Original research article

Impact of Domestication on the Endophytic Fungal Diversity Associated With Wild *Zingiberaceae* at Mount Halimun Salak National Park

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ABSTRACT

Mount Halimun Salak National Park is one of the tropical forest remnants in Java island. The national park has been recognized with high diversity of wild *Zingiberaceae*. Of that *Zingiberaceae*, two species namely *Alpinia malaccensis* (AM) and *Horstendia conica* (HC), were domesticated as garden plants in the surrounding area of the forest for medicinal use. The impact of domestication on the fungal endophytes associated with these two species of *Zingiberaceae* is reported here. Fungal endophyte diversity in the wild and domesticated AM and HC was analyzed based on the culturable fungi. Identification of species level used morphological and molecular approaches of ITS rDNA sequence. This study determined 19 species of fungal endophytes, namely *Arthrinium malaysianum*, *Aspergillus flavipes*, *As. sydowii*, *Chaetomium globosum*, *Cladosporium oxysporum*, *Cladosporium* sp., *Colletotrichum boninense*-complex, *Co. cliviae*-complex, *Co. gloeosporioides*-complex, *Diaporthe* sp., *D. anacardii*, *D. gardenia*, *Exophiala* sp., *E. lecanii-corni*, *Guignardia mangiferae*, *Ochroconis gallopava*, *Penicillium citrinum, Pyricularia costina*, and unsporulated *Sydowiellaceae*. Among them, *A. malaysianum*, *C. globosum*, *Co. cliviae*-complex, *D. gardenia*, and unsporulated *Sydowiellaceae* were only found in domesticated plants, while some others were absent. *Colletotrichum boninense*-complex was commonly found in both wild and domesticated plants. Domestication activity affected the diversity of endophytic fungi of AM and HC.

1. Introduction

Human activity, such as domestication of plant, causes a complex evolutionary process of plant species which may lead to morphological and physiological changes that distinguish the domesticated plant from its wild ancestor (Hancock 2005). During plant domestication process, genetic variation of the domesticated plant probably occurs due to genes shifting. Domestication for any agricultural purposes has had a profound impact on nature, particularly on biodiversity (Ceccarelli 2009). Microorganism communities associated with the plant, in particular fungi, are often ignored during the process of domestication of their host. These include changes in diversity of fungal endophytes on domesticated plant and their functional roles. Besides, endophytic fungal communities living within plant tissue produce significant impacts on plant communities through increasing fitness to abiotic and biotic stress, increasing biomass and decreasing water consumption, and so forth (Rodriguez *et al.* 2008). Moreover, a strong genetic differentiation between populations from wild and cultivated hosts can lead to marked shifts in the evolutionary dynamics of host-microbe interactions by environmental differences existing between natural and domesticated habitats (Lebarbenchon *et al.* 2008; Stukenbrock and McDonald 2008).

All plants associated with various endophytic fungal communities (Arnold 2007). Yet, little information has been known regarding the effects of domestication to the fungal communities living within the tissue of their host plants, and on how the fungal endophytes transmitted. Even though Schulz and Boyle (2005) thought that most endophytes are transmitted horizontally from plant to plant, rather than vertically as found in Clavicipitaceous endophytes (Saunders *et al.* 2010). Furthermore, Munkacsi *et al.* (2008) reported that *Ustilago maydis*, pathogen on maize, appeared to have followed their host along the domestication course through a process called host-tracking.

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Information regarding fungal endophyte assemblages on wild and domesticated plants is limited. Hoffman and Arnold (2008) had only described important effects of geographic locality on endophyte community structure, even among closely related host species. Therefore, it is necessary to examine differences on endophytic fungal communities associated with wild and domesticated plants to understand their functional role to the host plants.

Members of *Zingiberaceae* have traditionally been known for their medicinal uses in Indonesia. In the previous study, Priyadi et al. (2010) reported that, at least, 10 species of wild *Zingiberaceae* are distributed in Mount Halimun Salak National Park (MHSNP) and several species have been domesticated by local people. Previous studies on fungal diversity on zingiberaceous plants noted that a high diversity of fungi with more than 170 species from 17 genera have been described (Bussaban et al. 2002; Hyde et al. 2007). These include endophytes, saprobes and pathogens. No information was found on the impact of host domestication to their fungal endophytes. In this study, fungal endophytes assemblages associated with wild and cultivated zingiberaceous plants were examined to understand the impact of host domestication to the diversity of its fungal endophytes. It is expected that information from the current study would lead to a wider consideration of domestication strategy for herbaceous plants in Indonesia.

