M. acuminata* var. *flava*. a. plant general appearance, b. petiole base arrangement, c. pseudostem blotches, d. cross-section of petiole canal, e. leaf adaxial surface, f. leaf abaxial surface, g. inflorescence, h. male bud, i. male flowers, j. ovary and compound tepal, k. compound tepal, l. free tepal, m. ovary, style and filament, n. male bract, o. filament and anther, p. style and stigma, q. a bunch of fruits, r. a hand of fruits, s. mature fruits, t. longitudinal section of fruit.

Morphological characteristic studies of *M. acuminata* var. *flava* are useful for determining morphological variations of wild bananas in Indonesia, although other studies have shown that various *M. acuminata* have been identified but currently do not have economic value for the community (Hastuti et al. 2019). Neglected wild bananas will be a threat to the existence and reduction of banana gene variation in nature. Conservation is a strategy to saving wild bananas before they become extinct. Wild bananas are very valuable for future breeding programs. One of the important activities in plant breeding is selecting phenotypes that have the desired morphological characters. Pheno-

type diversity can be influenced by environmental factors while morphological characters are expressions of genetic and environmental factors. The diversity of *M. acuminata* from Indonesia confirms that this species are genetically diverse (Poerba et al. 2019).

Musa acuminata var. *flava* is a diploid banana. Based on the results of morphological characterization, the fruits of *M. acuminata* var. *flava* are quite good in quality with 10-12 hands per bunch, curved position upwards, 17 fruits per hand, 13-15 cm in length and 1.5-2.0 cm in diameter. The strategy of crossing diploid banana with good agronomic qualities with a triploid banana that has disease-resistant will produce a diploid hybrid with agronomic advantages, such as resistance to pests and diseases (Pedraza et al. 2005). Conventional sexual hybridization is often applied in most cultivated banana. Banana breeding efforts are focused on increasing selected wild diploid, semi -partenocarpic and parthenocarpic male parents (Sipen et al. 2011).

Seed morphological characteristics of *M. acuminata* var. *flava*

The seeds of *M. acuminata* var. *flava* weight was around 0.038 ± 0.001 g (mean ± standard deviation). The external morphological of the seeds comprised the seed coat (testa) and micropyle. They were angular in shape, dark brown to black in color, with size 2.19 ± 0.15 mm in diameter and 3.88 ± 0.10 mm in thickness (Figure 3a). The seed size is a plastic characteristic that can be altered within populations, individual plants, inflorescences and even in fruits due to environmental conditions in ripening, genetic factors, pollination rate, availability of nutrients, water, light and position of the fruit on the plant (Kaiser et al. 2016). Furthermore the seed had thick and hard coat with thickness of 0.46 ± 0.43 mm. The micropyle is located a the center of the seed, with size of 0.78 ± 0.03 mm (Figure 3b). The thick hard seed coat in wild bananas prevents the oxygen and water that are essential for germination from entering the seeds which subsequently leads to limiting factors of germination. However, it is still considered as water permeable, thus the process of water imbibition may occur (Kallow et al. 2021).

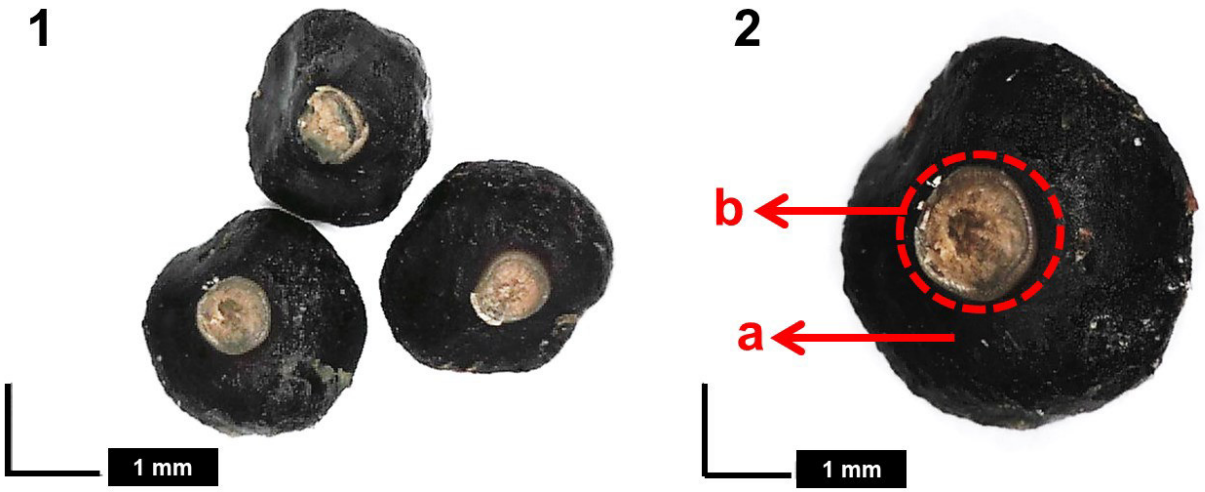

Figure 3. External seed morphology of *M. acuminata* var. *flava*. 1. seeds, 2a. seed coat, 2b. micropyle.

The longitudinal section of the seed showed the seed coat, outer and inner integument, embryo, endosperm, chalazal mass, micropyle cap and channel (Figure 4). Detail observation showed that the seed has two layers of integument (the outer multiple layers and the thin inner layer) which protect the seed during maturation, dispersal and dormancy (Silva et al. 2019). Furthermore, the seed has two chambers within the double-layered of the integument. The first chamber, which was larger, contained the embryo and endosperm. The embryo was small and undifferentiated, measuring 0.31 x 0.32 mm, and the embryonic axis extended to the micropyle collar. The embryo was surrounded by white flour-like (endosperm) served as food reserves for the embryo, with a size approximately of 1.04 x 0.35 mm. At the top of the embryo, there was the micropyle. The micropyle is where the shoot appears when seed germinated. The micropylar part of the seed coat develops into an operculum (a lid-like structure). During germination this lid is later displaced by the elongating radical-hypocotyl axis (Vineesh et al. 2015). In the second chamber, there was a brown chalazal mass measuring 2.65 x 1.57 mm, located at the basal part of the inner seed (Figure 4). The morphology and seed mass of *M. acuminata* var. *flava* observed in this study are in accordance to previous reports on other varieties and subspecies of *M. acuminata* (Puteh et al. 2011; Vineesh et al. 2015; Kallow et al. 2020) and also other wild *Musa* species (Burgos-Hernández et al. 2014; Bohra et al. 2020).

