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Tree Species Composition and Diversity in a Secondary Forest along the Sierra Madre Mountain Range in Central Luzon, Philippines: Implications for the Conservation of Endemic, Native, and Threatened Plants

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Abstract: The Sierra Madre Mountain Range (SMMR) is the backbone of the Luzon Islands that contains a high concentration of highly important ecological resources distributed among the 68 protected areas therewith. The present study aimed to assess the composition and diversity of tree species in a secondary forest within the SMMR. A 2.25 km transect with 10 900-m^2 plots were established to record tree species with a diameter at breast height of at least 10 cm. The findings revealed 148 individuals of trees from 38 morphospecies, 28 genera, and 20 families. Importance values unveiled the Aurora endemic *Macaranga stonei* Whitmore as the most important species in terms of the relative values of its abundance, frequency, and dominance. The area was also found to be home to 33 natives, 12 endemics, five IUCN threatened species, and nine Philippine threatened trees. Furthermore, the study site was also found to have considerably high diversity, with a Shannon–Weiner Index value of 3.269 and a relatively even distribution of individuals among species, as supported by the Simpson's Evenness index value of 0.9453. Significant correlational relationships were also found among species richness, Shannon–Weiner index, and Simpson's Evenness index, with correlation coefficients ranging from 0.881 to 0.934, with all significant at $p < 0.001$. Lastly, the study was able to produce a distribution map, which is necessary for implementing targeted conservation strategies. These findings provided valuable implications for future research and implementation of targeted and participatory biodiversity conservation and protection strategies.

Keywords: biodiversity; biodiversity hotspot; correlation analysis; distribution maps; Shannon–Weiner index; Simpson's Evenness index

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1. Introduction

The Philippines, an archipelagic country known for its rich biodiversity, stands proudly as one of the 18 megadiverse nations on Earth [1]. This designation signifies that it harbors over two-thirds of the world's biodiversity resources, which play a crucial role in supporting human well-being and survival while maintaining ecosystem stability [2,3]. Biodiversity generously provides us with essential resources like food, water, raw materials, and clean air. Moreover, it diligently regulates climate and protects us from natural disasters [4]. Ecologically speaking, biodiversity enables vital processes such as pollination, nutrient cycling, water filtration, and soil stabilization and erosion control—all working together to create balanced ecosystems and desirable environmental conditions [5–7].

Unfortunately, biodiversity has long been facing numerous threats that jeopardize its existence and the critical services it provides. Climate change, along with many undesirable human activities such as deforestation, habitat destruction, land use change, and overexploitation, primarily drive biodiversity loss globally [8]. Due to these, scientists were able to identify biodiversity hotspots that contain very high rates of endemism and

drastic loss of vegetation and habitat that threatens various key biodiversity species [9]. At present, there are already 36 biodiversity hotspots, including the Philippines [10]. This signifies the need for immediate planning and implementation of strategies to prevent total biodiversity loss.

In the Philippines, various conservation and rehabilitation efforts are continuously implemented. The establishment and monitoring of protected zones under the National Integrated Protected Areas Systems (NIPAS) Act is considered one of the most important tools in conserving the country's key biodiversity resources, as recommended by the Convention on Biological Diversity [11]. Other conservation and rehabilitation programs, such as the National Greening program under Executive Order No. 26 [12], community-based forest management under Executive Order No. 263 [13], and sustainable ecotourism [14], among others, are recognized as greatly contributing to biodiversity conservation while educating people about its values and services.

However, there were critical issues in some rehabilitation and conservation programs. One of these is the unsuitable choice of plant species to rehabilitate a degraded or disturbed area. Several efforts in the past used exotic and invasive species such as *Gmelina arborea* Roxb. And *Swietenia macrophylla* King [15] in many greening activities. Some used native species, but there was a lack of pre-assessment of the site–species relationships thus introducing the natives to inappropriate habitats and hindering their successful growth and survival [16]. This is where the importance of plant inventory and assessments comes in. The data and findings yielded by these studies provide essential information on the population structure, composition, and ecology of an area and its resources that are beneficial in recovery planning, such as biodiversity conservation and habitat rehabilitation [17].

This current study aims to contribute to the conservation of Philippine biodiversity by assessing tree diversity in the municipality of San Luis in the province of Aurora. The province is a part of the Sierra Madre Mountain Range, the longest mountain range in the country, which is considered a highly important area in terms of valuable ecological resources distributed among its 68 protected areas [18,19]. Furthermore, there are very few studies about the plant composition and diversity in the province, which only cover the tree species in the municipalities of Baler [20] and Dipaculao [21], as well as the diversity of ferns in the municipalities of Maria Aurora [22] and Baler [23]. Hence, this study will pioneer the assessment of plants in the municipality of San Luis, which is beneficial in identifying the area's key biodiversity resources, such as the endemic, native, and threatened species, which is a crucial step in biodiversity conservation. Specifically, the study aimed to determine the tree species composition, including ecological classifications (i.e., indigeneity, endemism, and conservation status), calculate the importance values and diversity indices and explore the underlying relationships among diversity parameters and ecological variables (i.e., elevation).

2. Materials and Methods

2.1. Study Site

The study was conducted in April 2023 in Barangay L. Pimentel in the municipality of San Luis, province of Aurora, situated approximately 15°41′2.94″ N and 121°30′1.23″ E (Figure 1). The barangay is composed of residential, agricultural, and mountainous forest lands. Specifically, the survey was carried out in mountainous forest lands, which is a portion of the Sierra Madre Mountain Range, the backbone of Luzon Island, that serves as a protector and barrier from typhoons coming from the Pacific Ocean [24]. The survey area had a moderately steep topography, with elevations ranging from 273 to 581 masl. Climate-wise, the municipality has average monthly temperatures ranging from 26 °C to 30 °C (high temperature) and 22 °C to 25 °C (low temperature), and average monthly rainfall ranging from 118.3 mm (March: average of 7 rainfall days) to 416.9 mm (October: average of 17 rainfall days) in 2023. In the past 10 years, the average annual temperatures have usually ranged from 26 °C to 28 °C, while rainfall has been 100.23 mm to 624.86 mm. During the study period, the area had an average temperature of 28 °C during daytime and

24 °C during nighttime and there were 8 rainy days, with precipitation of around 300 mm during the month of April [25].

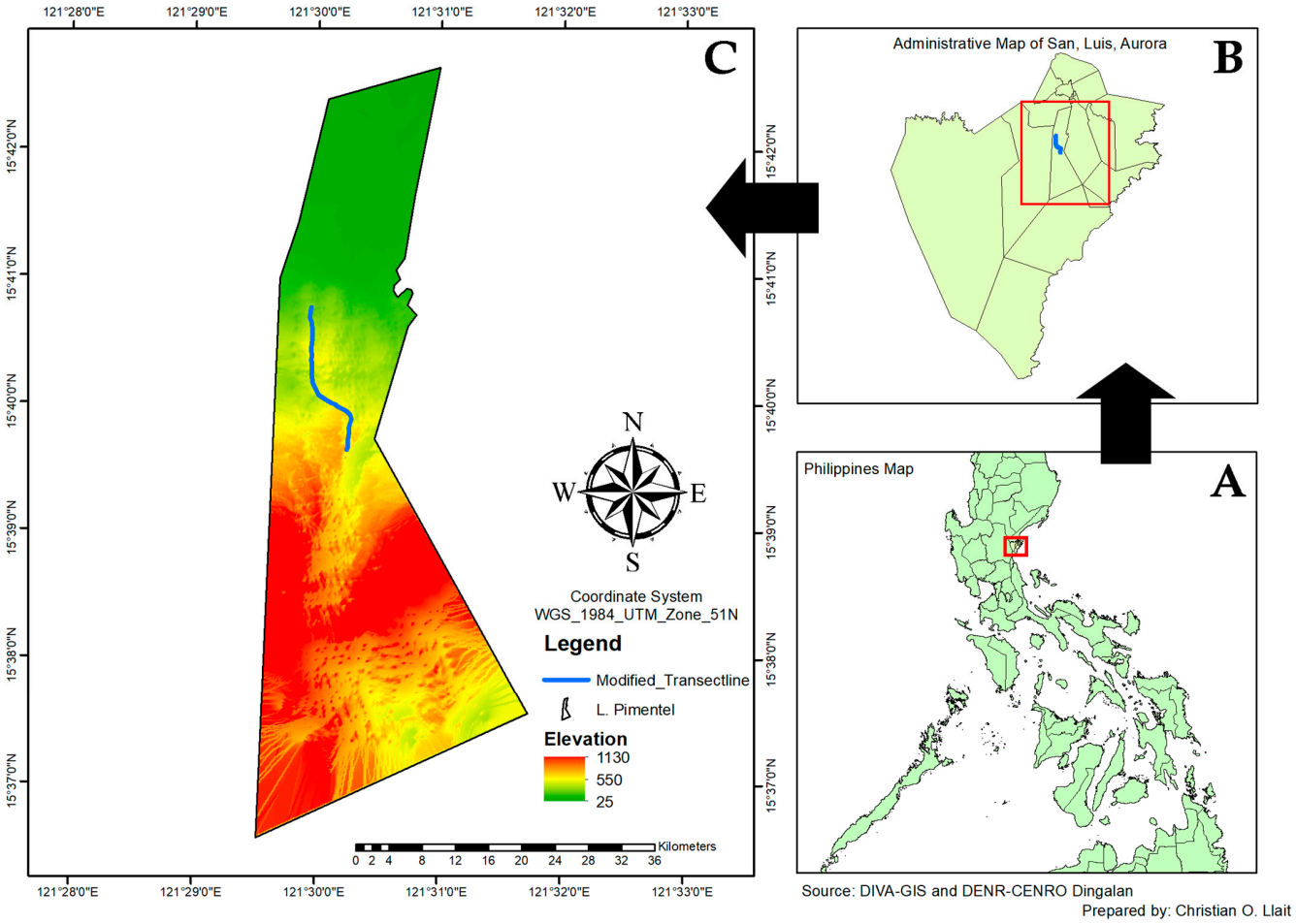

Figure 1. Location map of the study site in San Luis, Aurora: (**A**) study site pointed in the Philippine map, (**B**) location of the site pointed in the map of San Luis, Aurora, (**C**) elevation map of Barangay L. Pimentel showing the location of modified transect.

2.2. Survey and Mapping of Tree Species

The inventory of tree species was carried out along a 2.25 km transect line with 10 30 by 30 m quadrats established at every 250 m point (Figure 2). The transect line was established following the trail while the quadrats were positioned alternately at the left and right of the transect line, with an approximate distance of 5 m away from the trail. The total coverage of all the quadrats was 9000 m^2. The use of transect in conducting this plant inventory was used to ensure that the quadrats were evenly distributed throughout the forest stand [26].

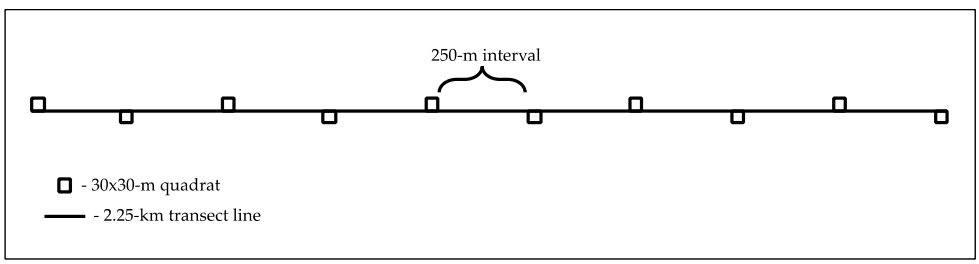

Figure 2. Arrangement of quadrats along the transect line.

After the establishment of the transect and quadrats, the plant survey was carried out. Trees with a diameter at breast height (DBH) of at least 10 cm were included in the study, following the DBH cut-off of many tree species inventories undertaken in the Philippines [27,28]. Plant identities were determined in the field using morphological characteristics. For individuals that were not identified in the field, photos were taken for further verification. References and databases such as Co's Digital Flora of the Philippines [29] and Revised Lexicon of Philippine Trees [30] were used to verify plant identities. Finally, the accepted scientific names of plants were determined using the Plants of the World Online database of the Kew Royal Botanic Gardens [31]. Significant ecological statuses (i.e., indigeneity, endemism, and conservation status) of all species were also assessed. Indigeneity and endemism were obtained from the Co's Digital Flora of the Philippines [29]. Meanwhile, conservation statuses were determined using the IUCN Red List of Threatened Species [32] for the global scale and DAO 2017-11 or the Updated National Checklist of Threatened Plants and their Categories [33] for the national scale.

Mapping was also carried out to visually present the location of each individual tree, which will also serve as the basis for the future implementation of targeted biodiversity conservation and management measures. Initially, the Locus map (a mobile outdoor navigation application) was used to record the location of the transect line and quadrats. Then, the geographic coordinates of each tree were recorded and encoded in Microsoft Excel. Geographic coordinates in decimal degree format were then converted into the Universal Transverse Mercator (UTM) format using the ArcGeek Coordinate Conversion Tool [34] before feeding it to ArcGIS software (v. 10.4). After that, the locations of all trees were plotted on the map. Lastly, final editing was undertaken to produce the final copy of the map in .jpeg format.