2. Materials and methods

2.1. Plant materials

Collection of samples was carried out in February 2013. Two species of zingiberaceous plants i.e. *Alpinia malaccensis* (AM; local name: Raja Gowah) and *Hornstedtia conica* (local name: Pinding Hijau) were selected for this study. Samples of wild and cultivated plants were collected from MHSNP and backyard in the village surrounding the forest.

2.2. Isolation of endophytic fungi

Endophytic fungi were isolated according to Okane et al. (2008). Dried samples were cut into 4 segments (0.5×0.5 cm^2) and put on half-strength malt extract agar and incubated at 25 °C for 1−2 weeks. Mycelia that was growing from each plant segments was isolated and purified by hyphal tip method using fine tungsten needle in the low carbon agar and then potato dextrose agar media.

2.3. Morphotyping

The fungi were firstly grouped on the bases of their colony appearance on potato dextrose agar such as colony shape, color, elevation, texture, mycelia type, edges, density, and diameter. Fungal colonies with similar characteristics were grouped into the same morphotypes. Microscopic observation was also carried out to verify the morphotyping by using OLYMPUS BX53 (Olympus, Japan).

2.4. Molecular identification: DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Representatives of each morphotype were selected for further morphological and molecular determination. Molecular identification of the fungal endophytes was based on analysis of sequence generated from ITS rDNA region. Fungal genomic DNA was isolated from mycelium grown in PDB for 7 days at room temperature. Mycelia were harvested and put into 2 mL plastic tubes by using a spatula. DNA extraction was performed by PhytoPure™ DNA extraction kit (Amersham International, Buckinghamshire, UK) according to the manufacture's protocol. Amplification was performed at a total volume 25 μL with the following composition: 10 μL nuclease free water, 12.5 μL Go taq green mastermix™

(Promega, USA), 0.5 μL of each ITS5 (5′-GGAAGTAAAAGTCGTAA-CAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), 0.5 μL dimethyl sulfoxide, and 1 μL of DNA template. Thermal cycle condition was set as follows: 90 seconds of initial denaturation at 95 °C, followed by 35 cycles of 30-second denaturation at 94 °C, 30 seconds annealing at 52 °C, 90 seconds of extension at 72 °C, and a final 5 minutes of extension at 72 °C. A similar protocol was used to amplify EF region using primer pairs of EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAG-GAACCCTT ACC3′). Amplification for EF-1α also performed at a total volume of 25 μL as follow: 2.5 μL of PCR products from each PCR reaction were examined by electrophoresis in a 1% (w/v) agarose gel in $1\times$ TAE buffer (0.4 M Tris, 50 mM NaOAc, 10 mM EDTA, pH 7.8) at 93 V for 25 minutes and visualized under ultraviolet light after staining with ethidium bromide for 20 minutes. The PCR products were sent to 1st BASE (Malaysia) for sequencing.

2.5. Phylogenetic analysis

BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the sequences were done to get the closest generic name. For species identification, ITS sequence data were aligned with the reference strains in the corresponding genus and outgroup, except for *Aspergillus*. The reference strains were cited from Asgari and Zare (2011); Brensch et al. (2012); Busssaban et al. (2005); Cannon et al. (2012); Crous and Groenewald (2013); Glienke et al. (2011); Gomes et al. (2013); Houbraken et al. (2010); Kruys and Castlebury (2012); Martin-Shanchez et al. (2012); Seyedmousavi et al. (2011); Suh et al. (2012); and Thompson et al. (2011). The phylogenetic trees were reconstructed on the bases of their maximum likelihood calculation in the MEGA version 5.0 (Tamura et al. 2011). All parameters were set as default with 1000 bootstrap replication. Phylogenetic tree using mixed data of ITS and EF was reconstructed for accurate identification.

2.6. Diversity and clustering analysis

Endophyte diversity is stated as frequency of fungal occurrence (one isolate is considered as one individual species) of each fungal endophytes species in different host ecosystems and in host organ. The diversity was also stated as Shannon-Wiener diversity index. This index was calculated using the following equation:

$$Hs = - \sum_{i=1}^{s} pi \cdot \ln pi$$

where *Hs* is species diversity, *s* is the number of species in the sample, *Pi* is the relative abundance of ith species $= n/N$, *N* is the total number of individuals of all kinds, *n* is the number of individuals of ith species, and ln is the log to base 2. The community in each host ecosystem and organ were clustered using PASW statistics 18 program.