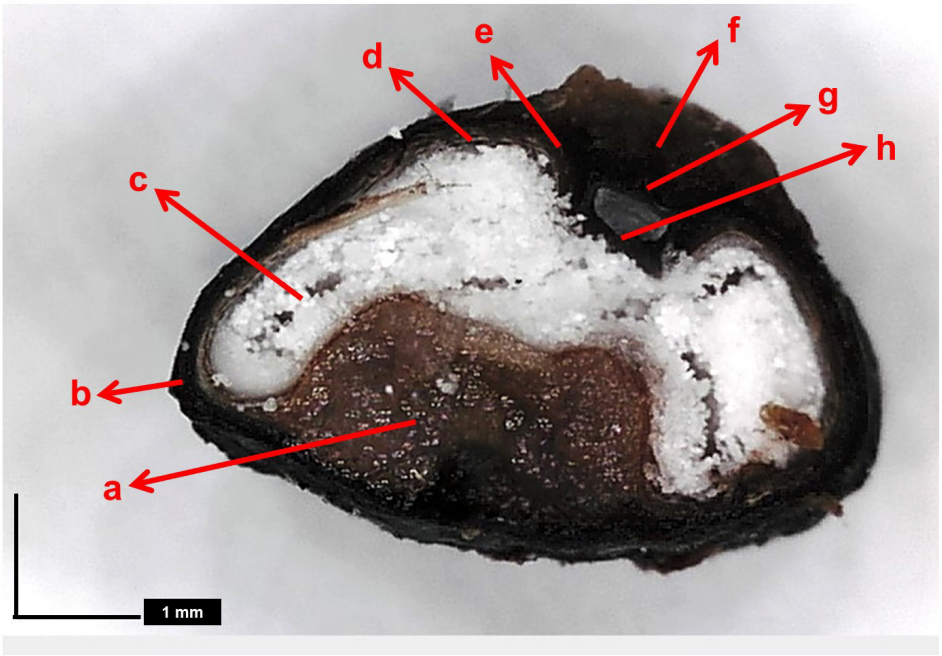

Figure 4. Longitudinal section of *M. acuminata* var. *flava* seed. a. chalazal mass, b. seed coat, c. endosperm, d. inner integument, e. outer integument, f. micropyle cap, g. micropyle channel, h. embryo.

Viability and quality of seeds can be determined by observing their morphological characters. Full mature of seeds are characterized by a darker color of the coat which indicated good seed development. The character of a healthy embryo is characterized by a compact mass in the seed (Figure 4H).

The morphological character of the embryo can be used as a measure of seed viability. Immature seeds show increased air space in the endosperm on drying since there are greater loss of structure during drying in embryos from less mature seeds (Kallow et al 2020). Full mature of seeds are estimated to have good seed viability (Figure 4). Some of their characteristics include compact embryo, white endosperm, darker seed coat and intact chalazal mass. Good quality seeds are characterized by successful germination approximately two weeks after being sown. Seeds with full maturity are characterized by a more powdery endosperm and are harvested from larger fruits with a softer flesh texture.

Seed germination of *M. acuminata* var. *flava*

Fresh and dry weight of seeds from fully-ripe fruit was significantly lower than those from under-ripe fruit. However, the seed moisture content of both categories were not significantly different, but seeds from fully-ripe fruit were slightly higher (Table 1). The seeds moisture content of *M. acuminata* var. *flava* from this study were less than 22%, thus predicted to have orthodox storage behaviour. Wild banana seeds are generally known to be orthodox or long-term storage under very low moisture and sub-zero temperature. However, several studies have shown that some banana species have intermediate seeds, such as *Musa indandamanensis*, where the seed viability decreases over time, especially after three months of storage (Bohra et al. 2020). Meanwhile Kallow et al. (2020) reported that *M. balbisiana* is considered as orthodox and indicated that *M. acuminata* subsp. storage behavior was between orthodox and intermediate. To confirm the storage behavior of *M. acuminata* var. *flava* seeds, further studies are required.

Furthermore the seed germination study showed that seeds from fully-ripe fruit germinated faster with a higher germination percentage than seeds from under-ripe fruits (Table 1; Figure 5 & 6). Banana seeds are generally known to germinate after 20-21 DAS, either with special treatment or not (Burgos-Hernández et al. 2014). From this study, seeds from fully-ripe fruits initially germinated on the 15th DAS, while seeds from under-ripe fruit germinated three days later on the 18th DAS. More than 50% of seeds from fully-ripe fruits germinated (61.33% ± 2.32%), meanwhile the germination percentage of the seeds from under-ripe fruits were lower (37.33% ± 1.01%). Germination began with the appearance of white shoots through the micropyle, followed by the growth of radicles which then developed into fibrous roots. The roots and shoots showed their optimum growth on the 36th DAS. Seedlings from fully-ripe fruits also had significantly higher root numbers and plant height than seedlings from under-ripe fruits (Table 1). The first leaf of seedlings from the fully-ripe fruit appeared on the 43rd DAS, with the number of roots of 8.76 ± 0.36 on the 50th DAS.

Heterogeneity of seed maturity between and within bunches is considered as important factors for germination potential (Kallow et al. 2021). The level of fruit ripeness is related to physiological quality of seeds (Kaiser et al.

Table 1. Comparison of seed germination result of *M. acuminata* var. *flava* from fully-ripe fruits and under-ripe fruits at 50th DAS.

Observed characters		Seeds from fully-ripe fruits	Seeds from under-ripe fruits
Seed variable	Fresh weight of seeds (25 seeds) (g)	0.94 ± 0.02[a]	1.11 ± 0.38[b]
	Dry weight of seeds (25 seeds) (g)	0.82 ± 0.44[a]	0.97 ± 0.32[b]
	Moisture content of seeds (%)	14.61±1.99[a]	13.69±0.06[a]
Germination & growth variable	Germination percentage (%)	61.33 ± 2.32 [a]	37.33 ± 1.01 [a]
	Number of roots	8.76 ± 0.36[a]	6.62 ± 0.29[b]
	Plant height (cm)	6.65 ± 1.07[a]	3.74 ± 0.72[b]

Note: The same letter in the same line shows no significant difference with independent t-test.

2016; Villa et al. 2019). The results of this study showed that with the advance of the ripening process, seeds extracted from fully-ripe fruits (yellow peel) generated seedlings with higher percentage and faster germination, also more vigorous in growth variables. The seeds from yellow fruits are considered to be fully developed (physiologically mature) compared to the seeds from green-yellow fruits (under-ripe). When the seeds were not completely mature, they could germinate, but did not result in seedlings as vigorous as those harvested at the appropriate ripening time. It was also observed from the lower amount of reserves deposited (endosperms) which may cause a limiting factor for the development of seedlings (Moiwend et al. 2015; Villa et al. 2019). The level of fruit maturity was also reported to affect *in vitro* seed germination percentage of *M. ornata* (Dayarani et al. 2014).