2.3. Data Analysis and Interpretation

2.3.1. Species Richness, Abundance, and Importance Values

Species richness, abundance, and importance values were either counted or calculated to discover the species composition in the area. Species abundance refers to the number of individuals of a species in an area [35], while species richness is the number of species or taxa present [36]. Hence, the number of species and its individuals were counted to determine the species richness and abundance. Lastly, importance values (IVs) serve as an index to measure how dominant a certain species is in a forest area through the relative values of its abundance, frequency, and dominance [37]. Thus, IVs were computed using the following equations [38]:

$$Density = \frac{number\ of\ individuals\ of\ a\ \ species}{total\ area\ sampled} \quad (1)$$

$$Relative\ Density = \frac{density\ of\ a\ species}{total\ density\ of\ all\ species} \quad (2)$$

$$Frequency = \frac{number\ of\ plots\ in\ which\ a\ species\ occur}{total\ number\ of\ plots\ sampled} \quad (3)$$

$$Relative\ Frequency = \frac{frequency\ of\ a\ species}{total\ frequency\ of\ all\ species} \quad (4)$$

$$Basal\ Area = 0.7854\ \left(DBH\ of\ a\ species^2\right) \quad (5)$$

$$Dominance = \frac{basal\ area\ of\ a\ species}{total\ area\ sampled} \quad (6)$$

$$Relative\ Dominance = \frac{dominance\ of\ a\ species}{total\ dominance\ of\ all\ species} \quad (7)$$

$$Importance\ Value = Relative\ Density + Relative\ Frequency + Relative\ Dominance \quad (8)$$

2.3.2. Diversity Indices

Biological diversity can be quantified using mathematical functions known as the diversity indices [39]. In this study, the widely accepted Shannon–Weiner (H') and Simpson's Evenness (E) were employed as the species diversity indices and computed through Paleontological Statistics (PAST v 3.18) software. The choice of these indices aligns with the standards set by previous biodiversity studies undertaken in the country and uses the Fernando Biodiversity Scale, which has been widely adopted in diverse ecological investigations in the Philippines to effectively facilitate the interpretation of computed values [40,41] (Table 1).

Table 1. Fernando Biodiversity Scale.

Interpretation	Shannon–Weiner	Simpson's Evenness
Very high	3.5 and above	0.75–100
High	3.0–3.49	0.5–0.74
Moderate	2.5–2.99	0.25–0.49
Low	2.0–2.49	0.15–0.24
Very Low	1.9 and below	0.05–0.14

2.3.3. Correlation Analysis

Exploring intricate relationships among key variables is essential in deeply understanding the dynamics of forest ecosystems. Therefore, Pearson correlation analysis was used to explore the underlying relationship (i.e., monotonic association) among important variables, namely, elevation, species richness, abundance, Shannon–Weiner, and Simpson's Evenness. This was computed at a significance level of $p < 0.05$ through JASP v. 0.16.1, an open-source statistical software package. The results were interpreted using the computed correlation coefficient values (r-values) and their associated p-values, as well as the conventional approach in interpreting r-values, contextualized as a direct or inverse relationship [42] (Table 2).

Table 2. Conventional approach in interpreting correlation coefficient [42].

Absolute Value of r	Interpretation
0–0.09	Negligible correlation
0.10–0.39	Weak correlation
0.40–0.69	Moderate correlation
0.70–0.89	Strong correlation
0.90–1.0	Very strong correlation

3. Results and Discussion

3.1. Tree Species Composition

The study recorded a total of 148 individuals of 38 morphospecies of trees from 20 families and 28 genera. In terms of the families, Dipterocarpaceae and Moraceae were the most speciose with seven and five species, respectively. The most abundant families were Euphorbiaceae, Dipterocarpaceae, and Moraceae, with 29, 28, and 21 individuals, respectively. These families are abundant in the Philippines, especially in tropical lowland evergreen forests that are dominated by dipterocarps [43]. Sadly, dipterocarps are among the most threatened plant species in the Philippines and in Southeast Asia due to deforestation, and their timbers have been massively exported in the past [44,45]. Species-wise, *Macaranga stonei* Whitmore was the most abundant, followed by *Parashorea malaanonan* (Blanco) Merr., with 24 and 9 individuals, respectively. Given that the study plots covered 9000 m^2, which is 9/10 of a hectare, it is estimated that these species, *M. stonei* and *P. malaanonan*, had 26 and 10 individuals in a hectare of the study area, respectively.

The importance values computation also revealed significant findings in terms of the species composition. Eleven (11) species had individual IVs of more than 10 (Figure 3). In

total, these 11 species contributed 55.50% of the total IV of all species in the area. Among them, *M. stonei* had the highest IV of 30.35, which is equivalent to 10.11% of the total IV of all the species recorded, followed by *Parashorea malaanonan* (Blanco) Merr, with 21.63 (7.21%). *M. stonei*'s high IV was related to its high abundance of 24, its occurrence in six plots out of all ten plots, and a total basal area of 136.73 m^2. *M. stonei* is Aurora province-endemic and a critically endangered plant species belonging to the family Euphorbiaceae [29,32]. This keystone species lacks focus in terms of research, thus dictating the need to study this species more and include it as one of the top priorities for conservation due to it being a species restricted to the province of Aurora.

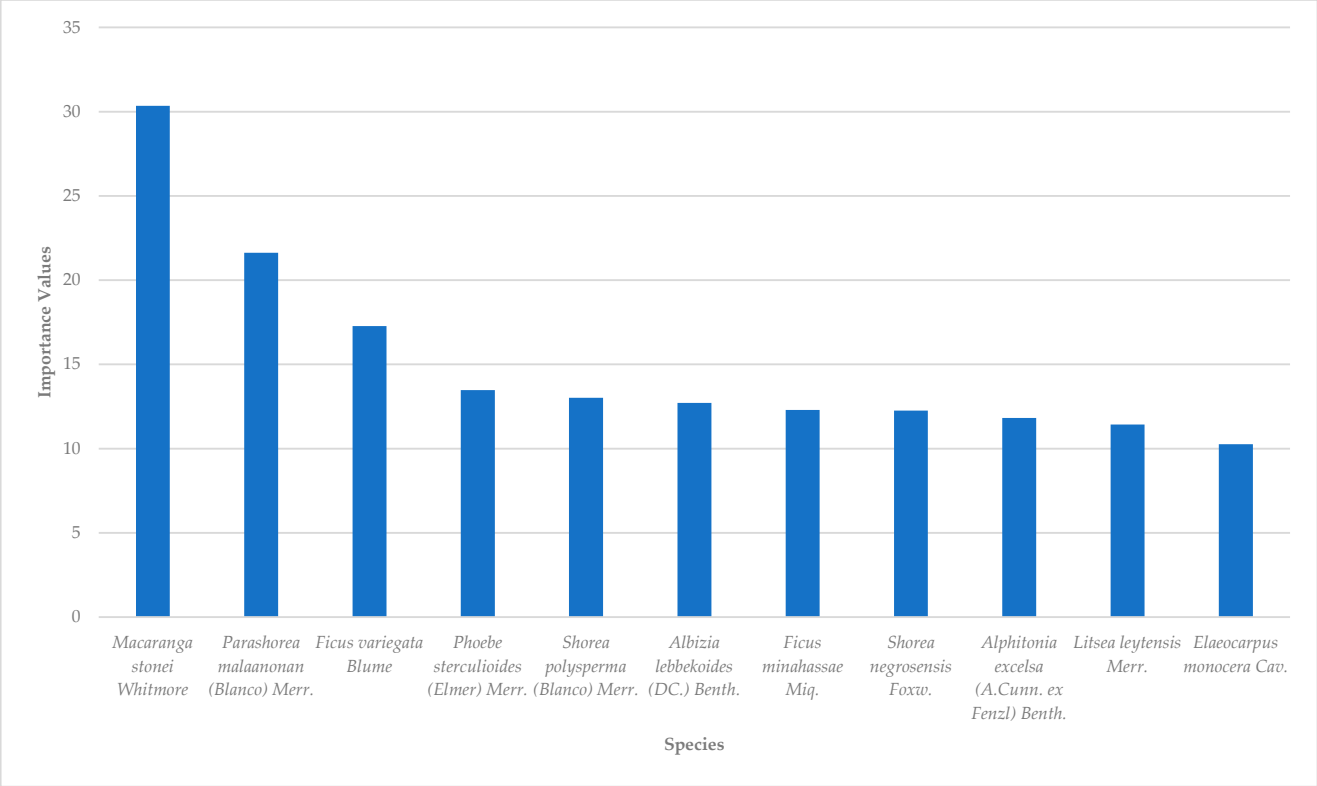

Figure 3. Top eleven species with highest importance values (IVs).

The surveyed forest was also found to be home to ecologically important species, namely, native, endemic, and threatened species (Table 3). Out of the 38 species found, 33 (86.84%) were natives, while five were exotics (with one invasive *Gmelina arborea* Roxb. ex Sm.). The native species were composed of 12 endemics, five IUCN threatened species, and 9 Philippine nationally threatened species. Specifically, there were one critically endangered, two endangered, and two vulnerable species found in the IUCN. Furthermore, there were two endangered, six vulnerable, and one other threatened species found in DAO 2017-11 or the Philippine Red List. The most notable among the Philippine endemic species were the IUCN critically endangered *M. stonei* and the IUCN vulnerable and DAO 2017-11 endangered *Hopea acuminata* Merr, and the IUCN endangered and DAO 2017-11 vulnerable Philippine national tree *Pterocarpus indicus* Willd. The presence of critically important plants in the area dictates the need for immediate action to conserve, protect, and even spread their population. It is emphasized that these species, particularly the endemics, have higher probabilities of extinction because of their narrow and restricted habitat than widespread species [46]. The native and endemic plant species also provide suitable habitats and enough food sources for native and endemic fauna species [47]. In fact, we were able to witness a couple of the Philippine endemic Luzon Rufous Hornbill (*Buceros hydrocorax* Linnaeus) during the survey. However, the presence of invasive species like the *G. arborea*

adds pressure to the survival and propagation of the native and endemic flora and fauna species due to the aggressive nature of most invasive plants [48]. Actual representative photos of some critically important plant species in the area and an image of *B. hydrocorax* individuals are shown in Figure 4.

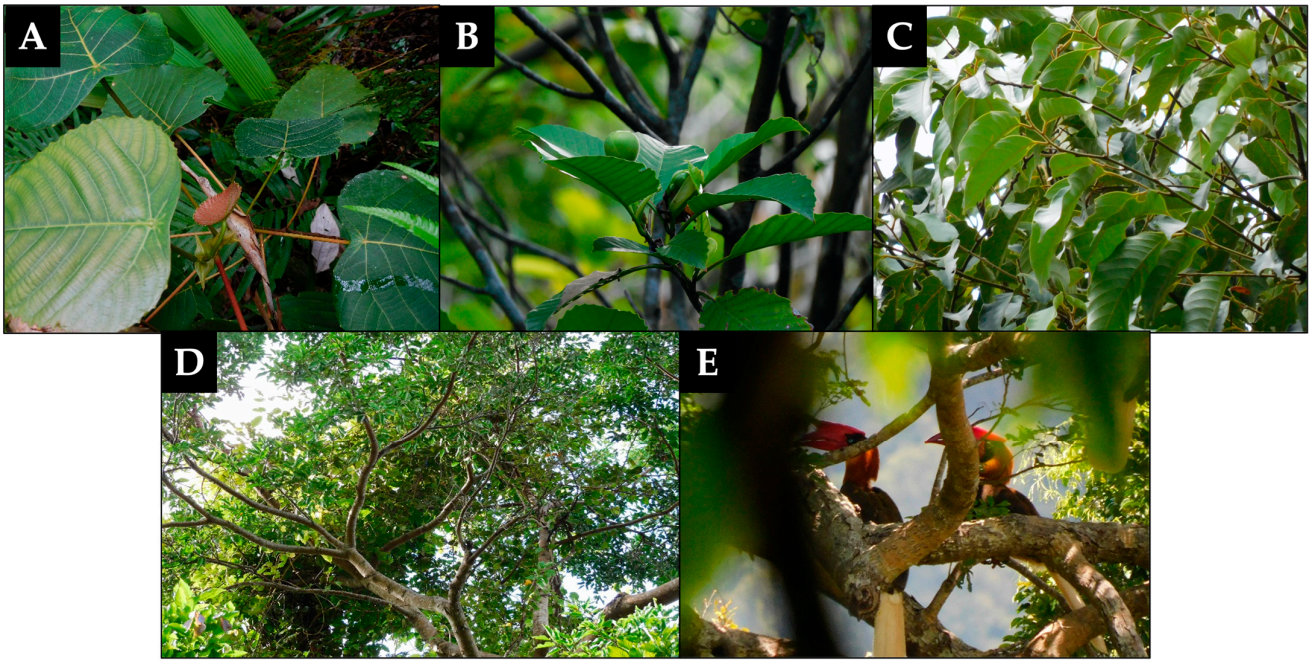

Figure 4. Representative photos of critically important species in the area. (**A**) *Macaranga stonei* Whitmore (endemic, IUCN critically endangered), (**B**) *Dillenia philippinensis* Rolfe (endemic, IUCN near threatened), (**C**) *Shorea polysperma* (Blanco) Merr. (endemic, DAO 2017-11 vulnerable), (**D**) *Shorea contorta* Vidal (endemic, DAO 2017-11 vulnerable), (**E**) a couple of *Buceros hydrocorax* Linnaeus (endemic, IUCN vulnerable, Philippine Red List endangered).

Table 3. Taxonomic list of native species recorded with their corresponding endemism and conservation statuses.