3. Results

The samples of *Zingiberaceae* were collected from two ecosystems, i.e. natural forest and semi-agricultural (Table 1). The environmental parameters of these ecosystems were slightly different, particularly in light intensity, and wind velocity. The *Zingiberaceae* in natural ecosystem is under tree canopy while that of in semi-agricultural ecosystem is in open area. In both ecosystems, the plant samples grew vigorously and healthy. Morphological differences among the observed plants in both ecosystems were not found.

A total number fungal endophytic colony appearing from one plant segment varied. Most segments harbored one colony of

Table 1. Environmental parameters of *Zingiberaceae* ecosystem

Parameters	Host in two different ecosystems			
	AMN	AMD	HCN	HCD
Latitude	S: 06°44.293′ N:106°31.606′	S: 06°44.333′ N:106°31.865′	S: 06°44.293′ N:106°31.606′	S: 06°44.327′ N:106°31.906′
Height (m ASL)	1104	1134	1104	1134
RH (%)	78	74	78	74
T (°C)	25	25	25	25
Light (Lux)	773	1203	773	1946
Wind (mph)	0	2.2	0	2.2

AM = *Alpinia malaccensis*; D = domesticated/semi-agricultural; HC = *Horstendia conica*; N = natural/wild.

fungal endophytes, but some segments were either free or contained more than one colony. It was also related to incubation period of the samples. The 1st fungal endophytic colony appeared after 5 days of incubation, and most of the colonies appeared between 5 and 14 days of incubation. The slow growing fungal endophytes emerged after 1 month of incubation. After purification, 148 fungal endophytes were obtained in total.

Based on colony appearance and microscopic characters examination, the fungal endophytes were divided into 19 morphotypes. Phylogenetic analysis of the representative of each morphotypes showed that there were 19 species. These include *Arthrinium*

malaysianum, *Aspergillus flavipes*, *As. sydowii*, *Chaetomium globosum*, *Cladosporium oxysporum*, *Cladosporium* sp., *Colletotrichum boninense*-complex, *Co. cliviae*-complex, *Co. gloeosporioides*-complex, *Diaporthe* sp., *D. anacardii*, *D. gardenia*, *Exophiala* sp., *E. lecanii-corni*, *Guignardia mangiferae*, *Ochroconis gallopava*, *Penicillium citrinum*, *Pyricularia costina*, and unsporulated *Sydowiellaceae*. All these species were found in anamorphic stage, including *Diaporthe* spp. and *Guignardia mangiferae*.

The distribution of endophytic fungal community on AM and *Horstendia conica* (HC) were varied within different host species. Frequency of occurrence (FO) of endophytes in domesticated plants, even in the two host species, was higher than that of wild plants (Figure 1). HC domesticated/semi-agricultural (D) contained the highest FO (33.11%) and followed by AMD (25.68%), HC natural (N) (22.30%), and AMN (18.92%) (Figure 1). The FO was in contrast to their diversity as shown by Shanon-Wiener index (Table 2). The most diverse group of fungal endophytes hosted by AMN (2.14), followed by AMD (1.90), HCN (1.67) and HCD (1.53) (Table 2).