The seed germination type of *M. acuminata* var. *flava* was observed as hypogeal. In this type of germination, the cotyledons remain below the germination media due to rapid elongation of epicotyl (part of the stem above the cotyledon), while the hypocotyl (part of the stem below the cotyledon) remains the same in length. Then, the epicotyl pushes the plumule above the germination media, and followed by the formation of leaves. Most of monocots species considered to have hypogeal germination type (Tillich 2007), including wild banana in this study. In addition, the seeds germinated gradually over time if not simultaneously. It was started to germinate on the 15th DAS and continued until the 27th DAS in almost all replications (Figure 5 & 6).

In the wild, banana seeds buried in the soil might survive for years and germinate due to disturbances, especially after logging in the forest (Chin 1996). The germination may be stimulated by micro-climate changes in relation to disturbance, such as sunlight (due to the opening of forest canopy), moisture regimes, temperature, as well as changes in the ecological community such as predators and dispersers (Kallow et al. 2020). The germination rate is also affected by genetic factors, pollination rate, population size, pollinators availability and environmental conditions during fruit ripening (Kaiser et al. 2016; Fidalgo et al. 2019).

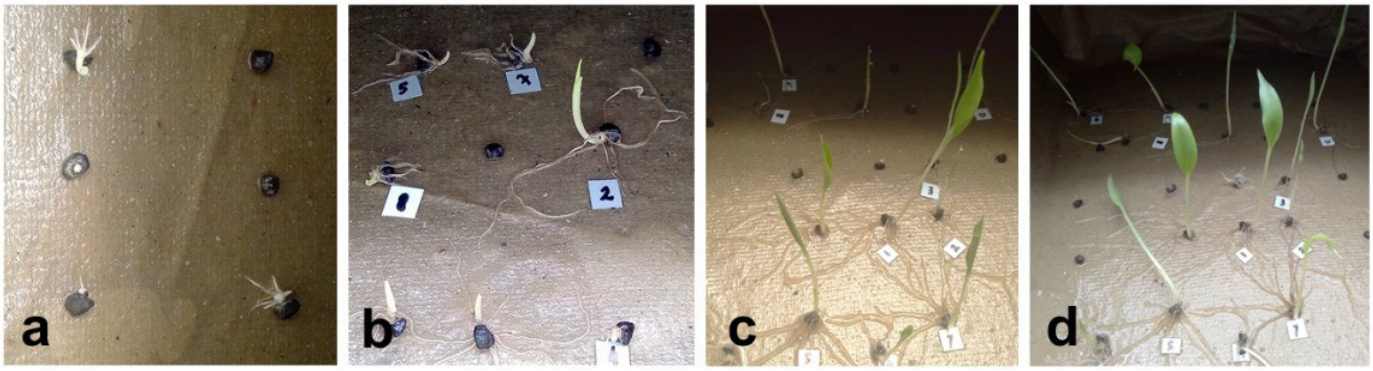

Figure 5. The germination of *M. acuminata* var. *flava* seeds extracted from fully-ripe fruits, a. 22nd DAS, b. 31st DAS, c. 43rd DAS, d. 50th DAS (DAS=Days After Sowing).

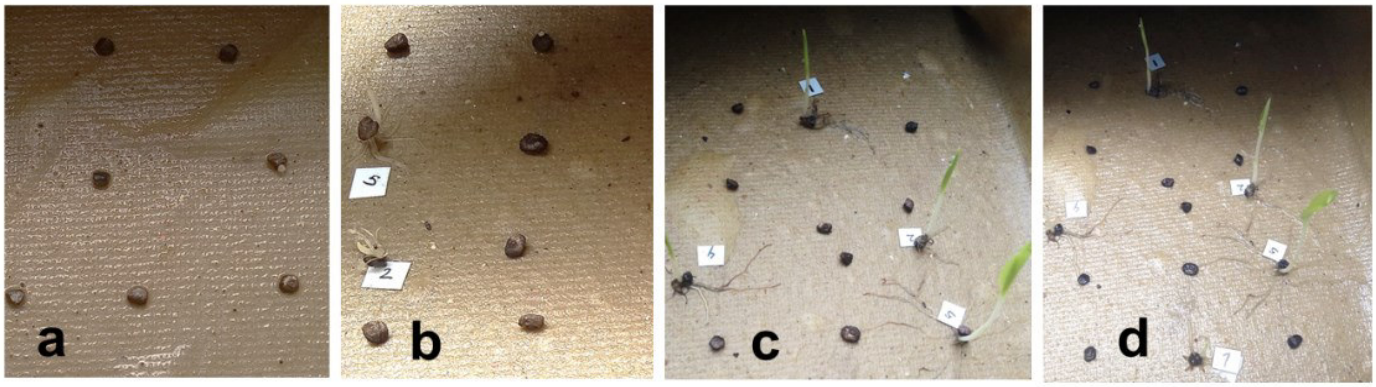

Figure 6. The germination of *M. acuminata* var. *flava* seeds extracted from under-ripe fruits. a. 22nd DAS, b. 31st DAS, c. 43rd DAS, d. 50th DAS (DAS=Days After Sowing).

Implications for further seed conservation efforts and studies of wild bananas

A complete plant morphological characterization is necessary specifically on core collection to confirm the species identity. Because the morphological characteristics of wild bananas are very varied, especially at the infraspecific level, subspecies of *M. acuminata* is also difficult to identify. Therefore the collectors must prioritize to conduct the detail morphological characterization on site preferably using full descriptor for bananas (IPGRI-INIBAP 1996) or minimum descriptor (at least). Considering the time limit during the collecting mission or fieldwork, it is also important to take plenty of photographic documentation of various plant parts, populations and surrounding habitats for further identification and supporting information. Molecular analysis is also required for more advanced study to confirm genetic fidelity by taking leaf samples of each distinctive specimen or population (Hapsari et al. 2020). In addition, since wild bananas can be both propagated generatively and vegetatively, collectors should collect both seeds and suckers to complement each other for *ex-situ* conservation effort.