Family	Species	Endemism [1]	Conservation Status [2]	
			IUCN Red List	DAO 2017-11
Anacardiaceae	*Koordersiodendron pinnatum* (Blanco) Merr.	NE	ND	OTS
Brownlowiaceae	*Diplodiscus paniculatus* Turcz.	PE	LC	ND
Cannabaceae	*Celtis philippensis* Blanco	NE	LC	ND
Dilleniaceae	*Dillenia philippinensis* Rolfe	PE	NT	ND
Dilleniaceae	*Tetracera scandens* (Linn.) Merr.	NE	ND	ND
Dipterocarpaceae	*Dipterocarpus grandiflorus* (Blanco)	NE	EN	VU
Dipterocarpaceae	*Hopea acuminata* Merr.	PE	VU	EN
Dipterocarpaceae	*Parashorea malaanonan* (Blanco) Merr.	NE	LC	ND
Dipterocarpaceae	*Shorea contorta* Vidal	PE	LC	VU
Dipterocarpaceae	*Shorea negrosensis* Foxw.	PE	LC	VU
Dipterocarpaceae	*Shorea polysperma* (Blanco) Merr.	PE	LC	VU
Dipterocarpaceae	*Shorea squamata* (Turcz.) Benth. & Hook.	PE	LC	ND
Elaeocarpaceae	*Elaeocarpus cumingii* Turcz.	NE	LC	ND
Elaeocarpaceae	*Elaeocarpus monocera* Cav.	PE	ND	ND
Euphorbiaceae	*Macaranga grandifolia* (Blanco) Merr.	NE	VU	ND
Euphorbiaceae	*Macaranga stonei* Whitmore	PE	CR	ND
Euphorbiaceae	*Macaranga tanarius* (L.) Muell. Arg.	NE	LC	ND
Euphorbiaceae	*Mallotus paniculatus* (Lam.) Müll. Arg.	NE	LC	ND

Table 3. *Cont.*

Family	Species	Endemism [1]	Conservation Status [2]	
			IUCN Red List	DAO 2017-11
Fabaceae	*Albizia lebbekoides* (DC.) Benth.	NE	LC	ND
Fabaceae	*Pterocarpus indicus* Willd.	NE	EN	VU
Hypericaceae	*Cratoxylum sumatranum* Blume	NE	LC	ND
Lauraceae	*Litsea leytensis* Merr.	PE	NT	EN
Lauraceae	*Phoebe sterculioides* (Elmer) Merr.	PE	LC	ND
Meliaceae	*Aglaia luzoniensis* (Vidal) Merr. & Rolfe	NE	NT	ND
Moraceae	*Artocarpus blancoi* (Elmer) Merr.	PE	LC	ND
Moraceae	*Ficus minahassae* Miq.	NE	LC	ND
Moraceae	*Ficus nota* (Blanco) Merr.	NE	LC	ND
Moraceae	*Ficus variegata* Blume	NE	LC	ND
Myrtaceae	*Syzygium nitidum* Benth.	NE	ND	VU
Myrtaceae	*Syzygium tripinnatum* (Blanco) Merr.	NE	ND	ND
Rhamnaceae	*Alphitonia excelsa* (A.Cunn. ex Fenzl) Benth.	NE	LC	ND
Sterculiaceae	*Sterculia ceramica* R.Br.	NE	ND	ND
Urticaceae	*Leucosyke capitellata* (Poir.) Wedd.	NE	LC	ND

[1] Endemism classifications: PE—Philippine endemic; NE—Not endemic. [2] Conservation status classifications: CR—Critically endangered; EN—Endangered; Vu—Vulnerable; OTS—Other Threatened Species; NT—Near threatened; LC—Least concern; ND—No data.

3.2. Tree Species Diversity

The diversity indices of the secondary forest in San Luis are presented in Figure 5. The Shannon–Weiner index values per quadrat ranged from 1.626 to 2.3384 and were interpreted as very low to low based on the Fernando Biodiversity Scale. In terms of Simpson's Evenness, the values ranged from 0.8182 to 0.9619, which were interpreted as very high. Quadrat 2 had the highest diversity ($H' = 2.384$ and $E = 0.9619$). Overall, the study area had a high Shannon–Weiner index ($H' = 3.269$) and a very high Simpson's Evenness index ($E = 0.9453$), which means that the trees in the area were relatively diverse and had a considerably even distribution of individuals among species. In most ecological studies in the Philippines, H' values generally range from 1.5 to 3.5, wherein higher values dictate higher species diversity [49]. The overall H' value of the present study falls within this range and was interpreted as high, which can possibly be attributed to the variety of native and endemic species that still thrive therewith. This is comparable with some studies undertaken in the Philippines, such as in a lowland forest in Agusan del Sur ($H' = 3.32$, $E = 0.52$) [50], in a secondary forest in Benguet ($H' = 2.40$) [49], and in a secondary forest in Pampanga ($H' = 2.2807$, $E = 0.8549$) [51], which were all categorized as having low to moderate diversity based on the Shannon–Weiner index. Similarly, these study sites were either under the management of upland communities or near their residential or agricultural sites. In contrast, the values are lower than the studies in a private mountainous forest in Baler, Aurora ($H' = 4.096$; $E = 0.9735$) [18], in the Quezon Protected Landscape ($H' = 3.90$, $E = 0.81$) [52], and in the Mt. Makiling Forest Reserve ($H' = 3.50$, $E = 0.91$) [53]. The common characteristics that possibly caused these high values were their classifications as private property, with strict monitoring and considerably high protection for the site in the first study and being classified as protected areas under the law of the second and third study sites, relating to the monitoring and protection activities of the government.

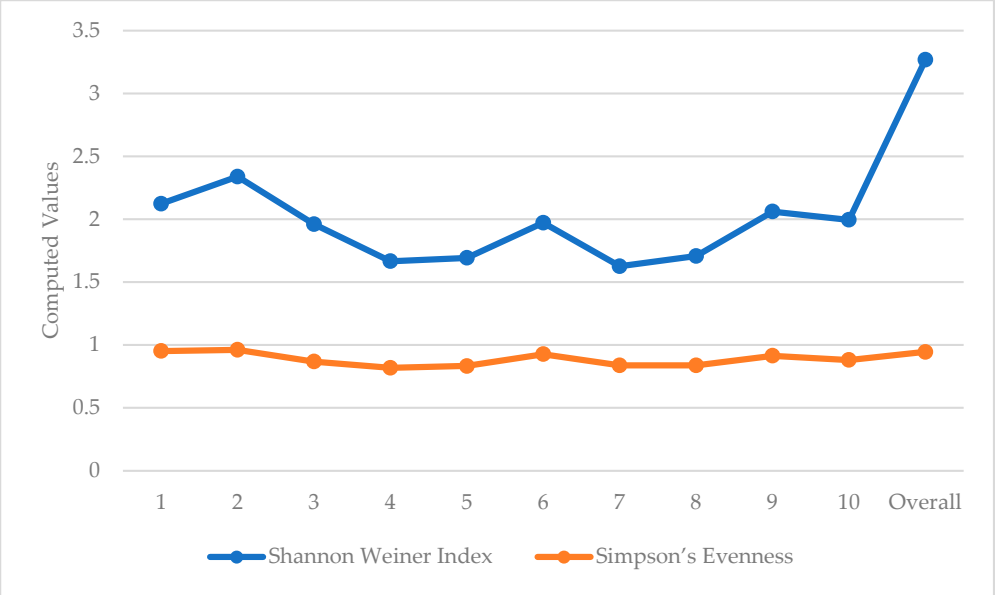

Figure 5. Computed diversity indices per quadrat and for the entire survey area.

3.3. Correlation among Parameters

This study also tested the correlations among elevation, species richness, abundance, Shannon–Weiner, and Simpson's Evenness values. As a result, significant correlational relationships were only observed for the following: (a) Species Richness and Shannon–Weiner (r = 0.881, $p < 0.001$); (b) Species Richness and Simpson's Evenness (r = 0.885, $p < 0.001$); and (c) Simpson's Evenness and Shannon–Weiner (r = 0.934, $p < 0.001$) (Figure 6). Based on the r-values, there was a strong positive correlation between species richness and Shannon–Weiner index as well as between species richness and Simpson's Evenness, as supported by a very high significance value of p that is less than 0.001. This relationship suggests that as species richness increases, the values of the Shannon–Weiner and Simpson's Evenness indices also tend to increase. Thus, this observation indicates that having a greater variety of species can lead to a higher diversity, as measured by using the mentioned indices. Furthermore, there was a very strong positive correlation found between the Simpson's Evenness index and Shannon–Weiner index based on the obtained r-value, which is backed up by a very high statistical significance with $p < 0.001$. This indicates that as the value of Shannon–Weiner index increases, the value of Simpson's Evenness also tends to increase. The findings are corroborated by the study of DeJong, which also found a very strong correlation among species richness, Shannon–Weiner index, and Simpson's Evenness index, with correlation coefficients of more than 0.96 [54]. However, no significant correlational relationships were found between the following: (a) elevation and other variables, and (b) abundance and other variables. A similar finding was found in a study at a mountain range in Southern Mindano, suggesting that elevation did not greatly affect biodiversity parameters such as the diversity indices [55]. In essence, these results are beneficial in understanding the dynamics of an ecosystem, which can be the foundation for implementing management and rehabilitation strategies in different areas within the study site with the goal of improving biodiversity.

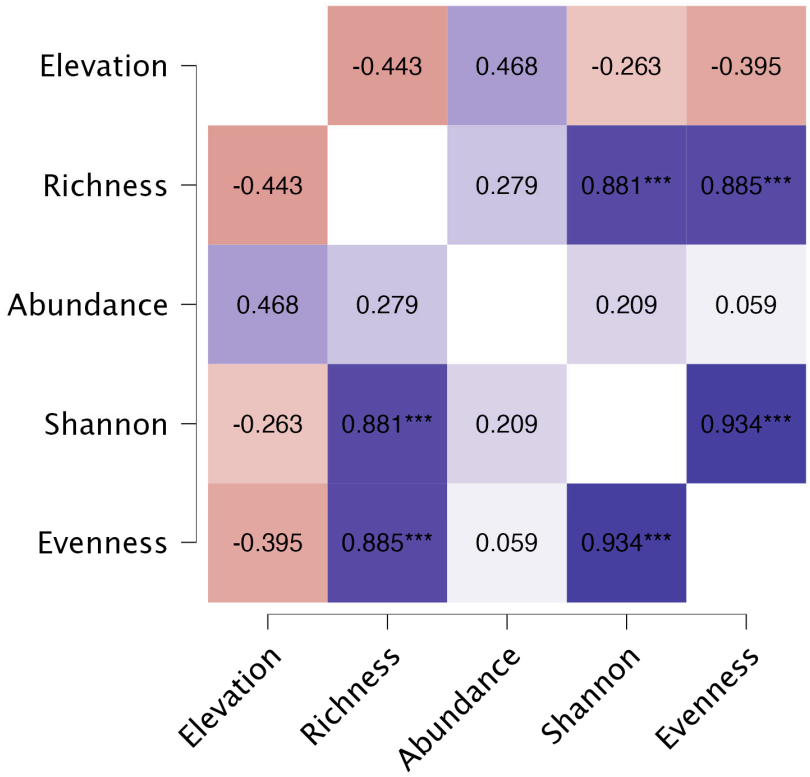

Figure 6. Heatmap of Pearson's *r* correlation among elevation and biodiversity parameters (*** $p < 0.001$).

3.4. Spatial Distribution of Trees

Figure 7 shows the spatial distribution of trees across the sampling plots in the secondary forest of San Luis in Aurora, Philippines. This map shows the position of the transect line and the approximate location of each individual tree, represented by colored dots (legend placed on the right-hand side), based on the recorded coordinates. As observed in the map, the plots were zoomed in to show the locations of the trees more clearly. We can also see in the background of the zoomed image of the plots the actual image of the forest cover in the area, as reflected in the base map used. Mapping the spatial distribution of trees is a crucial element in devising strategies for the sustainable management and conservation of natural resources [56]. For instance, locating the trees can help us identify areas with possible sources of mother trees of the targeted species that we aim to propagate [57]. For example, if we are looking for a source of planting materials for a high-priority species such as *M. stonei*, which is a very important species in the area due to the fact that it is an endemic and critically endangered species, we can refer to the map and see that it can be seen in plots 3, 5, 7, 8, 9, and 10. Furthermore, distribution maps can visually present areas needing attention and immediate measures, such as in the case of our study, the presence of invasive *G. arborea* that poses a threat to the native biodiversity. Knowing the location of its recorded individuals (present in plots 2 and 3) will allow the forest managers to perform targeted measures in managing specific portions of the area where invasion issues arise [48]. Lastly, we can identify micro-biodiversity hotspots among the sampling plots in the study area by determining the number of critically important species [58].