Co. boninense-complex, *Cladosporium* sp., and *E. lecanii-corni* were found to dominate the fungal endophytes community in all hosts (16.89%–24.32%). This indicates that these fungal endophytes are common in AM and HC, and their occurrence in both hosts are not affected by the ecosystem. Another species, *Co. gloeosporioides*-complex (13.51%) was also common on AM and HC. In contrast, *D. anacardii* was host and ecosystem specific as it was only found on
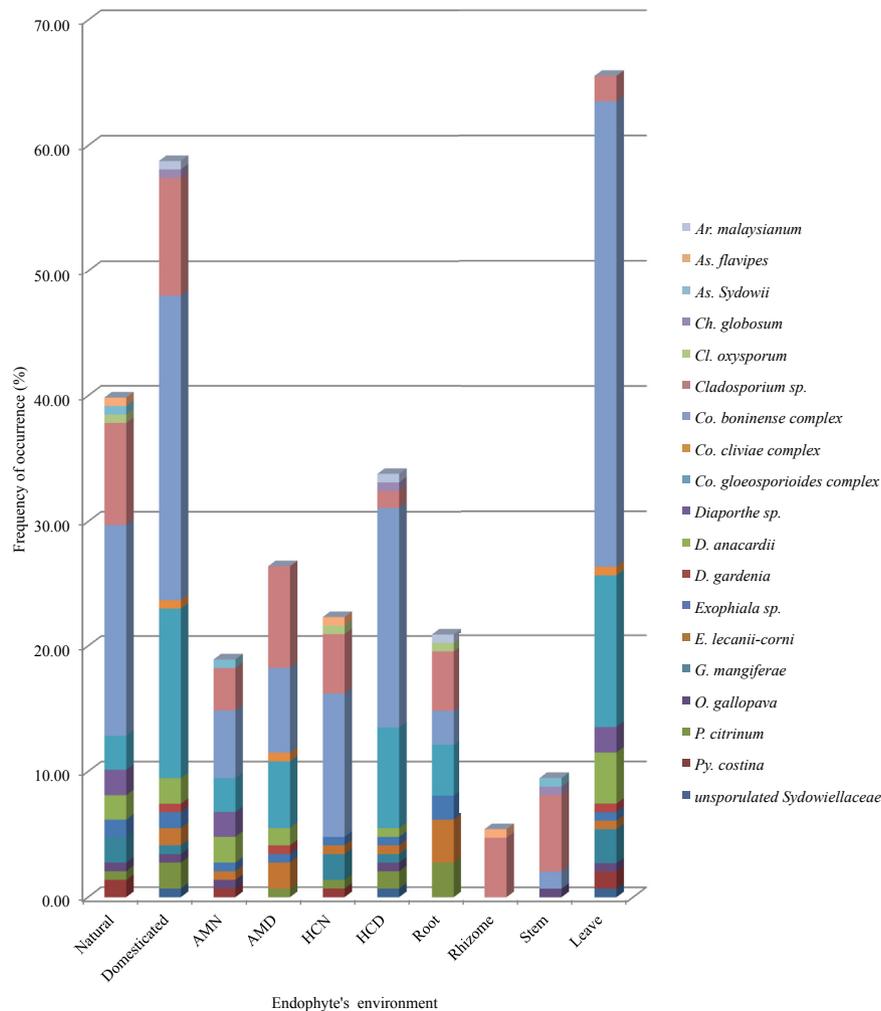

Figure 1. Occurrence frequency of fungal endophyte in various ecosystems. AM = *Alpinia malaccensis*; D = domesticated/semi-agriculture; HC = *Horstendia conica*; N = natural/wild.

Table 2. Diversity of endophytic fungi on various habitats

Habitat	Shanon–Wiener (Hs)	No. of species	Existing species in nature	Lost species from nature	Newly found species in domesticated
Natural	2.12	14	14	5	–
Domesticated	1.90	14	–	–	5
AMN	2.14	10	10	4	–
AMD	1.90	9	–	–	3
HCN	1.67	9	9	4	–
HCD	1.53	11	–	–	6
Leaf	1.54	12	8	2	6
Stem	1.29	6	2	2	3
Rhizome	0.68	3	1	1	4
Root	1.84	7	7	2	1

AM = *Alpinia malaccensis*; D = domesticated/semi-agricultural; HC = *Horstendia conica*; N = natural/wild.

the domesticated AM, while *G. mangiferae* and *P. citrinum* were only found on the domesticated HC. The fungal communities in one ecosystem were closely related regardless of the host (Figure 2). HCN was closer to AMN, and HCD was closer to AMD.

The distribution of the fungal species within the host also varied depending on their microhabitat. Roots were the most preferred microhabitat by fungal endophytes as shown by Shanon-Wiener index (1.84), followed by leaves (1.54), stems (1.29) and rhizomes (0.68). Most fungal endophytes (14 of 19 species) were microhabitat (organs) specific, and the remaining species showed to be common and had wide microhabitats. *Colletotrichum cliviae*-complex, *Diaporthe* spp., *G. mangiferae*, *Py. costina* were specific to the leaf, three species (*Ar. malaysianum*, *Cl. oxysporum*, *P. citrinum*) and one unsporulated *Sydowiellaceae* to the root and two species (*As. sydowii* and *Ch. globosum*) to the stem and one species (*As. flavipes*) to the rhizome. Fungal endophytes that had wide microhabitats were *Cladosporium* sp., *Co. boninense*-complex, *Co. gloeosporioides*-complex, *E. Lecanii-corni*, *O. gallopava* and *Cladosporium* sp. *Colletotrichum boninense*-complex preferred to inhabit leaves (37.16%). It was also found in root and stem but never found in rhizome. *E. lecanii-corni* was found on root and leaf, while *O. gallopava* was found on stem and leaf.