In seed conservation efforts, physical variables are generally adopted to harvest forest seeds, such as change in fruits and seeds color, size, odor, presence of predators, dispersers and dehiscence of fruit as indicators of ripening (Kaiser et al. 2016). Generally in wild bananas, seeds from fruits of basal

hands are produced and matured first, so that when harvested they are already in a more advanced state compared to the distal hands. The banana fruits from basal hands are faster in changing peel color to yellow and cracking, also the pulp may be softened and rotting with some aromatic odor (Hapsari 2014; Kallow et al. 2020). However, obtaining perfectly mature bunch of fruits during collecting missions is very challenging. Whereas, collecting mature fruits from field germplasm collections is more manageable in term of harvest time. Thus, if possible, initial survey should be conducted to manage the readiness of the population for seed collection. For seed collection management, it is suggested to apply different accession numbers of each bunch from different individual plants. Furthermore, to lower the heterogeneity in further studies (as highlighted from this study), the seeds from fully-ripe fruits (yellow peel) are suggested to be separated from under-ripe fruits (green, green-yellow peel) within a bunch, or fruits from basal hands are separated from distal hands (at least). It is not recommended to bulk or mix up the seeds within a bunch of wild banana fruits.

CONCLUSION

The most prominent plant morphological characteristics of wild banana *M. acuminata var. flava* are the green-yellow male bud and bracts. The seeds are 20-170 seeds per fruit, wrinkled surface and dark brown-black when ripe. The seed had a thick and hard coat with a small embryo; and was classified as having orthodox storage behavior. Fresh seeds were germinated in two weeks after sowing, with hypogeal type and gradual germination pattern. The level of fruit ripeness was significantly affected the seed germination percentage, and growth variables *i.e.* plant height and number of roots. The seeds from fully-ripe fruit were considered more mature physiologically than the seeds from under-ripe fruits. When the seeds were not completely mature, they could germinate, but did not result in seedlings as vigorous as those harvested at the appropriate ripening time. For further conservation, storage, propagation, and related studies on seeds of wild bananas; it is suggested to clearly define and separate the seeds from the fully-ripe fruits out of the under-ripe fruits to lower the heterogeneity. Further studies on germination of periodically storage seeds of this species are required to confirm the behavior character of the storage. Germination studies by embryo culture method are also suggested in order to overcome the medium-low germination percentage of this species by conventional seed germination method.

AUTHORS CONTRIBUTION

All authors contributed equally from conceptualization, writing, review and editing of the final manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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RESEARCH ARTICLE

In Silico Study of the Active Compounds of *Lindera aggregata* (Sims) Kosterm as Anti-coronavirus

Elok R. Firdiana[1,*], Elga Renjana[1], Linda W. Ningrum[1], Melisnawati H. Angio[1], Muhamad Nikmatullah[2] and Syaiful Rizal[2]

[1]*Research Center for Plants and Botanic Garden Conservation, Indonesian Institute of Sciences, Bogor, Indonesia;*
[2]*Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia*

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Abstract: ***Background*:** CoVID-19, caused by a new type of coronavirus named SARS-CoV-2, has become a pandemic. Together with SARS-CoV and MERS-CoV, CoVID-19 is a large global outbreak of coronavirus infection; however, its rate of spread is much higher. Since the vaccines and anti-SARS-CoV-2 have not been found, a faster control mechanism is much needed. Traditional herbs have shown the potential for this purpose, as has been demonstrated by the Chinese Government with a high success rate. One of the herbs used was *Lindera aggregata*, which is part of the collection in Purwodadi Botanic Gardens.

***Objectives*:** Through *in silico* study, this research aims to reveal the secondary metabolites contained in *L. aggregata* that have the potential to serve as anti-SARS-CoV-2 medication as well as showcase their inhibitory mechanisms.

***Methods*:** The research was conducted through molecular docking analysis of terpenoids and alkaloids contained in the root of *L. aggregata*, with target proteins 3CLpro, PLpro, Spike, and ACE 2 playing a role in SARS-CoV-2 infection.

***Result*:** All analyzed compounds tended to interact with all four target proteins with different binding affinity values, but the interaction seemed stronger with 3CLpro and Spike. Terpenoids, linderane and lindera lactone had the strongest interaction tendency with 3CLpro, PLpro, and Spike; the compound norboldine, an alkaloid, had the strongest interaction with ACE 2, with a binding affinity value of -8.2 kcal/mol.

***Conclusion*:** Terpenoids and alkaloids contained in the root of *L. aggregata*, which caused inhibition of adsorption and replication of SARS-CoV-2, could serve as anti-SARS-CoV-2.

Keywords: Active compound, anti-coronavirus, CoVID-19, *in silico*, *Lindera aggregata*, molecular docking.

1. INTRODUCTION

Coronavirus 2019 (CoVID-19) has become a pandemic in more than 100 countries within a few weeks [1]. As of May 3, 2020, it caused 3,349,786 positive cases with 238,628 deaths in 212 countries [2]. In Indonesia, since the first report in February 2020, there have been 11,587 positive cases, and 864 deaths, leading to a mortality rate of 7.4% in just two months [3].

CoVID-19 was first reported in Wuhan, China, with the main symptom being fever of more than 38°C followed by breathing difficulties and other symptoms resembling pneumonia [4]. The sequencing analysis of samples obtained from the lower respiratory tract of patients indicated that the cause was a new type of coronavirus, named 2019 novel coronavirus (2019-nCoV) [5].

Coronavirus is an RNA virus belonging to Nidovirales family Coronaviridae and spreads in humans and other mammals [6]. Sequencing analysis shows that 2019-nCoV has a unique coronavirus genome structure and is included in the betacoronavirus cluster with Bat-SARS-like (SL) -ZC45, Bat-SL ZXC21, Severe acute respiratory coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) [7]. Based on these data and other in-depth support studies of phylogeny and taxonomy, the Coronaviridae Study Group gave 2019-nCoV the name SARS-CoV-2 [8].

Although most coronavirus infections are self-limited diseases and bear only mild symptoms, the epidemic of two betacoronaviruses, SARS-CoV [9] and MERS-CoV [10, 11], had caused more than 10,000 cumulative cases in the last two decades, with a mortality rate of 9.6% for SARS-

* Address correspondence to this author at the Research Center for Plants and Botanic Garden Conservation, Indonesian Institute of Sciences, Jalan Ir. H. Juanda No. 13, Bogor 16122, Indonesia; Tel: (0251) 8311362 - 8336871; E-mail: elok.firdiana@gmail.com

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CoV and 34.4% for MERS-CoV [12, 13]. Similar to SARS-CoV and MERS-CoV, CoVID-19 has been a large global outbreak of coronavirus infection; however, its rate of spread is faster than SARS-CoV and MERS-CoV: SARS-CoV spread in 32 countries in a period of nine months and MERS-CoV spread in 27 countries in a period of eight years, while CoVID-19 spread in 27 countries in only two months [14].