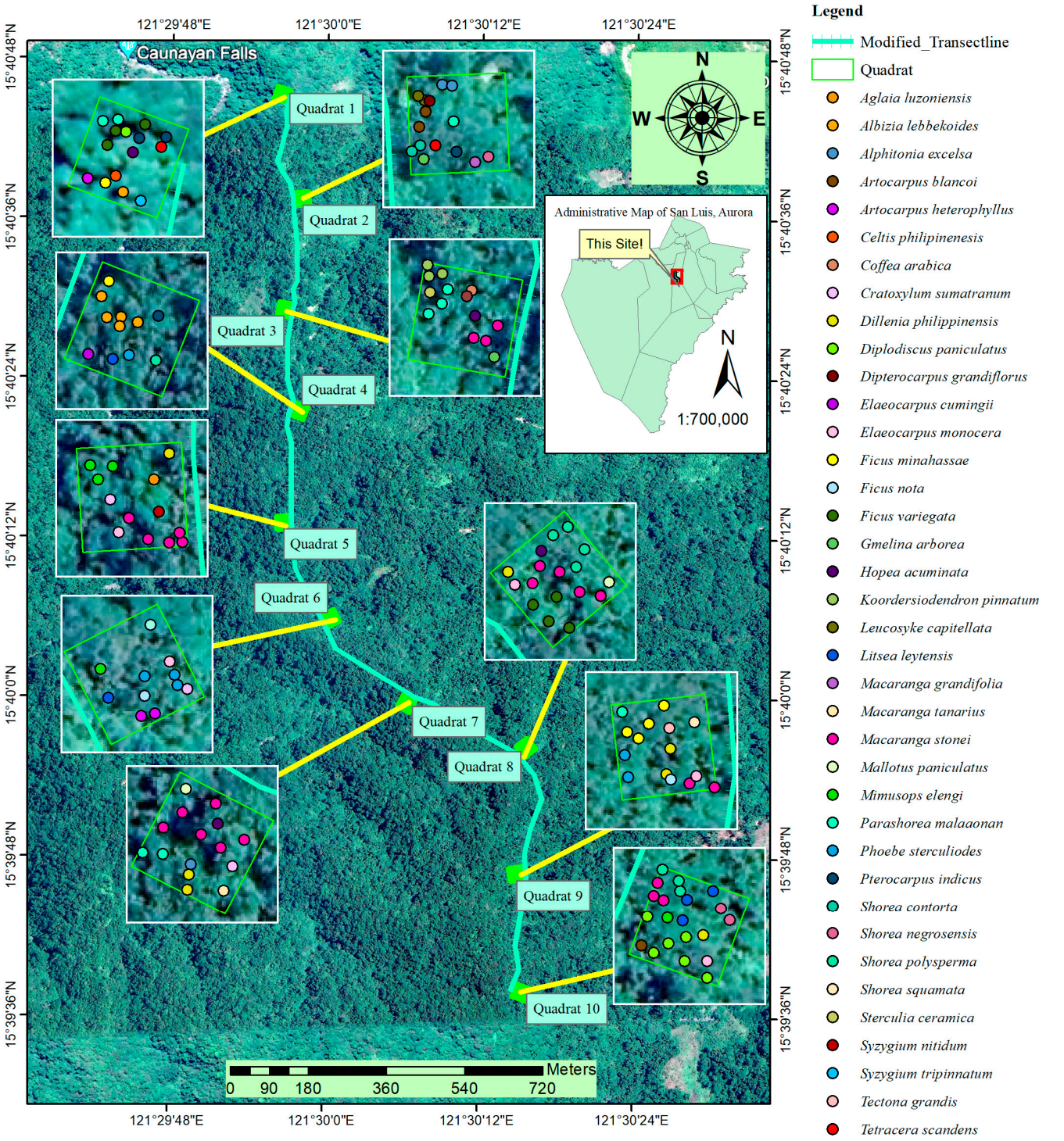

Figure 7. Distribution map of all trees across sampling plots in the secondary forests of San Luis, Aurora.

3.5. Research Limitations

This study provides valuable insights into the composition and diversity of tree species in the San Luis area of the SMMR. However, certain limitations were acknowledged. The research scope was limited to a short duration, and only a specific portion of the secondary forests of San Luis, Aurora, were covered by the transect, where variations in topography, exposure, and elevations, as well as forest dynamics, were not fully explored. Thus, these issues limit the generalizability of the findings in terms of larger ecosystems and dictate

the need for long-term monitoring and the eventual establishment of a protected area. Furthermore, tree species were the only life forms included in the study, opening the door for a more comprehensive assessment of other biodiversity components, such as understorey and ground vegetation, wildlife, and soil characteristics, which were beyond the scope of this study. These limitations are crucial in interpreting the results to guide future research directions and in planning a holistic and more effective biodiversity management and conservation.

4. Conclusions and Implications

This study yielded valuable findings and insights regarding the species composition and diversity of a secondary forest in San Luis, Aurora. Overall, the area had a relatively high diversity and significant conservation, as signified by the recorded 148 individuals of 38 morphospecies belonging to 20 families and 28 genera, with 33 natives, 12 endemics, five IUCN threatened, and nine Philippine threatened species. Furthermore, diversity was found to be high in terms of the Shannon–Weiner index ($H' = 3.269$) and very high in terms of the Simpson's Evenness index ($E = 0.9453$). Significant correlational relationships were also found among species richness, Shannon–Weiner index, and Simpson's Evenness index. Lastly, individual trees were mapped to serve as a guide for targeted conservation measures. These findings are critical in the following applications for the conservation of native, endemic, and threatened species:

1. The presence of many native, endemic, and threatened species underscores the immediate need to prioritize the conservation of these species through the aid of the map produced in locating the micro-biodiversity hotspots in the area. Furthermore, many endemic species lack scientific studies, highlighting the need to conduct focused studies to explore the ecology and distribution of these critically important species. Furthermore, this can serve as a basis for the Department of Environment and Natural Resources to include the forest as one of the high conservation priorities or to expand protected areas to cover the area surveyed.
2. The relatively high diversity values and even distribution of plants calculated for the area somehow indicate a relatively healthy ecosystem. Thus, this underscores the need for intensified law enforcement to protect the remaining forests that serve as habitats for native and endemic wildlife, such as *Buceros hydrocorax* Linnaeus.
3. The presence of introduced and invasive species such as *Gmelina arborea* Roxb. poses a very significant threat to local native biodiversity. Targeted and participatory invasive species management is needed to control and eventually eradicate the impact of invasive plants in the ecosystem.
4. All the implications and conservation strategies discussed above will need the participation of locals and other stakeholders due to the fact that the area is adjacent to residential communities. Thus, information and educational campaigns, as well as a participatory approach in implementing conservation strategies, are ideal tools to ensure more effective biodiversity conservation and protection.

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Climate-Resilient Agri-fisheries (CRA) Assessment, Targeting & Prioritization for the Adaptation and Mitigation Initiative for Tarlac Province

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Abstract. This study is part of the Adaptation and Mitigation Initiative in Agri-fisheries (AMIA) Project of the Department of Agriculture (DA) and International Center for Tropical Agriculture (CIAT) to operationalize the goal of making agriculture and its stakeholders adapt and mitigate the effects of climate change. The outputs of this study were vital in the implementation of the next phase of AMIA: climate-risk vulnerability (CRVA) map and investment brief. The CRVA map was developed to identify the vulnerable areas in Tarlac. The CRVA map considered three factors: climate-risks sensitivity, exposure, and adaptive capacity given a 15-15-70 weighted percentages, respectively. The municipalities of Ramos, La Paz, Bamban and Victoria were identified as highly vulnerable to the effects of climate change. Three investment briefs were prepared for policy makers for possible funding and implementation. They were developed by identifying climate-resilient agricultural practices in the province which are the use of climate smart varieties, crop rotation-minimum tillage combination, and alternate wetting and drying method. These practices were evaluated using the CBA Tool and were found to be sustainable. Social net present value was also determined taking into account the externalities, which was given a value through interview of experts.

INTRODUCTION

The Philippines is highly vulnerable to the adverse impacts of climate change as the country's backbone is agriculture [1]. The effect of intense and longer droughts, increase in temperature, erratic rainfall distribution and natural hazards that affects earth's ecosystem as well as health, livelihood, social systems and economy. Agriculture is one of the most vulnerable sectors on the impact of climate change and farmers who are directly dependent on their lands for survival are the most affected. Climate change mitigation and adaptation to climate change is crucial particularly in agriculture sector, thus, different adaptive strategies are being done to cope up with its impacts. One of the activities in the strategic actions of food security for 2011-2028 is to enhance site-specific knowledge on the vulnerability of agriculture and fisheries [2].

The Adaptation and Mitigation Initiative in Agriculture (AMIA) seeks to enable the Department of Agriculture (DA) to plan and implement strategies to support local communities in managing climate risks – from extreme weather events to long-term climatic shifts. Spearheaded by the DA System-wide Climate Change Office (DA SWCCO), AMIA Phase 1 in 2015-16 to implement activities to strengthen DA's capacity to mainstream climate change adaptation and mitigation strategies locally and in national scale. With AMIA Phase 2, making climate-resilient agri-fisheries (CRA) an operational approach through field-level action that directly involves, and influences on the livelihoods of farming communities is one of the next big task for the program. AMIA2 aims to invest in the launching of CRA communities in Tarlac province as the initial target site for action learning, supported by an integrated package of climate services within a broader food value chain setting. The program is launching an integrated and multi-stakeholder effort to operationalize CRA at the community level in 10 target regions. Successful implementation of AMIA2 at the regional level requires the strong collaboration and support of key research and development institutions within the region. This proposed project enables AMIA2 to establish and mobilize regional teams, each led by a local State University/College (SUC), and in partnership with the corresponding Department of Agriculture - Regional Field Office (DA-RFOs).

Climate-resilient agriculture/agri-fisheries (CRA) aims to achieve food security and broader development goals under a changing climate and increasing food demand [3]. With the three pillars: productivity, adaptation, and mitigation, CRA initiates sustainably increase productivity, enhance resilience, and require planning to address tradeoffs and synergies [4]. To enable to assess, plan and pilot climate-risk prone agri-fisheries communities in pursuing sustainable livelihoods while effectively managing the impacts of climate variability. Thus, one of the Region to initiates the CRA strategic framework was Region 3 or Central Luzon specifically in

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Tarlac province to identify key climate risk and vulnerable areas and to assess the current status of CRA as well as the cost and benefits of these practices and technologies. Therefore, the study aims to establish and mobilized team in Region III for AMIA 2 in order to operationalized AMIA strategies in managing climate risk in Tarlac province. Specifically is to enhanced capacities of AMIA partner organizations in the Region, developed geospatially referenced data on climate-risks in Tarlac, generate profile on community's CRA strategies, and perform costs-benefits & trade-offs for these CRA practices. The result of the study will serve as guide in piloting community action research in establishing community-level research and development interventions.

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MATERIALS AND METHODS

Study Area

The study was established in the province of Tarlac. The study covered an area of 273,660 hectare and is located between latitude 15º 10'15" N to 15º 52'52" N, longitude 120º 8'4" E to 120º 46'27" E. The study area has flat to undulating topography, with the eastern part of the province being plain and the western part to be hilly to mountainous. Tarlac has two distinct seasons, the wet and the dry seasons. It has unimodal rainfall pattern, having high monsoon peaks in the wet season (WS) and negligible rainfall in the dry season (DS). Recorded annual rainfall varies from 2,030 mm to 4,060 mm in the northwestern portion [5].

Tarlac is basically an agriculture-based economy, located in the heart of Central Luzon with a total land area of 305,345 ha, constitutes 16.75% of the regional land area and 1.0 % of the total national land area with 112,997.57 hectares concentrated on agricultural production. Rice and corn are the top 2 commodities planted in the province planted in 2 to 3 cropping a year. There are 102,178.06 ha planted to rice, which are irrigated, rainfed and in upland areas. On the other hand, there are 16,458.98 ha planted to corn. Of these, only a small portion, are planted with white corn while the rest are planted with the yellow corn. With this vast track of land concentrated in agriculture, Tarlac likewise grows lowland vegetables and root crops. Of the lowland vegetables grown in the region, tomato occupies the largest area with 215.81 has while sweet potato is the largely grown rootcrops with a production area of 3,641.58 ha. Both crops are grown after rice usually during the onset of the dry season when rice has been harvested. Orchard occupies 10,498.65 hectares planted with our local fruit trees. The most common is mango that is planted in an area totaling to 25,660.03 hectares [6].

Framework of AMIA 2 Project

The project seeks to contribute to the overall AMIA2 program framework, by contributing specific outputs to targeted national-level research projects. It has four key components: (1) Capacity strengthening for CRA research & development, (2) Geospatial assessment of climate risks, (3) Stakeholders' participation in climate adaptation planning, and (4) Documenting & analyzing CRA practices. These project components were designed to be directly aligned with the research agenda of three AMIA2 projects: 1) climate-risk vulnerability assessment (CRVA), 2) decision-support platform for CRA, and 3) institutional and policy innovations. Figure 1 shows the framework for this study.

Component 1 - Capacity strengthening for CRA research & development

The regional project team participated in a series of trainings, workshops and learning events organized by AMIA2 projects. These were focused on three key methodologies: 1) CRVA, 2) CRA prioritization, and 3) CRA M&E. The project provided training support to key research and development stakeholders in the region, by organizing an intra-regional training that covers key learning contents from the national-level trainings. The CRA monitoring and evaluation was later included in the phase two of the AMIA2 which is currently handled by the Department of Agriculture Regional Field Office 3 in Victoria, Tarlac. The town was one of the identified vulnerable areas in the province.

Component 2 - Geospatial assessment of climate risks

The project team collected and organized geo-referenced data on vulnerability to climate risks of the region's agri-fisheries sector. These datasets, from both primary and secondary sources, were used on the methodological guidelines provided by the AMIA2 CRVA project – covering climate-risk exposure, sensitivity and adaptive capacity. Preliminary analysis – using GIS software and climate modelling tools – was undertaken at the regional level. The project team also participated in a national-team level joint analysis of cross-regional data.

Component 3 - Stakeholders' participation in climate adaptation planning

The regional project team organized a series of stakeholders' meetings and focus group discussions to collect supplementary data and validate preliminary results of CRVA, as well as in identifying CRA prioritization and planning. These activities were guided by process facilitation using the MaxEnt and CBA Tool developed by the AMIA2 projects on CRVA and CRA decision-support platform.

Component 4 - Documenting & analyzing CRA practices

A semi-structured survey with local stakeholders was conducted to identify and document CRA practices, as well as collect existing CRA-relevant statistical and other secondary data. Focus group discussion with farmers, farmer-leaders, technician, municipal and provincial agriculturists, and representative of provincial government of Tarlac were done on four separate occasions to gather data, validate and present the results, and revalidation of results. These data was systematized and analysed using cost-benefit and trade-off analyses tools as input to AMIA2 CRA prioritization and investment planning. A CBA Tool was made available online by the CIAT to facilitate the computation of cost, benefits, and others. These contributed to developing knowledge products, such as searchable online portal, under the AMIA2 project on CRVA decision-support platform.