4. Discussion

There were 148 fungal isolates that distributed into 19 morphotypes. Phylogenetic analyses showed that these morphotypes represented 19 species. All these species were found in anamorphic stage, including *Diaporthe* spp. and *G. mangiferae*. Previous studies reported that endophytic fungi belonging to Ascomycetes often observed in anamorphic stage, and rarely form teleomorphic structure (Davies *et al.* 2003; Khan *et al.* 2010; Pressel *et al.* 2008; Rodriguez *et al.* 2009).

The light intensity and wind velocity in two kinds of ecosystem in MHSNP were slightly different. The wind velocity apparently determined the relative humidity and has influence on fungal development (Thomas *et al.* 1988). However, the plant sampled in both ecosystems grew vigorously, so it was assumed that the plant health was not affected by the environmental condition of the ecosystems. Host plant health was apparently not affected by diffrences in FO. Interaction among fungal endophytes may contribute to the plant health.

Domestication of plants affects significantly the FO in AM and HC. The FO in domesticated environment was higher than that of natural ecosystem. Thrall *et al.* (2007) stated that domestication/agro-ecosystem favors for fungal infection rather than in natural ecosystem. Gerard *et al.* (2006) stated that domesticated ecosystem represented as monoculture agriculture system has lower species diversity, higher host density, and uniform genes of fungal plant pathogen. However, factors affecting different fungal community on wild and cultivated plants are unknown.

FO in AM as well as in HC from natural ecosystem differ from those of domesticated environment. Differences of fungal community within the same species of wild and cultivated plants are
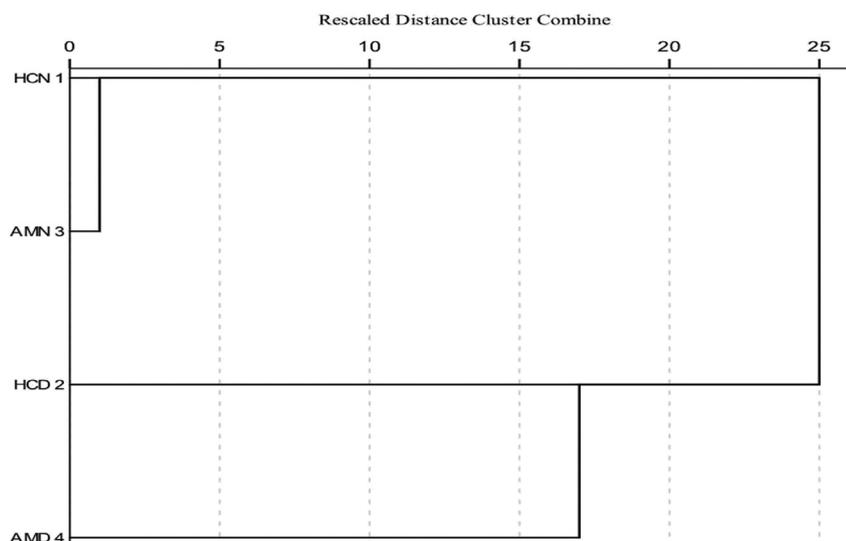

Figure 2. Clustering analysis of fungal endophytic community from different host ecosystems. AM = *Alpinia malaccensis*; D = domesticated/semi-agriculture; HC = *Horstendia conica*; N = natural/wild.

Table 3. List of existing, lost, and newly found species

Habitat	Existing species in natural ecosystem	Lost species from nature	Newly found species in domesticated
Natural	Asf, Ass, Cl, Clo, Cob, Cog, Dsp, Da, E, El, G, P, Pyc, Sh	Asf, Ass, Clo, Dsp, Pyc	—
Domesticated	—	—	Arm, Ch, Coc, Dg, US
AM	Ass, Cl, Clo, Cob, E, El, G, P, Pyc, Ogv	Ass, Clo, Dsp, Pyc	Coc, Cog, Dg
HC	Asf, Cl, Cob, Dsp, Da, E, El, P, Pyc	Dsp, E, El, Pyc	Arm, Ch, Cog, Da, US, Ogv
Leaf	Cl, Cob, Cog, Dsp, Da, G, Pyc	Dsp, Pyc	Coc, Dg, E, El, US, Ogv
Stem	Ass, Ogv	Ass, Ogv	Ch, Cl, Cob
Rhizome	Asf	Asf	Ch, Cl, Cob, P
Root	Cl, Clo, Cob, Cog, E, El, P	Cl, Clo	Arm