Thus far, the most effective effort to deal with viral infections is the provision of specific vaccines and antiviral agents. However, to date, neither of these has been available for the CoVID-19 outbreak. The development of this treatment requires months, even years, while CoVID-19 is spreading at a fast pace. Therefore, a rapid control mechanism is necessary [15]. Traditional herbs are a potential source for achieving this goal. The successful use of traditional herbs in overcoming the SARS outbreak in 2002 has inspired the Chinese government to implement the same policy in dealing with CoVID-19. This is supported by the homology in SARS-CoV with SARS-CoV-2. Furthermore, Yang *et al.* [16] stated that this policy of handling CoVID-19 was more than 90% effective.

Lindera aggregata (Sims) Kosterm. is a traditional Chinese herb species. It belongs to the family Lauraceae and is distributed over South China, Japan, and Southeast Asia [17]. Its dried roots have been used in China and Japan for hundreds of years to treat heart, kidney, cyst, and rheumatic diseases [18]. Phytochemical investigation showed that *L. aggregata* has antitumor, anti-inflammatory, antiviral, antimicrobial, and antidiabetic activities [19]. More specifically, an *in vitro* study conducted by Li *et al.* [20] proved that *L. aggregata* was able to inhibit SARS-CoV propagated on Vero E6 cells, but its inhibitory mechanism is not yet clearly known.

The SARS-CoV-2 genome sequence has been published, and it demonstrates that SARS-CoV-2 has a high level of similarity with previously known SARS-CoV and MERS-CoV [21, 22]. Thus, most of the proteins used for its adsorption and replication are also similar to SARS-CoV and MERS-CoV. The proteins in coronavirus that are studied extensively hitherto include papain-like protease (PLpro), 3C-like protease (3CLpro), and Spike protein. PLpro and 3CLpro play an important role in coronavirus replication [23-25], while Spike is used by coronavirus to insert genetic material into host cells through angiotensin-converting enzyme 2 (ACE 2) as the receptor [26].

The anti-coronavirus potential of compounds contained in *L. aggregata* can be explored through the *in silico* method. This preliminary method is often used to ascertain the potential of an active compound through computer simulations. The predictions, hypotheses, new findings, or new advances in discovering therapeutic drugs could be provided [27]. Previous studies have shown that *in silico* studies helped screen and confirm direct inhibition of PLpro, 3CLpro, and Spike in SARS and MERS [28-34]. Therefore, *in silico* analysis can also be applied to determine the antiviral activity of compounds contained in the roots of *L. aggregata*, and hence would strengthen the basis for using *L. aggregata* in the handling of CoVID-19.

In Indonesia, although the use of *L. aggregata* as a medicinal plant has not yet been reported, it is among the collection of Purwodadi Botanic Gardens, as a part of its effort towards *ex situ* conservation. Thus, this study aims to uncover the types of compounds contained in *L. aggregata* that have the potential to serve as anti-coronavirus medication as well as to learn about their inhibitory mechanism. This study is expected to increase the value of one of the collections of Purwodadi Botanic Gardens, thereby improving its conservation efforts.

2. MATERIALS AND METHODS

Establishment of *L. aggregata* as a source species for potential anti-SARS-CoV-2 compounds was based on the research of Li *et al.* [20]. Its existence in Purwodadi Botanic Gardens' collection was confirmed by the catalog of living collections. The subsequent stages are mentioned below:

2.1. Search for Chemical Compounds of *Lindera aggregata*

The chemical compounds contained in *L. aggregata* were searched through the website http://www.knapsackfamily.com/ [35] and a literature study was conducted through Google Scholar with the keywords "*Lindera aggregata*" and "natural compound". A web search was also conducted on https://pubchem.ncbi.nlm.nih.gov/ [36] to obtain the structure of the chemical compounds contained in *L. aggregata*. The 3D structure of each compound was downloaded and saved in a structure data file (SDF) format.

2.2. Determination of Target Proteins

The composition of each target protein used in this study, namely 3CLpro (1Z1J_B), PLpro (4MM3_B), Spike (APF29063.1), and ACE 2 (BAB40370.1), was obtained from https://www.ncbi.nlm.nih.gov/protein [37]. The FASTA format of each target protein was downloaded and saved in the text document (txt) format. Afterward, the preparation of the 3D structure of each target protein was carried out through https://swissmodel.expasy.org/ [38] by inputting the FASTA target protein file. The 3D structure modeling of the protein was then downloaded and saved in a protein database (PDB) format.

2.3. Anti-coronavirus Screening of Chemical Compounds of *Lindera aggregata*

The screening was conducted by the molecular protein docking analysis using PyRx software version 0.8 [39]. Before docking, the 3D structure of each chemical compound was minimized in the same software and saved in PDB format. Then, the docking process was performed with the chemical compounds of *L. aggregata* as ligand and the target proteins as macromolecule. The results of the molecular docking protein analysis were saved in PDB format.

2.4. Data Analysis

The results of molecular docking were analyzed using PyMOL software (32 bit) version 1.3.0.0 to determine the interaction between each chemical compound of *L. aggregata* and each target protein. Thereafter, the best interactions were determined by the best binding affinity value.

3. RESULTS

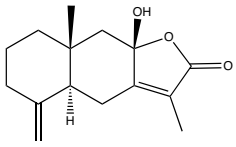
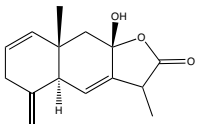
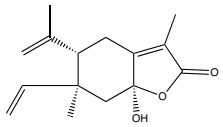
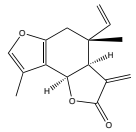
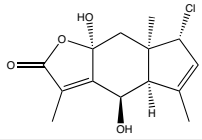
Based on the information acquired from http://www.knapsackfamily.com/, the root of *L. aggregata* contains 14 terpenoid compounds. However, a previous study [19] stated that the antiviral property of *L. aggregata* was due to its alkaloid contents such as norboldine, pallidine, and reticuline. Therefore, docking molecular protein analysis was undertaken on 14 terpenoids and three other alkaloids. The results of the molecular docking protein analysis showed that all compounds of *L. aggregata* interacted with the four target proteins with different binding affinity values Table **1**.