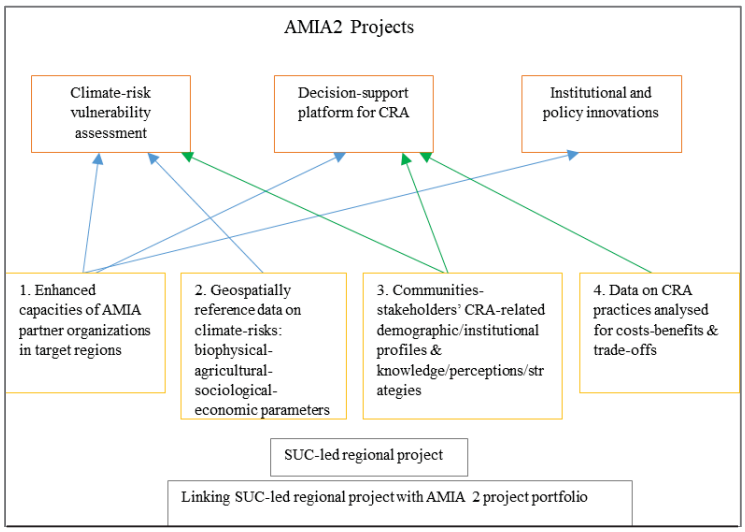


FIGURE 1. Framework of the Study

Data Acquisition

Collection of secondary data for exposure-sensitivity and secondary-primary data for adaptive capacity was done from different agencies such as the Provincial Agriculture Office, Municipal Agriculture Offices, and data from the AMIA2 outputs. Data collection started with gathering of primary and secondary data, that includes those from focus group discussion (FGD), key informant interviews (KII) and municipality surveys.

CRVA Framework

The vulnerability mapping was done which follows the Climate-Risk Vulnerability Assessment (CRVA) framework. It starts with the identification of the vulnerability determinants (hazard, sensitivity, and adaptive capacity) and their respective indicators. This framework was the standardized procedure followed by different SUCs involved in the project. Data collection started in gathering the primary and secondary data, that includes those from focus group discussion (FGD), key informant interviews (KII) and municipality surveys. The identified priority crops of the province are rice, corn, tomato, and mango. Sweet potato was an additional crop prioritized by the team because it is one of the crops planted on the second cropping season after rice and corn.

CRA Practices

Key informant survey on climate-resilient agri-fisheries (CRA) practices in the province of Tarlac. These were gathered from a series of Focus Group Discussions with municipal agriculturists, representatives of the Office of the Provincial Agriculturists and Local Farmer Technicians. From the list of CRA practices, key

informants identified three priority practices. The interview guide provided by CIAT was modified to fit the scope of the study before it was given to the group of key informants. Data gathered were used in the evaluation of CRA practices using the CBA Tool. CRA practices with the incorporation of externalities was given priority in the study.

RESULTS AND DISCUSSIONS

Enhanced Capacities of AMIA Partner Organizations in the Region

Series of trainings, workshops and learning events were organized for AMIA2 project teams in an intra-regional training that covers key learning contents from the national-level trainings as shown in Table 1.

Climate Risk Vulnerability Assessment (CRVA)

The vulnerability mapping was based on the Climate-Risk Vulnerability Assessment (CRVA) framework. It started with the identification of the vulnerability determinants (sensitivity, hazards, and adaptive capacity) and their respective indicators.

Sensitivity Analysis

In developing the impact of climate change to crop suitability, a crop distribution model was used and the factors associated are the 20 bioclimatic variables and the existing crop location. Sensitivity index was used in the sensitivity analysis to determine the sensitivity of crops to changes in temperature and precipitation.

The aggregated sensitivity of crops for rice, maize/corn, tomato, mango, and sweetpotato are given equal weights of 20% as shown in Figure 2 (a). It appears that the sensitivity index of the province generally ranges from -5% to -50% which means that it is sensitive to highly sensitive as influenced by the bioclimatic variables. On the other hand, improved varieties of crops, water conservation and soil conservation technologies that are being practiced by farmers mitigate the effect on crop yield.

Hazards Vulnerability

The natural hazards that were added to come up with the hazard vulnerability index are tropical cyclone/typhoon, flood, drought and erosion which are given the corresponding weights of 35%, 35%, 27% and 3%, respectively. The areas of San Manuel, Anao, Moncada, and La Paz are highly vulnerable to hazards as shown in Figure 2 (b). These areas experiences flooding on the onset of monsoon rain especially during typhoon because of geographically low areas of the province and become the catchment basin of the nearby provinces.

Adaptive Capacity Analysis

The adaptive capacity indicators considered in the assessment of the province's readiness to withstand the effects of climate change. These seven capitals are economic, natural, human, physical, anticipatory, social and institutional. Figure 2 (c) on the adaptive capacity illustrates the economic, natural, human, physical, anticipatory, social and institutional capital of every municipality. These seven (7) capitals of the adaptive capacity were given the same weight to come up with the aggregated adaptive capacity map.

The adaptive capacity of the province shows the readiness to adapt to climate risk. Concepcion and Gerona were found to have very high adaptive capacity while the municipalities of Ramos, San Jose, La Paz, and Bamban have very low adaptive capacity.

Climate-Risk Vulnerability

The climate-risk vulnerability map was developed by adding the sensitivity index, hazard index and the adaptive capacity index with their corresponding weights. A national experts' meeting composed of agriculturists, policy makers, and scientist, agreed the 15-15-70 percentage of weight for the sensitivity, hazard and adaptive capacity, respectively. The formula used in the development of climate-risk vulnerability map is stated in Equation 1:

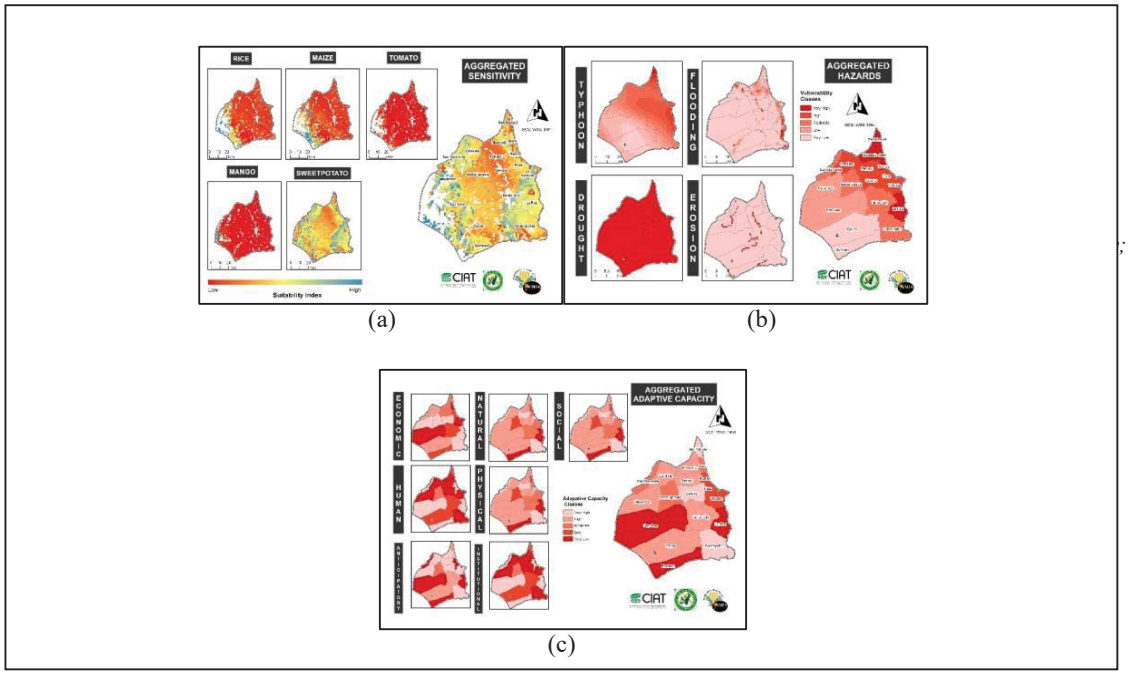
$$\begin{aligned}\text{Climate - risk vulnerability} = {} & \text{Sensitivity index} * 0.15 + \\ & \text{Hazard index} * 0.15 + \\ & \text{Adaptive capacity index} * 0.70\end{aligned} \quad (1)$$

Figure 3 show the vulnerability to climate-risk is very high in the municipalities of Ramos, Bamban and La Paz; high in San Jose, Victoria and Pura; lowest in Tarlac City. The factor that has the major contribution in the vulnerability assessment is the adaptive capacity given a weight of 70% compared to sensitivity and hazards with 15% weights each. The perspective of giving a high percentage to the adaptive capacity is the thought of the ability of every municipality being able to cope with extreme events like temperature, rainfall, typhoon, flood, drought, erosion and other natural hazards because these municipalities are equipped with facilities and structures, and services for the adaptation.

The maps developed in the CRVA assessment was presented to a focus group discussion (FGD) with the stakeholders from the Provincial Agriculture Office (PAO), Municipal Agriculture Office (MAO), Farmers and other agencies. From the FGD and field visit conducted, the participants agreed that the maps developed are similar to the actual situation in their municipality. Vulnerable areas due to risks of climate was mentioned in the study of Dikitanan, et al. (2017).

TABLE 1. Capability Building Training, Seminars and Workshop Attended by the Regional Team

Title	Date	Venue
Training on Climate Risk Vulnerability Assessment	June 6-8, 2016	Torre Venezia Hotel, Quezon City
Cost- Benefit Analysis (CBA) on Climate Resilient Agriculture Practices	August 6-8, 2016	Torre Venezia Hotel, Quezon City
Methodology for Evaluating Social and Environmental Benefits, in Agricultural Systems	December 2, 2017	Tarlac Agricultural University, Camiling, Tarlac
Climate Risk Vulnerability Assessment (CRVA) Mapping & and Adaptive Capacity Mapping	January 10-12, 2017	SEARCA, UPLB, Los Banos, Laguna
AMIA2-CIAT Project: Results Sharing and Validation Workshop on CRVA & CRA Decision Support	February 6-7,2017	Parklane International Hotel, Cebu City
Workshop on Finalizing Results on CRA and Prioritization and Extended CBA	March 1-3, 2017	B Hotel, Quezon City
Completion Review of BAR Funded Climate Change Projects	May31-June 2, 2017	Partido State University, Goa, Camarines Sur
AMIA2-CIAT Project: Workshop on Outcome Monitoring and Evaluation of Community-Based Action Research	June 21-22, 2017	Sequoia Hotel, Quezon City



(a) (b)

(c)

FIGURE 2. Maps on sensitivity, hazards and adaptive capacity to produce CRVA map: (a) Aggregated Sensitivity; (b) Aggregated Hazards; and (c) Aggregated Adaptive Capacity

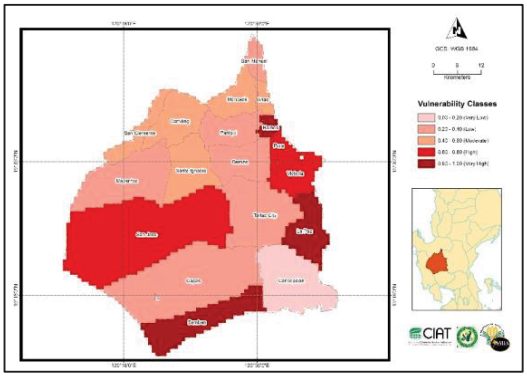


FIGURE 3. Climate-Risk Vulnerability Map of Tarlac

Cost-Benefit Analysis (CBA) of CRA Practices

The identified climate-resilient practices in the province of Tarlac which are the following: Organic agriculture, climate smart varieties/lines, water conservation technology, adaptive crop calendar/crop switching, soil conservation technology, intercropping/crop rotation, community-based management, crop insurance/weather adverse condition insurance, and improved housing for animals. The identification of CRA practices were gathered through interview. The list was trimmed and validated through a series of FGDs with municipal agriculturists, representatives of the Office of the Provincial Agriculturists, local farmer technicians and farmers. CRA practices have also been priorities in the study of Dikitanan, et al. (2017).

From the list of CRA practices, key informants identified three priority practices: climate smart varieties, crop rotation, and water conservation technology particularly the alternate wet and dry method (AWD). The interview guide provided by CIAT was modified to fit the scope of the study before it was given to the group of key informants. Data gathered were used in the evaluation of CRA practices using the CBA Tool. Validation of data was done by seventy one (71) municipal agriculturists, farmer leaders and farmer technicians participated in the Focus Group Discussions. The cost and benefits derived from the CRA practices were confirmed. These data were used to analyse the profitability and sustainability of the CRA practice. The three prioritized practices are the climate smart varieties, crop rotation and water conservation technology because of the immediate effects and perceived potential benefits to the farmers, food security and, mitigation and adaptation to climate change. Investment prioritization brief were also prepared for policy makers to support the climate-resilient agriculture practices in the provinces as shown in Figure 4. Researchers and extensions' personnel must use the investment brief for dissemination and reference for further validation or research in a specific area.

Climate Smart Varieties

Yield of crops is affected by extreme changes in climatic conditions such as flooding, and drought and attack of insect pests and diseases. Farmers and agricultural technologists favor climate smart varieties because of its high yield and its capacity to withstand varied climatic conditions. The Green Super Rice lines (GSR 8, 15, 21, and 22) earned special mention among farmers who have experienced growing it due to its resiliency especially during typhoons and floods, drought, and attack of insect pests and diseases. Farmers who planted GSR lines attested the resilience of these lines to typhoon and submergence. Yield was not likewise affected.

Crop Rotation

Crop rotation was chosen mainly because farmers could switch from rice to other crops (corn, sweet potato, etc.) depending on availability of water, soil conditions, and other climactic factors. Crop rotation also means reduction in pest occurrence due to non-availability of the host.

Water Conservation Technology

To address problems on scarcity of water supply or limited access to water sources in the upland, rainfed, and other areas, water conservation technologies are resorted to. The Alternate Wet and Dry (AWD) Method was specially mentioned because it utilizes materials readily available and can be constructed easily by the farmer. Some parts of the province depend on shallow tube wells and Tarlac Ground (TG) water as their main

source of irrigation. In these areas, one of the main expenses incurred by farmers is gasoline that is used to run pumps. This practice was chosen because it allows farmers to irrigate at the proper time. The intermittent drying of fields enables the farmers to save on time and money.