AM = *Alpinia malaccensis*; Arm = *Arthrinium malaysianum*; Asf = *Aspergillus flavipes*; Ass = *Aspergillus sydowii*; Chg = *Chaetomium globosum*; Cl = *Cladosporium* sp.; Clo = *Cladosporium oxysporum*; Coc = *Colletotrichum cliviae*-complex; Cob = *Colletotrichum boninense*-complex; Cog = *Colletotrichum gloeosporioides*-complex; Dsp = *Diaporthe* sp.; Dg = *Diaporthe gardenia*; Da = *Diaporthe anacardii*; E = *Exophiala* sp.; El = *Exophiala lecanii-corni*; G = *Guignardia mangiferae*; HC = *Horstendia conica*; Ogv = *Ochroconis gallopava*; P = *Penicillium citrinum*; Pyc = *Pyricularia costina*; US = unsporulated Sydowiellaceae.

probably related to variety in their adaptability to such environmental condition. Whether this fungal endophytic diversity shifts from providers to protectors as suggested by Thrall *et al.* (2007), should be confirmed by further research. In addition, Rodriguez *et al.* (2009) noted that some endophytes have the "habitat-adapted" ability and hypothesized that this ability allows them and their host to establish and survive in high-stress habitats. Pan and May (2009) noted that species pools, habitat effects, and inter-specific interaction among fungal endophytes affect the fungal endophytic community in maize. In addition, fungal-fungal endophytes interaction within the plant tissue seems to play a key factor in affecting the structure of the endophytic fungal community. "Habitat-adapted" ability of fungal endophytes is also related to the ecosystem specificity of some fungal endophytes. The data clearly showed that there is specificity of endophytic fungi in host (plant). Some previous reports had investigated specificity of endophytes in single plant species, and they strongly suggest the existence of some degree of host specificity among fungal endophytes (Cannon and Simmons 2002; Suryanarayanan *et al.* 2000).

In clustering analyses, fungal community in AM and HC from natural ecosystem should rather be similar. However, these fungal communities were found to change after domestication. However, this is in constrast to Higgins *et al.* (2007) and Sun *et al.* (2008) who reported that fungal endophytic communities were conspicuously affected by their hosts. Even though they then stated that ecosystem type (wild and domesticated) also contributes to the endophytic fungal community structure. Wilbeforce *et al.* (2003) also hypothesized that the diversity of endophytic fungi on reseeded agricultural grassland would be changed by the extreme and uniform anthropogenic disturbance imposed within that area.

The endophytic fungi found in this study may be host and organ specific, but they were usually not belonging to the dominant species. This study also found that root were the most preferred microhabitat. However, Arnold and Lutzoni (2007) previously proved that leaf was the hot spot for fungal endophyte diversity in tropical area. Leaves are a favorable environment for endophyte development as leaves are the most active photosynthesis organ on plant so nutrition supply will support endophyte development. In addition, the presence of endophytes also alters the metabolism of many plant species including enhancement of photosynthesis (Marks and Clay 1996). Recently, Moricca *et al.* (2012) reported that endophytes tended to be specific to each host, and to the organs of that host. They also stated that interaction between plant species and the environment and continued competitive interaction between endophyte species may have led to niche diversification, with selection favoring host specific and organ-specific endophytes.

Although most of endophytes collected from MHNSP were microhabitat (organ) specific, the relationship between the specificity with the anatomical structures of the plant organ is not known. Differences in endophyte assemblages in different tissue types might indicate a signal of tissue recurrences for dominant taxa, and might reflect their capacity for utilizing nutrients within a specific substrate (Schardl *et al.* 2004).

The current study recorded newly found species in domesticated plant, while some others were lost probably due to domestication (Tables 2 and 3). Generally, there were five of 14 species lost from natural ecosystem. The highest number of newly found species was on HCD and leaf. Anthropogenic impacts on all levels of biological organization in agricultural systems that are occurring through fragmentation and simplification of natural ecosystems has been discussed by Thrall *et al.* (2011). Even though strong indication of impact on endophytic fungal assemblage associated with the two zingiberaceous plants (AM and HC) domestication was shown in this study. Further study involving metagenomic approach within timescale is necessary to confirm the current results. More samples (repeated sampling) should be considered to minimize the bias of interpretation, including the number of individual plants (as replication). The isolation techniques should also be considered to reduce errors during analyses.

Conflict of interest

The authors declare no conflict of interest. All experiments in this study comply with the current law of the country where they were performed.

Acknowledgment

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