On comparing the binding value of each compound with all four target proteins, it was found that in the terpenoid group, only linderagalactone A and linderane tended to interact more strongly with 3CLpro, and 12 other compounds tended to interact more strongly with Spike; while in the alkaloid group, norboldine tended to interact more strongly with Spike and pallidine with 3CLpro. It was interesting to find that reticuline tended to interact with 3CLpro and Spike with the same binding affinity value of -7.5 kcal/mol. Although the binding values were low, all compounds showed interactions with PLpro and ACE 2 Table **1**.

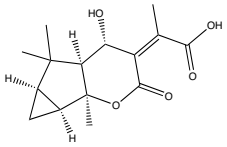
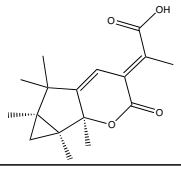
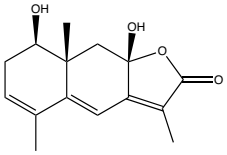
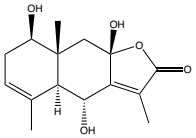
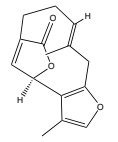
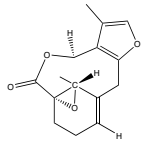
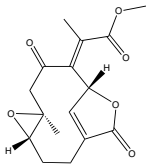
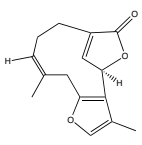
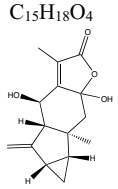
By comparing the binding affinity value of each target protein with all the compounds, it was inferred that the two terpenoids, linderane and linderalactone had the strongest interaction tendency with 3CLpro, PLpro, and Spike, while the compound norboldine, an alkaloid, had the strongest interaction with ACE 2, with a binding affinity value of -8.2 kcal/mol Table **1**.

The four best interactions of a compound and target protein were then visualized. Linderane formed the best conformation with 3CLpro and PLpro. In 3CLpro, conformation occurred through the binding of hydrogen bonds to the amino acids ASN-151, GLN-110, THR-292, and THR-111 (Fig. **1A**), while in PLpro, linderane formed conformation through the binding of hydrogen bonds to the amino acid ARG-167 (Fig. **1B**). Linderalactone had the strongest interaction with Spike, with a binding affinity of -9.5 kcal/mol. However, there seemed to be no hydrogen bond formed in

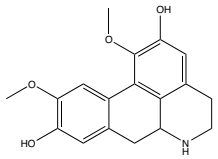
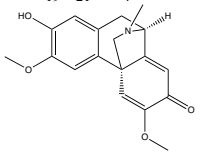
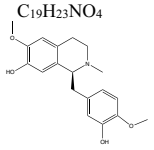
Table 1. The binding affinity values of compounds of *L. aggregata* with proteins in docking analysis.

Type	Compound (IUPAC name)	References	Molecular Formula & Chemical Structure	Pubchem CID	Binding Affinity (kcal/mol)			
					3CLpro	PLpro	Spike	ACE 2
Terpenoid	8beta-hydroxyasterolide (4*aS*,8*aR*,9*aS*)-9*a*-hydroxy-3,8*a*-dimethyl-5-methylidene-4,4*a*,6,7,8,9-hexahydrobenzo[f] [[1]]benzofuran-2-one)	[35]	$C_{15}H_{20}O_3$ 	155948	-7.4	-6.3	-8.5*	-7.5
	8-hydroxylindestenolide (4*aS*,8*aS*,9*aS*)-9*a*-hydroxy-3,8*a*-dimethyl-5-methylidene-3,4*a*,6,9-tetrahydrobenzo[f] [1]benzofuran-2-one)	[35]	$C_{15}H_{18}O_3$ 	46883415	-7.7	-6.2	-8.7*	-7.7
	Hydroxyisogermafurenolide (5*S*,6*S*,7*aS*)-6-ethenyl-7*a*-hydroxy-3,6-dimethyl-5-prop-1-en-2-yl-5,7-dihydro-4*H*-1-benzofuran-2-one)	[35]	$C_{15}H_{20}O_3$ 	14038425	-6.6	-6.2	-7.9*	-6.8
	Isolinderagalactone (3*aS*,4*R*,8*bR*)-4-ethenyl-4,8-dimethyl-3-methylidene-5,8*b*-dihydro-3*aH*-furo[2,3-e] [[1]]benzofuran-2-one)	[35]	$C_{15}H_{16}O_3$ 	5318587	-7.1	-5.9	-9.1*	-7.4
	Linderagalactone A ((4*R*,4*aR*,7*R*,7*aR*,8*aS*)-7-(chloromethyl)-4,8*a*-dihydroxy-3,5,7*a*-trimethyl-4,4*a*,7,8-tetrahydrocyclopenta[f] [[1]]benzofuran-2-one)	[35]	$C_{15}H_{19}ClO_4$ 	44254425	-7.2*	-5.9	-6.5	-6.5

(Table 1) contd....

Terpenoid	Linderagalactone B ((2*Z*)-2-[(1*S*,2*S*,4*R*,6*S*,7*S*)-7-hydroxy-1,5,5-trimethyl-9-oxo-10-oxatricyclo[4.4.0.0^{2,4}]decan-8-ylidene]propanoic acid)	[35]	$C_{15}H_{20}O_5$ 	44254423	-6.9	-6.4	-8.0*	-6.9
	Linderagalactone C ((2*E*)-2-[(1*S*,2*S*,4*R*)-1,5,5-trimethyl-9-oxo-10-oxatricyclo[4.4.0.0^{2,4}]dec-6-en-8-ylidene]propanoic acid)	[35]	$C_{15}H_{18}O_4$ 	102597495	-7.2	-6.0	-8.4*	-7.4
	Linderagalactone D ((8*R*,8*aR*,9*aS*)-8,9*a*-dihydroxy-3,5,8*a*-trimethyl-8,9-dihydro-7*H*-benzo[f][[1]]benzofuran-2-one)	[35]	$C_{15}H_{18}O_4$ 	44254427	-7.7	-6.2	-8.7*	-7.2
	Linderagalactone E ((4*R*,4*aS*,8*R*,8*aR*)-4,8,9*a*-trihydroxy-3,5,8*a*-trimethyl-4*a*,7,8,9-tetrahydro-4*H*-benzo[f][[1]]benzofuran-2-one)	[35]	$C_{15}H_{20}O_9$ 	44254424	-7.3	-6.5	-8.2*	-7.3
	Linderalactone ((1*R*,8*E*)-3,8-dimethyl-5,14-dioxatricyclo[10.2.1.0^{2,6}]pentadeca-2(6),3,8,12(15)-tetraen-13-one)	[35]	$C_{15}H_{16}O_3$ 	6450191	-7.4	-6.3	-9.5*	-7.7
	Linderane ((1*S*,4*E*,12*S*,13*S*)-5,10-dimethyl-8,14,16-trioxatetracyclo[10.2.2.0^{1,13}.0^{7,11}]hexadeca-4,7(11),9-trien-15-one)	[35]	$C_{15}H_{16}O_4$ 	6915739	-8.0*	-6.6	-7.3	-7.4
	Neolindenenonelactone (methyl (2*E*)-2-[(4*S*,6*S*,10*R*)-6-methyl-8,12-dioxo-5,11-dioxatricyclo[8.2.1.0^{4,6}]tridec-1(13)-en-9-ylidene]propanoate)	[35]	$C_{16}H_{18}O_6$ 	102595622	-7.6	-6.1	-8.2*	-6.4
	Neolinderalactone ((1*R*,8*Z*)-3,8-dimethyl-5,14-dioxatricyclo[10.2.1.0^{2,6}]pentadeca-2(6),3,8,12(15)-tetraen-13-one)	[35]	$C_{15}H_{16}O_3$ 	12311262	-7.2	-6.1	-8.9*	-7.4
	Strychnistenolide ((1*S*,2*R*,9*S*,10*R*,12*S*)-2,7-dihydroxy-4,9-dimethyl-13-methylidene-6-oxatetracyclo[7.4.0.0^{3,7}.0^{10,12}]tridec-3-en-5-one)	[35]	$C_{15}H_{18}O_4$ 	15484712	-7.9	-6.3	-8.7*	-7.5