FIGURE 4. Investment Brief for Climate Smart Agriculture Practices

CONCLUSION

The two major output of this study are: climate-risks vulnerability map and investments briefs. The map was used to identify pilot area of climate smart village. The Department of Agriculture Regional Field Office 3 chose the municipality of Victoria as the pilot climate-smart village in the province of Tarlac. DA RFO3 based the selection on the result of the climate-risk vulnerability mapping and willingness of the local government unit to support the project. Victoria is considered as one of the high vulnerable municipalities that is sensitive to changes in climate, vulnerable to hazards and has less adaptive capacity to cope up with the phenomenon. Victoria is a 2nd class municipality of the province of Tarlac with 26 barangays and is located at the eastern part of the province. Majority of the total land area is devoted to agricultural activities and livestock production.

The climate resilient agriculture practices identified in the prioritization is being adopted in areas in the province for further testing and evaluation in the suitability of a CRA practices in an area. The investment briefs were presented to Department of Agriculture and Office of the President as a support for decision- and policy-making.

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Validation of suitable reference genes for normalization of quantitative reverse transcriptase- polymerase chain reaction in rice infected by *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a costly disease in rice that threatens global rice production. Gene expression analysis by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) allows the study of the underlying mechanisms of both BB pathogenesis and resistance. In relative quantification, reference genes are often used to normalize the results to remove technical variations allowing the determination of true biological changes in a pilot experiment. However, variations in the expression of these reference genes can lead to erroneous and unreliable results. Thus, choosing the most stable reference genes for any specific experimental condition is of utmost importance in qRT-PCR experiments. Here, we used geNorm, NormFinder, Bestkeeper, Delta-Ct and RefFinder programs and/or methods to analyze the stability of the expression of eleven candidate reference genes namely: *18S ribosomal RNA* (*18S rRNA*), *Actin-1* (*ACT1*), *ADP-Ribosylation Factor* (*ARF*), *Endothelial differentiation factor* (*Edf*), *eukaryotic Elongation Factor-1α* (*eEF-1α*), *eukaryotic Initiation Factor-4a* (*eIF-4a*), *Profilin 2* (*Prof2*), *Nucleic Acid Binding Protein* (*NABP*), *Triosephosphate Isomerase* (*TI*), *Ubiquitin 5* (*UBQ5*) and *Ubiquitin 10* (*UBQ10*) in cDNA samples from BB-susceptible and Xa21-mediated resistant rice cultivars collected at various times after *Xoo* inoculation. Under our experimental conditions, *Edf* and *TI* were the most stable reference genes while the common housekeeping genes *18S rRNA*, and *UBQ5* were among the least stable genes. Though using either *Edf* or *TI* as internal control is adequate for gene expression analysis, we suggest using both genes to normalize the data of qRT-PCR assays for rice subjected to *Xoo* inoculation.

1. Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a threatening rice disease responsible for the large percentage of yield reduction in all rice growing areas. Its symptom includes a vascular wilt at the seedling stage, a leaf blight, and unfilled panicles in mature plants which resulted from the invasion of the vascular system by *Xoo* bacteria (Mew, 1987). At the molecular level, *Xoo* secretes transcription activator-like (TAL) effectors which invade and highjack the host cells by activating the transcription of genes that enhance plant susceptibility and support bacterial virulence (Boch and Bonas, 2010; Römer et al., 2010). To date, more than 30 BB resistance genes have been identified in *Oryza sativa* and its closely related species. Among them, *Xa21* has probably been the most commonly used for rice variety improvement as it provides high level and broad-spectrum BB resistance (Nguyen et al., 2018; Singh et al., 2001; Zhang et al., 2006). *Xa21* codes for a plasma membrane receptor which recognizes the tyrosine-sulfated protein RaxX and triggers the Xa21-mediated immunity (Pruitt et al., 2015). It is known that this response involves Xa21-binding proteins (Chen et al., 2010; Park et al., 2010; Wang et al., 2007) as well as the direct interaction of a cleaved Xa21 subunit with the WRKY62 transcription factor (Park and Ronald, 2012; Peng et al., 2008), nonetheless, the precise mechanisms of the resistance are still not yet completely elucidated.

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Studying the variations in the expression of candidate genes provides perspectives of the mechanisms of plant responses to BB. The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) remains a tool of choice to quantify mRNA expression and thus confirm the involvement of various candidate genes in a specific cellular response due to the sensitivity, precision and reproducibility of its results (Derveaux et al., 2010; Hellemans et al., 2007). However, the veracity and reliability of qRT-PCR results are essentially altered by the variations between samples, RNA quality and quantity, and the different reverse transcription and PCR efficiencies (Bustin et al., 2009; Derveaux et al., 2010). To cope with this variability, expression levels of the genes of interest (GOIs) are usually normalized against a stably and uniformly expressed reference genes according to the $\Delta\Delta CT$ method in order to provide reliable relative expression data (Rao et al., 2013). Though, in this method, the selection of reference genes is critical since variations in their expression can completely overturn the final data especially when the variations in GOI expression are restrained. The expression of the reference genes used in qRT-PCR analysis should remain stable across all experimental samples and under different conditions (Derveaux et al., 2010; Huggett et al., 2005). Yet, previous reports on the stability of the usual reference genes has shown that no universally stable reference gene could be found, thus implicating that reference genes' stability in qRT-PCR experiments needs to be validated for specific and suitable experimental conditions and purposes (Kozera and Rapacz, 2013; Laurent et al., 2008).

Several software and programs have been established to evaluate the stability of the reference genes expression. Among the most commonly used are the geNorm (Vandesompele et al., 2002a), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms, and the Delta-Ct method (Silver et al., 2006) and RefFinder system (Xie et al., 2012). Using these tools, various studies on rice have highlighted that proper validation of reference genes is essential to determine their stability and thus recommending the suitability of each reference gene for various experiments in rice. For example, *Ubiquitin 5* (*UBQ5*), *Ubiquitin-conjugating enzyme E2* (*UBC-E2*), *Endothelial differentiation factor* (*Edf*) and *eukaryotic Elongation Factor-1α* (*EF-1α*) were found to be suitable for growth and development, environmental conditions, and organ-specific gene expression analyses (Auler et al., 2017; Narsai et al., 2010; Wang et al., 2016). For hormonal and abiotic treatments and stresses, *18S ribosomal RNA* (*18S rRNA*), *25S ribosomal RNA* (*25S rRNA*), *Ubiquitin 10* (*UBQ10*) and *Ubiquitin conjugating enzyme (UBC)* were the most stable reference genes (Almas and Kamrodi, 2018; Jain et al., 2006; Kim et al., 2003; Moraes de Freitas et al., 2015) while for a few biotic stress like blast infection *18S rRNA, Actin, and 40S 27a ribosomal subunit (40S 27a)* were found to be the most suitable reference genes (Bevitori et al., 2014; Che Omar et al., 2016). To our knowledge, no resources are currently available on the suitable reference genes for gene expression analysis involving *Xoo* inoculation experiments. Hence, in this paper, we analyzed 11 candidate reference genes in terms of their expression stability in both BB-susceptible rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21/Xa21*) (Sagun, Suachaowna et al., unpublished) at different times post *Xoo* inoculation.

2. Materials and methods

2.1. Plant material and growth conditions

Rice (*Oryza sativa* L. ssp. *indica*) cultivars RD47 and IRBB21 were provided by the Bureau of Rice Research and Development, Phitsanulok, Thailand. The *Xa21* gene originated from the wild species *O. longistaminata* was transferred through wide hybridization in IR24, resulting in the near-isogenic line, IRBB21. In tests for disease resistance, IRBB21 has been reported to be resistant to many *Xoo* strains from the Philippines and India (Khush et al., 1990). Rice cultivar RD47, an elite Thai cultivar, was derived from three-line cross between Suphanburi 1 and IR64 then with CNT86074–25–9-1 at Chainat Rice Research Center, Thailand (the Rice Department, Ministry of Agriculture and Cooperatives, Thailand).

The *Xa21* gene from IRBB21 was introgressed in RD47 through backcross breeding and Marker Assisted Selection (data not shown) until homozygous-*Xa21* BC_3F_3 lines were obtained.

2.2. Xoo isolation and inoculation test

Since Thailand biosafety regulations limit the import of living microorganisms especially those causing diseases in major commodities like rice, the authors made use of the local strains of *Xoo* in this study and validated them through PCR using specific universal primers.

BB infected leaves were collected from paddy fields in Phitsanulok province and *Xoo* was isolated on nutrient agar (peptone-bovine-agar). The isolated bacteria, xoo16PK002, was identified as *Xoo* through PCR assays using *Xoo* specific primers TXT (Sakthivel et al., 2001) and *Xoo* specific primers Xoo80 (Lu et al., 2014). Furthermore, preliminary pathogenicity tests on 60 days old rice plants were done and had shown that RD47 plants were rather susceptible to highly susceptible to xoo16PK002 with clear BB lesions lengths (LL) ranging 25-29 cm ± 1.28 at 21 days after inoculation, whereas RD47's near isogenic lines BC_3F_3 and the IRBB21 cultivar were more resistant to the *Xoo* strain with LL ranging from 5 to 9 cm ± 0.26 (Sagun, Suochawna, Puttasem et al., unpublished). For the infection experiments presented here, the *Xoo* isolate xoo16PK002 was re-streaked and incubated at 28 °C for 48 h. A *Xoo* inoculum (OD600 of 0.2) was prepared and used to inoculate 60-day-old plants according to the clipping method (Kauffman, 1973). Mock (water) inoculation was used as a control. Samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0, 2, and 24 h post inoculation, respectively, and leaf samples were frozen in liquid nitrogen immediately.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from each 100 mg leaf sample using RNAprep Pure Kit (Tiangen Biotech LTD., China) following manufacturer's instructions. Total RNAs were quantified using Synergy H1 microplate reader (Biotek, USA) and their integrity was assessed through agarose gel electrophoresis. The qScript™ XLT cDNA synthesis kit (QuantaBio, USA) was used to reverse transcribe 1 μg of total RNA templates in order to synthesize first strand cDNAs according to manufacturer's protocol.

2.4. Quantitative Real-time PCR

For qPCR analyses, the fast SYBR Green Master Mix (Quantabio, USA) was used to prepare 20 μl qRT-PCR reactions containing 1 μl of the cDNA templates and 0.5 μM of each primer pairs. Technical triplicates and No Template Controls (NTCs) were run through the Eco48 Real-Time PCR system (PCRmax, UK) for 35 cycles (95 °C for 10s, 60 °C for 10s and 72 °C for 20s) followed by a melting curve analysis. Ct values were determined using the Eco™ 48 Study Software installed in the Eco48 Real-Time PCR system. The Eco™ 48 Study software allows for normalized data combination from multiple Eco 48 experiments into a single analysis, and supports standard curve analysis and relative quantification experiments, which were used in this study. The comparative Ct method was used to transform generated Ct values in relative quantities with the highest relative quantity for each gene set up to 1.0. All data were subjected to analysis using geNorm v3.4 (Vandesompele et al., 2002a), NormFinder v20 (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), Delta-Ct method (Silver et al., 2006) and RefFinder (Xie et al., 2012) programs.

Table 1
List of candidate reference genes validated in this study.

Candidate reference gene	Primer sequence	Reference
Endothelial differentiation factor(Edf)	5′-TCCGAACCAGCAGATCATCG-3′	Wang et al., 2016
LOC_Os08g27850	5′-GCATGGTATCAAAAGACCCAGC-3′	
Triosephosphate Isomerase (TI)	5′-CGACATCATCAACTCCGCCAC-3′	Wang et al., 2016
LOC_Os01g05490	5′-CCTCTTCAGACATCTTCCCACG-3′	
eukaryotic Initiation Factor-4a (eIF-4a)	5′-TTGTGCTGGATGAAGCTGATG-3′	Wang et al., 2016
LOC_Os03g08020	5′-GGAAGGAGCTGGAAGATATCATAGA-3′	
Profilin-2 (Prof2)	5′-CCAACTGGTCTTTTCCTTGGG-3′	Wang et al., 2016
LOC_Os06g05880	5′-GGGGTCATCGGCTCATCATAG-3′	
ADP-ribosylation factor (ARF)	5′-ATGAAAGGAAGACATGGCGG-3′	Wang et al., 2016
LOC_Os05g41060	5′-TGGTGGTGGAACCTAAAGAGC-3′	
Nucleic acid binding protein (NABP)	5′-GGAATGTGGACGGTGACACT-3′	Narsai et al., 2010
LOC_Os03g25980.1	5′-TCAAAATAGAGTCCAGTAGATTTGTCA-3′	
eukaryotic Elongation Factor-1α (eEF-1α)	5′-TTTCACTCTTGGTGTGAAGCAGAT-3′	Jain et al., 2006
LOC_Os03g08020	5′-GACTTCCTTCACGATTTCATCGTAA-3′	
Ubiquitin 10 (UBQ10)	5′-TGGTCAGTAATCAGCCAGTTTGG-3′	Jain et al., 2006
LOC_Os02g06640	5′-GCACCACAAATACTTGACGAACAG-3′	
Actin-1 (ACT1)	5′-CTTCATAGGAATGGAAGCTGCGGGTA-3′	Narsai et al., 2010
LOC_Os05g36290.1	5′-CGACCACCTTGATCTTCATGCTGCTA-3′	
18S ribosomal RNA (18SrRNA)	5′-CTACGTCCCTGCCCTTTGTACA-3′	Jain et al., 2006
Locus ID: AK059783	5′-ACACTTCACCGGACCATTCAA-3′	
Ubiquitin-5 (UBQ5)	5′-CCAGTACCTCAGCCATGG A-3′	Jain et al., 2006
Locus ID: AK061988	5′-GGACACAATGATTAGGGATC-3′	

Gene names and all their details are presented the way they are reflected in the reference cited.