(Table 1) contd....

Alkaloid	Norboldine (1,10-dimethoxy-5,6,6*a*,7-tetrahydro-4*H*-dibenzo [de,g]quinoline-2,9- diol)	[40]	$C_{18}H_{19}NO_4$ 	628557	-7.9	-6.2	-8.3*	-8.2
	Pallidine ((1*S*,9*S*)-5-hydroxy-4,13-dimethoxy-17-methyl-17-azatetracyclo [7.5.3.$0^{1,10}$.$0^{2,7}$]heptadeca-2,4,6,10,13-pentaen-12-one)	[41]	$C_{19}H_{21}NO_4$ 	12313923	-7.7*	-6.5	-7.2	-5.9
	Reticuline ((1*S*)-1-[(3-hydroxy-4-methoxyphenyl)methyl]-6-methoxy-2-methyl-3,4-dihydro-1*H*-isoquinolin-7-ol)	[41]	$C_{19}H_{23}NO_4$ 	439653	-7.5*	-6.1	-7.5*	-7.1

Notes:
* The best binding affinity values of interaction of each compound with all four-target proteins.
[a] The best binding affinity values of interaction of each target protein with all compounds.

the conformation (Fig. **1C**); hence, it was assumed that the interaction formed was non-covalent or non-bonded interaction. According to Knegtel & Grootenhius [42], a non-bonded interaction occurred between ligands and proteins owing to electrostatic and van der Waals interactions, thereby affecting the binding affinity. Meanwhile, norboldine, compound of *L. aggregata*, formed the best conformation with ACE 2, where the hydrogen bonds interacted with amino acids ASN-674, LEU-675, ARG-482, and HIS-493 (Fig. **1D**).

4. DISCUSSION

Previous studies stated that *L. aggregata* contained four main bioactive compounds, *i.e.* alkaloids [43-45], terpenoids [46], flavonoids [47], and tannins [48]. Although Iwasa *et al.* [19] stated that among the four groups of compounds, the antiviral property of *L. aggregata* was caused by the presence of alkaloids, this study showed that the terpenoids also increased the potential of *L. aggregata* as an antiviral, especially anti-SARS-CoV-2. Moreover, the two terpenoid compounds, linderane and lindera lactone, had the highest inhibitory activity on the 3CLpro, PLpro, and Spike found in SARS-CoV-2. Conversely, the alkaloids of *L. aggregata* played an important role in the inhibition of ACE 2 receptors as the attachment site for SARS-CoV-2. Thus, both compounds contributed significantly to anti-SARS--CoV-2 activity.

As mentioned by Li *et al.* [20], the aqueous root extract of *L. aggregata* showed antiviral activity of SARS-CoV *in vitro* ; however, the mechanism was not explained in detail. By observing this study's screening results, the anti-coronavirus activity specifically anti-SARS-CoV-2 in *L. aggregata* compounds might be predicted to occur through two mechanisms. In the first mechanism, antiviral activity occurred through the blocking of Spike and ACE 2 receptors, which made them unable to interact with each other, thereby preventing the attachment and penetration of the genetic material of SARS-CoV-2 into the host cell. In the second mechanism, antiviral activity occurred through the inhibition of SARS-CoV-2 replication by inactivating 3CLpro and PLpro. Thus, the two inhibitory mechanisms might restrict SARS-CoV-2 mobility both inside and outside the host cell, thereby suppressing the viral infections. In addition, the immobilization of SARS-CoV-2 would also help the immune system's performance in recognizing the virus to allow antibodies to be formed immediately.

In addition to the research conducted by Li *et al.* [20] using *L. aggregata* extracts on Vero E6 cell lines, there have been no *in vitro* studies of *L. aggregata* compounds regarding their activity as anti-coronavirus. However, the compounds having the greatest potential as anti-coronavirus in this study, namely linderalactone, linderane, and norboldine, have been shown to have other benefits as well. Linderalactone was reported to inhibit the proliferation of lung-cancer cells A-549 with IC50 of 15 μM [49]; linderane played a role in anti-inflammatory [50] and pancreatic protection from oxidative damage caused by streptozotocin [51]; and norboldine showed the potential as a drug for pancreatic cancer as it was cytotoxic for BxPC-3 pancreatic cancer cell lines with IC50 of 27.060 ± 1.037 μM [52].

To date, only the root of *L. aggregata* is frequently used in traditional medicine and research for its antiviral activity; however, other parts also have the potential as a drug, owing to the presence of active compounds. Wang *et al.* [53] proved that aqueous leaf extract of *L. aggregata* contains quercitrin which is useful as an antihyperlipidemic agent. In addition, Gu *et al.* [54] also reported that *L. aggregata* leaf extract contains flavonoids, which act as antioxidants and hepatoprotective agents. In addition to roots and leaves, it turns out that *L. aggregata* stems also contain tannin that is proven to inhibit the enzyme integrase in HIV [55].

This study also revealed the potential of plants as medicines (herbs). Herbs have long been used as the main source

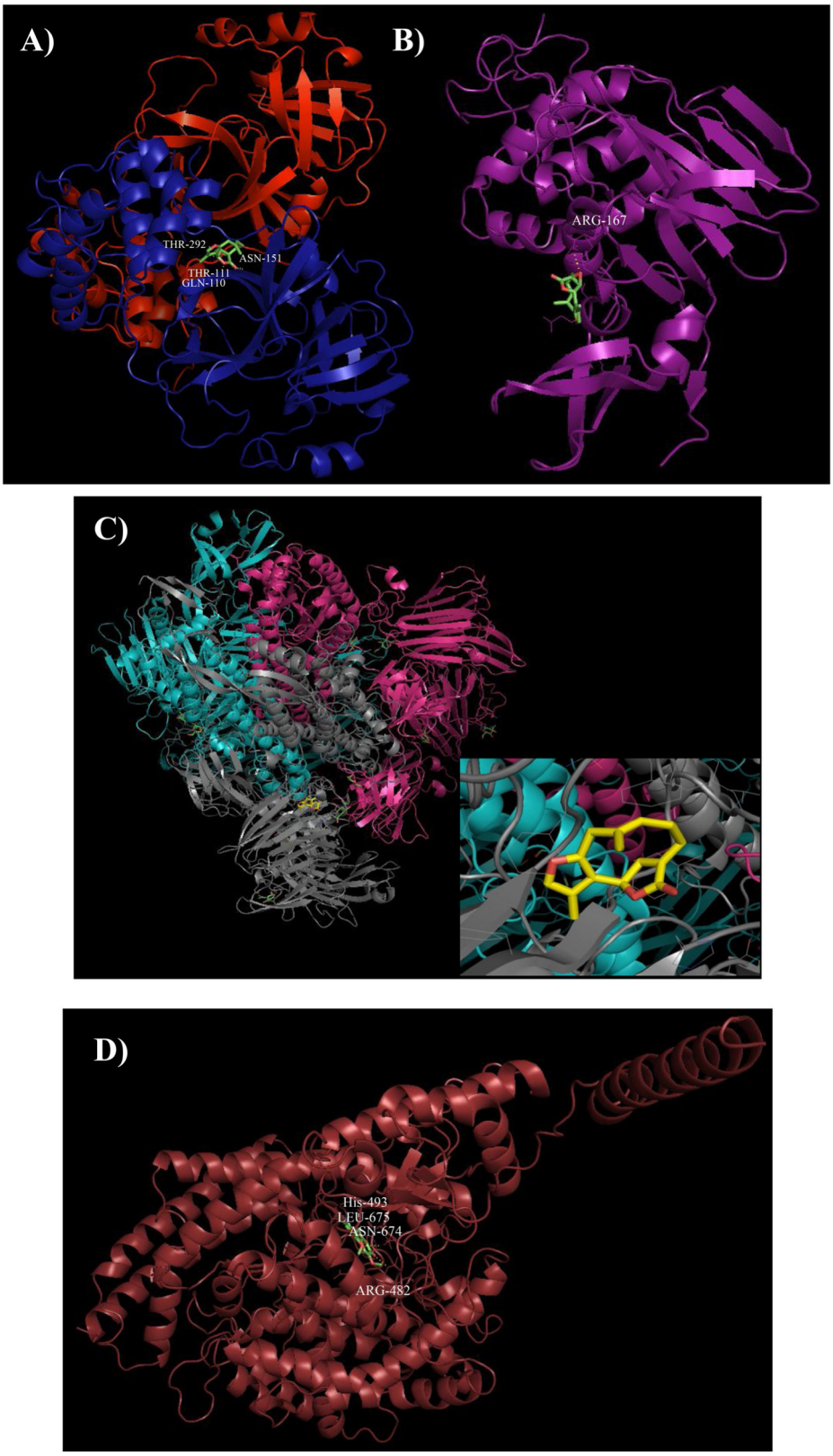


Fig. (1). Visualization of molecular docking between the compounds of *L. aggregata* and target proteins. (**A**) linderane and 3CLpro; (**B**) linderane and PLpro; (**C**) linderalactone and Spike; (**D**) norboldine and ACE 2. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

of disease management around the world, owing to the active substances they contain [56-61]. For example, in the case of *L. aggregata* used in this study. This plant with the habitus of shrubs and small trees, colloquially called Wu Yao in Chinese, has a very broad spectrum of utilization [62]. Besides being used as a stomachache medicine by the local community of Huanjiang Maonan, Southwest China [63], this plant and other *Lindera* species have also been reported to improve blood circulation and to prevent liver damage [64] as well as to treat various diseases such as arthritis, stroke, cholera, diarrhea, and toothache [65-67]. All of these benefits are on account of their biological activities including cytotoxic [68], antihypertensive [69], and antioxidants [70, 71].

In the living collections catalog of Purwodadi Botanic Gardens [72], it is mentioned that *L. aggregata* is one of the living collections of the Purwodadi Botanic Gardens, planted in Vak XVII.B with collection number 004-004a-004bcde. It is one of the 2,140 species of the collection whose potential uses have not been known. With the screening results of antiviral potential in this study, it is expected that the utilization and conservation of *L. aggregata* might be enhanced, and it might also pave the way for the screening of other Purwodadi Botanic Gardens collections.

CONCLUSION

The terpenoids and alkaloids contained in *L. aggregata* showed the potential inhibition of target proteins that have a role in SARS-CoV-2 infection. Two terpenoids, linderane and linderalactone, showed the strongest inhibition of 3CLpro, PLpro, and Spike, while one of the alkaloids, norboldine, showed the strongest inhibition against ACE 2. Thus, *L. aggregata* has the potential to be anti-SARS-CoV-2 owing to the prevention of SARS-CoV-2 adsorption and replication. In keeping with this research, the mechanism of anti-SARS-CoV-2 activity possessed by *L. aggregata in vitro* could be further explored in depth, and it is expected that clinical trials could be performed on animals to identify other effects. In addition, the disclosure of the potential of *L. aggregata* has paved the way for further research seeking to explore the potential of other plant collections of Purwodadi Botanic Gardens.

AUTHORS' CONTRIBUTION

All individuals listed as authors have significantly contributed to the writing of this manuscript. Their responsibilities were as follows: ERF and ER designed and wrote the manuscript; ER and SR conducted the molecular docking; ERF, ER, and SR analyzed data; LWN, MHA, and MN edited the manuscript and added the content in accordance with their field of interest; ERF had the primary responsibility for the final content of the manuscript; and all the authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors have no conflicts of interest, financial or otherwise.

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Declared none.

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