3. Results

3.1. qRT-PCR of candidate reference genes

A set of 11 candidate reference genes and their specific primers was selected from previous studies on reference gene validation in rice (Table 1). The expression levels of these genes were measured by qRT-PCR in 8 different samples corresponding to the leaves from the BB susceptible RD47 (no *Xa21*) and its BB resistant progeny BC_3F_3 (homozygous *Xa21*) collected at 0, 2 and 24 h after *Xoo* inoculation and 2 h after mock inoculation. For all the tested candidate genes, NTCs showed no amplification, and the sample melting curve analysis generated single peaks indicating that a specific PCR product for each gene was amplified. Moreover, electrophoresis of qRT-PCR products showed a single band of the expected size for each candidate gene (Fig. 1). After confirming the specificity of the qRT-PCR primers, the Ct values were determined for each technical triplicate in all samples. The mean Ct values (Supplemental Table 1) were then transformed into relative quantity values, which was later used in the geNorm and NormFinder programs by fixing the highest relative quantity for each candidate gene to 1 and using the comparative Ct method. For Bestkeeeper, Delta-Ct and RefFinder analyses, the raw Ct values were used.

3.2. Stability of candidate reference genes using geNorm analysis

The geNorm software (Vandesompele et al., 2002a) uses the principle that the expression ratio of perfect reference genes should remain constant across different experimental treatments. It determines the gene expression stability measure (M) of reference genes as well as the average pairwise variation for that gene as compared to other tested reference genes. Stepwise exclusion of the gene with the highest M value allows to select the two most stable genes. The two most stable genes are determined by sequentially removing the least stable gene with the highest M value (Vandesompele et al., 2002a). Initially, analysis of the samples from the BB susceptible RD47 (Fig. 2a) and the BB resistant BC_3F_3 plants (Fig. 2b) was undertaken separately. For the RD47 samples, *Edf* and *eIF-4a* had the lowest M values, hence, they were the most stable reference genes, and followed by *TI*. Meanwhile, *ARF*, and *18S rRNA* yielded the highest M values and were the least stable genes (Fig. 2a). In the BC_3F_3 samples, *Edf* and *TI* were the most stable genes while *eIF-4a* was ranked fourth. Meanwhile, *ARF*, *UBQ5*, and *18S rRNA* were among the three least stable reference genes (Fig. 2b). Finally, when all the samples were analyzed together for the expression stability of the candidate genes (Fig. 2c), *Edf* and *TI* were the most stable genes, followed by *eIF-4a* while *ARF*, *18S rRNA*, and *UBQ5* were still among the least stable genes.

3.3. Determination of the optimal number of reference genes by geNorm

Although most published studies on gene expression suggest a single internal control for qRT-PCR normalization, it is also known that increasing the number of reference genes results in more reliable and more accurate data (Jain et al., 2018; Vandesompele et al., 2002b; Zhao et al., 2016). However, there is a trade-off between accuracy and practical considerations when selecting reference genes to be used. Pairwise variation (V) analysis calculates between two sequential normalization factors containing an increasing number of reference genes; geNorm also provides the tool in generating the optimal reference genes to be used. The program suggests that if the value of V is below the 0.15 cut-off value, the last added reference gene may not need to be included for the data normalization. In this study, the V2/3 value of 0.136 indicates that the third most stable reference gene, which is *eIF-4a*, is not required and thus, the use of the two most stable reference genes, *Edf* and *TI*, is already optimal for accurate normalization (Fig. 3).

3.4. Stability of candidate reference genes using NormFinder analysis

To validate the results of the geNorm analysis, we also assessed the expression stability of the candidate genes in our samples with the NormFinder software. NormFinder directly calculates for each gene a stability value based on its inter- and intragroup variations of expression which can prevent the selection of co-regulated genes (Andersen et al., 2004). As the NormFinder software is limited to only 10 genes for analysis, the top 10-ranked stable genes from the geNorm analysis were used for the calculation. Results of the analysis showed that *Edf* and *TI*, with the lowest individual stability values of 0.036 and 0.059, respectively, were again selected as the most stable genes with a combined stability value of 0.029. The reference gene *Edf*, in particular, was deemed to be the best reference gene as its variation values for both intra- and inter- groups were lowest while *TI* still showed more variation than *eIF-4a* in the RD47 group (Table 2). The *UBQ5* and *18S rRNA* genes, respectively, were ranked 8th and 10th among the 10 tested

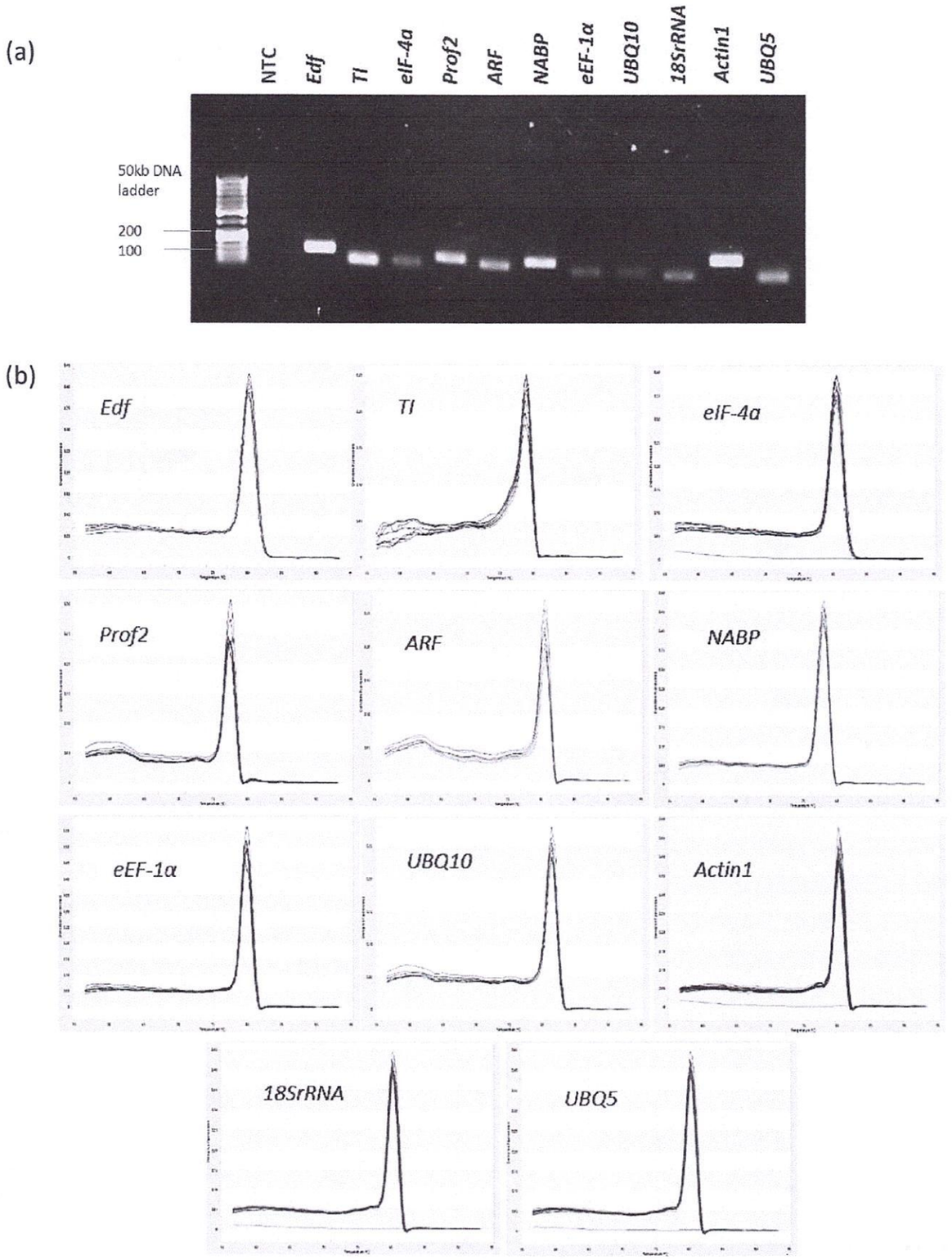

Fig. 1. Specificity of gene amplification products tested on the 6-leaf stage rice cultivar RD47 at 0 h after *Xoo* inoculation. (a) Gel migration of qRT-PCR products. A single DNA amplicon of the projected size is shown for each gene tested in this study. Agarose gel (2%). NTC-Non-Template Control; and (b) Dissociation curves of qRT-PCR products for all candidate reference genes validated in this study. Analysis of dissociation curves, also called melt curves, was used to assess whether the candidate reference genes produced single, specific products. The single peaks of dissociation curves in each candidate reference gene represented a pure, single

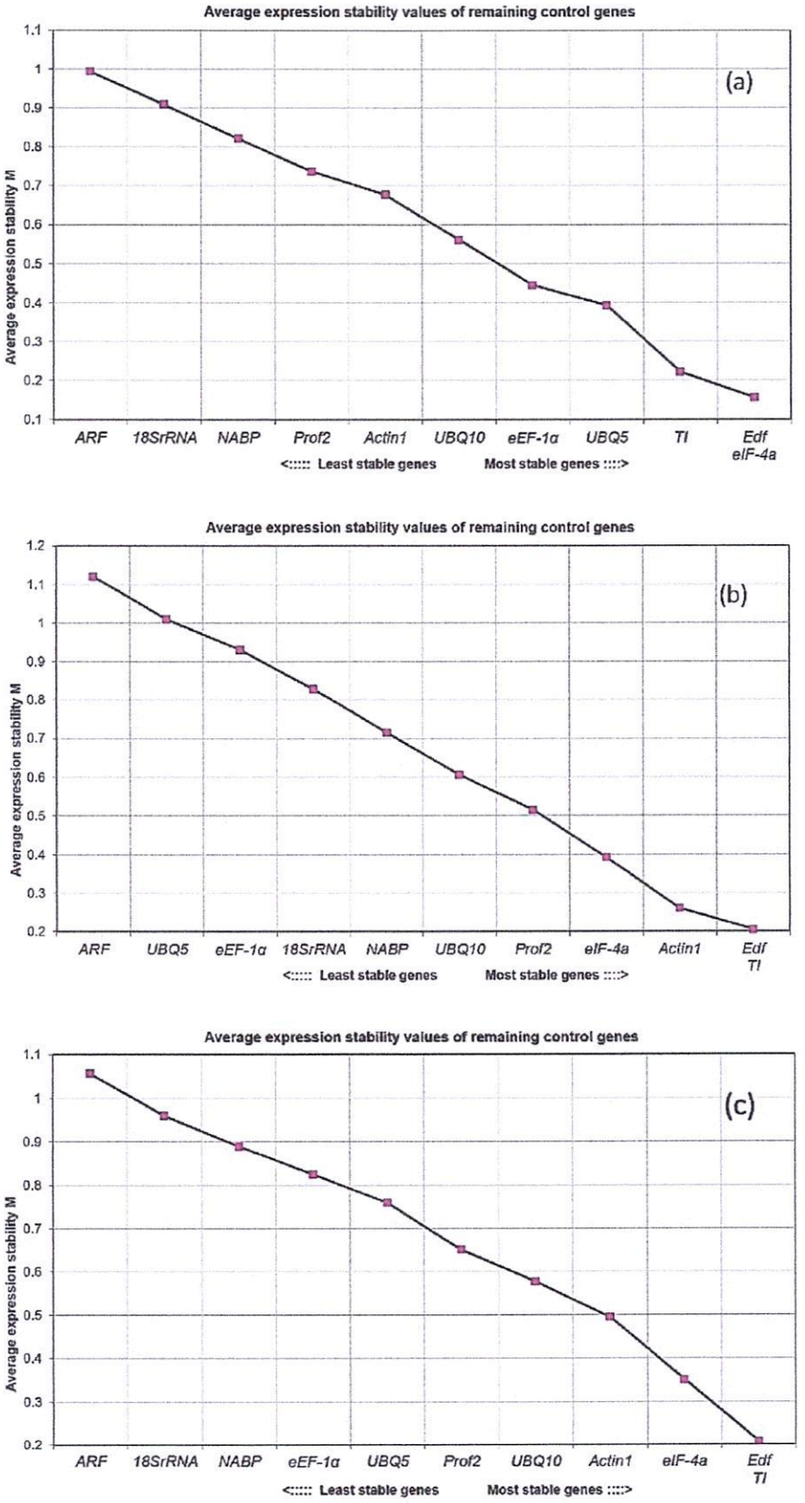

Fig. 2. Expression stability and ranking of reference genes by geNorm: (a) RD47; (b) BC_3F_3 (*Xa21-Xa21*) progeny; and (c) all samples. Gene(s) with lower average expression stability M denotes more stable expression.

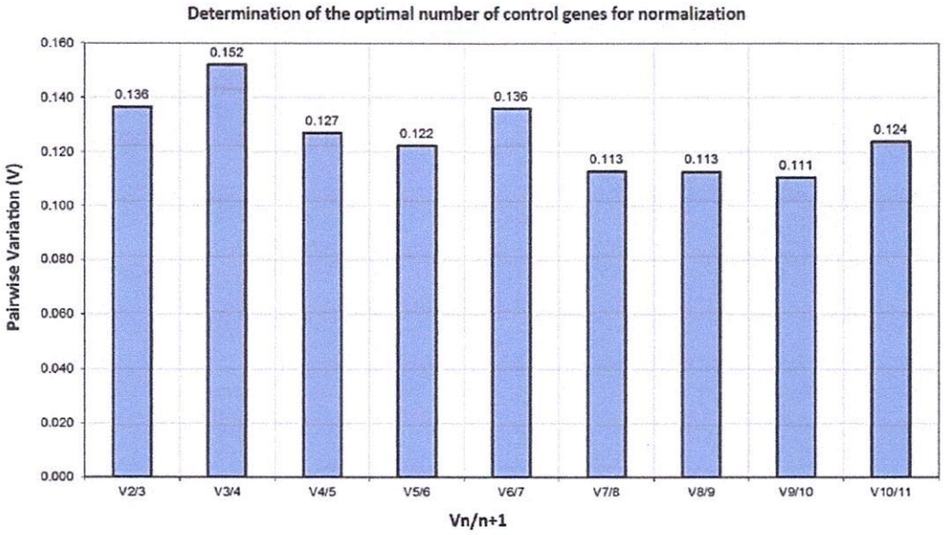

Fig. 3. Determination of the optimal number of control genes for accurate normalization by geNorm pairwise variation analysis. Pairwise variation analysis (Vn/n + 1) between the normalization factors NFn, including the n most stable reference genes, and NFn + 1, corresponding to the stepwise inclusion of the next most stable reference gene, were calculated. A large variation means the last added reference gene has a significant effect and should be included while Vn/n + 1 with a value below the 0.15 cutoff indicates n as the optimal number of reference genes.

Table 2
NormFinder analysis of top 10-ranked stable reference genes from the geNorm analysis. Gene(s) with lower stability value denotes more stable expression.

Candidate reference gene	Stability value	Intra-group variation		Inter-group variation
		RD47	BC_3F_3	
Edf	0.031	0.003	0.005	0.022
TI	0.048	0.015	0.005	0.032
eIF-4α	0.072	0.003	0.054	0.169
ACT1	0.184	0.319	0.029	0.061
UBQ10	0.208	0.135	0.217	0.037
Prof2	0.289	0.386	0.286	0.130
eEF-1α	0.322	0.280	0.575	0.297
UBQ5	0.326	0.242	0.659	0.178
NABP	0.339	0.396	0.530	0.033
18S rRNA	0.390	0.625	0.595	0.178
Best gene				*Edf*
Stability Value for Best Gene				0.031
Best combination of two genes				*Edf and TI*
Stability value for best combination of two genes				0.029

candidate reference genes indicating very low expression stability.

3.5. Stability of candidate reference genes using BestKeeper analysis

The BestKeeper software determines the best suited standards of reference genes and combines them into an index. The index is used to decide whether reference genes are differentially expressed under an applied treatment. All data processing for this software is based on crossing points and determines the optimal housekeeping genes employing the pair-wise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the 'best' suited ones (Pfaffl et al., 2004). In this study, though *18S rRNA* and *ARF* were among the least stable reference genes selected by geNorm and NormFinder, the BestKeeper software identified and placed these two genes in rank 1 and 2 as the most stable genes since they have the lowest variation compared to the other genes as determined by their standard deviation (SD) of the crossing point values (CV) at 0.58 and 0.59, respectively. On the other hand, *Prof2* and *eEF1α* were among the least stable reference genes with SD values of 1.39 and 1.78, respectively (Table 3). For BestKeeper analysis, any SD values higher than 1 is considered unstable.

Table 3
Crossing point data of candidate reference genes by BestKeeper. Results of data analysis were taken from raw Ct values of RD47 and BC_3F_3 rice samples under 0, 2 and 24 h post inoculation, respectively, including mock inoculation.

	18S	*ARF*	*UBQ10*	*NABP*	*TI*	*Edf*	*UBQ5*	*ACT1*	*eEF4α*	*Prof2*	*eIF1α*
N	24	24	24	24	24	24	8	24	24	24	24
Geo mean [CP]	9.19	17.35	18.66	27.59	20.14	20.4	22.35	23	23.64	22.7	23.03
AR mean [CP]	9.21	17.37	18.69	27.62	20.18	20.4	22.39	23.1	23.69	22.8	23.12
Min [CP]	8.33	15.91	17.21	26.05	18.16	18.2	20.28	20.2	21.21	19.7	20.1
Max [CP]	10.51	18.24	20.58	29.59	21.92	22.2	25.33	25	26.12	25.1	26.63
Std dev [+/− CP]	0.58	0.59	0.89	0.97	1.09	1.12	1.22	1.26	1.28	1.39	1.78
CV [% CP]	6.3	3.39	4.77	3.49	5.43	5.47	5.44	5.46	5.41	6.09	7.7
Min [x-fold]	−1.81	−2.72	−2.74	−2.92	−3.95	−4.56	−4.18	−7.13	−5.42	−8.25	−7.6
Max [x-fold]	2.5	1.85	3.79	3.99	3.43	3.52	7.89	4.01	5.57	5.13	12.16
Std dev [+/− x-fold]	1.5	1.5	1.85	1.95	2.14	2.17	2.33	2.4	2.43	2.62	3.43

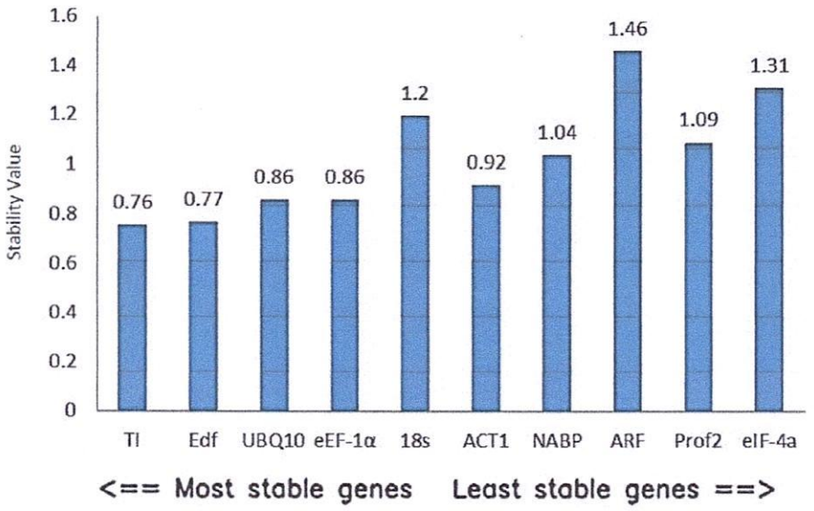

Fig. 4. Expression stability and ranking of candidate reference genes as determined by Delta-Ct method. Low gene stability values denote more stable expression.

3.6. Stability of candidate reference genes using the Delta-Ct (ΔCt) method

The ΔCt method compares relative expression of gene pairs within each sample to identify useful housekeeping genes. If the calculated value between the two tested genes remains constant in different RNA samples, it means that the expression of either both genes are stable among those samples, and if the ΔCt changes, then either one or both genes are inconsistently expressed (Silver et al., 2006). In our results, *TI* (0.77) and *Edf* (0.772) were identified as the most stable reference genes, the same genes identified by geNorm and NormFinder, while *ARF* (1.272) and *eEF1α* (1.487) were among the least stable genes (Fig. 4).

3.7. Stability of candidate reference genes using RefFinder analysis

To come up with a more comprehensive report on the expression stability of reference genes relative to our experimental conditions, we subjected our data series for RefFinder analysis to pull out a general recommendation. RefFinder is a web-based comprehensive tool developed to evaluate and screen reference genes from a widespread experimental dataset. It integrates the currently available major computational programs such as geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method. The system compares and ranks the tested candidate reference genes based on assigned appropriate weight of each individual gene and calculated geometric mean of their weights for the overall final ranking.

Results of the analysis showed that *TI* and *Edf* were among the most stably reference genes since they have the lowest stability values at 1.778 and 1.861, respectively. These two genes were consistent in all validation analyses except for BestKeeper. The *18S rRNA* (5.335) and *ARF* (7.183) which were the most stable genes determined by BestKeeper were ranked 6th and 8th, respectively. *Prof2, UBQ5,* and eEF1α were identified as the least stable reference genes with gene stability values of 7.364, 7.483, and 9.685, respectively (Table 4).

Table 4
Ranking of candidate reference gene expression stability by RefFinder.

Candidate reference gene	geNorm	NormFinder	BestKeeper	Delta Ct	RefFinder	Rank
TI	0.208	0.048	1.09	0.76	1.5	1
Edf	0.208	0.031	1.12	0.77	2.21	2
UBQ10	0.576	0.208	0.89	0.86	3.66	3
eEF-1α	0.351	0.322	1.28	0.86	4.12	4
18S rRNA	0.962	0.39	0.58	1.2	4.9	5
ACT1	0.494	0.184	1.26	0.92	5.14	6
NABP	0.891	0.339	0.97	1.04	5.63	7
ARF	1.057	N/A[a]	0.59	1.46	6.69	8
Prof2	0.65	0.289	1.39	1.09	7.17	9
UBQ5	0.76	0.326	1.22	1.2	7.48	10
eIF-4a	0.827	0.072	1.78	1.31	8.97	11

[a] N/A - not applicable.

4. Discussion

Earlier studies reported that the expression stability of various reference genes in rice vary under different experimental conditions and emphasized the need for a proper validation of the stability of reference genes for any gene expression analysis to come up with accurate and reliable results. The geNorm method was the first released algorithm in evaluating the stability of the expression of candidate reference genes and has established itself as the golden standard with more than 10,000 citations to date. However, as the expression stability value M for a gene generated from geNorm is dependent upon the other tested genes, concern has been raised about the possible selection of co-regulated genes instead of the stable ones. In this case, the NormFinder software (Andersen et al., 2004) uses a different approach which can cope with this problem. Thus, it is not rare that both of these methods alongside with BestKeeper, Delta-Ct method and RefFinder programs are usually used together to determine the best reference genes for a specific gene expression assay (Auler et al., 2017; Bevitori et al., 2014; Wang et al., 2016). In several studies, the genes selected by these algorithms are often slightly different even in most cases, the most stable reference genes selected by one program still belongs to the relatively stable genes category in the other analysis. In our study, *Edf* and *TI* were found to be the best reference genes across all approaches except for Bestkeeper thereby suggesting that their expressions are stable under *Xoo* inoculation.

Traditional reference genes like the *Actin1* (*ACT1*), *eEF-1α, β-tubulin, UBQ10, UBC-E2, UBQ5, 18S rRNA,* and *25S rRNA* were commonly used as internal controls in various experiments in rice focusing on growth and development stages, different tissue samples, and various treatments due to their recognized stability. Among these reference genes, *UBC* and *UBQ10* were found to be the most stable in rice plant responses to heavy metal stress (Almas and Kamrodi, 2018); *UBC* was the

most stable in rice treated with different nitrogen levels (Benemann et al., 2017); *18S rRNA* was the most suitable reference gene under various growth stages of etiolated seedlings, different cultivars, and various times after UV-irradiation treatment compared to *glyceraldehyde-3-phosphate dehydrogenase, actin,* and *tubulin* (Kim et al., 2003). However, other studies have also stressed that the transcription levels of these traditional reference genes may change depending on the plant developmental processes, environmental conditions and treatment sets, and expression differences of the genes of interest could be attributed to the expression variation of inappropriate reference genes (Gutierrez et al., 2008a; Gutierrez et al., 2008b; Wang et al., 2016). In the report of Jain et al. (2006), *UBQ5* and *eEF-1α* were found to be the most stable whereas *18S rRNA* and *UBQ10* were among the least stable in these commonly used reference genes when analyzed across all their samples and in developmental series. Li et al. (2009) also analyzed the stability of these commonly used reference genes during rice seed development and found out that *eIF-4a* and *ACT1* were the most suitable reference genes while again *18S and 25S rRNAs* were among the least stable in almost all the tested samples from two rice varieties at different developmental stages, and a total of 6 reference genes was optimal for qPCR calibration using most of their tissue groups.

As no holistic stable reference gene could be found among the traditionally used housekeeping genes, significant efforts have been created to find novel and more stable reference genes (Jain, 2009; Narsai et al., 2010). With the increasing number of analyses of large sets of microarray data, *Edf* and *TI* were selected as potential reference genes for rice gene expression analysis. In a recent article, the expression stabilities of *Edf*, *TI*, and other novel reference genes in rice were compared to those of the more traditional ones like *UBQ5* for 22 different experimental conditions (Wang et al., 2016). The results of these experiments showed that novel reference genes were globally more stable and *Edf* and *TI* were often among the most stable genes. Our study revealed similar results for the *Xoo* inoculation condition as *Edf* and *TI* were also found to be the best reference genes while the traditional housekeeping genes *18S rRNA* and *UBQ5* including *ARF* and *eEF1-α* were among the least stable genes. Though *18S rRNA* has probably been the most frequently used reference genes for gene expression analyses, increasing number of reports that it can have very low expression stability under blast infection and drought tolerance (Bevitori et al., 2014) and in different developmental and environmental conditions (Jain et al., 2006; Li et al., 2009). The present study for two different rice cultivars under *Xoo* inoculation also showed that *18S rRNA* was the least stable and therefore, should only be used as an internal control with the highest caution. Moreover, in the report of Wang et al. (2016), the stability of *Edf* and *TI* were found under hormone treatments including salicylic acid (SA), which plays an important signaling role in the activation of various plant defense responses following pathogen attacks as highlighted in the report of Dempsey et al. (2010). And since the expression of *Edf* and *TI* were found to be stable under SA treatment, these findings indicate why the expression of these two genes under *Xoo* inoculation were also stable as presented in this study.

5. Conclusion

In our study, *Edf* and *TI* were found to be the most stably expressed among all reference genes validated, thus should be suitable internal controls for the normalization of gene expression analysis in rice inoculated with *Xoo*. While the use of either of the two genes as internal control is adequate for gene expression analysis, using both genes as presented in this study, is suggested to produce more accurate and reliable results. Besides, due to the variability of the TAL effectors between *Xoo* strains, confirming the stability of these reference genes when using a new *Xoo* strain would also be advisable.

Declaration of Competing Interest

All authors declare that they have no conflict of interest and all ideas reflected in this manuscript have been agreed upon.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2019.100217.

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