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DNA Barcoding of Red Jungle Fowls (*Gallus gallus philipensis* Hatchisuka) from Different Mountains Areas in the Philippines

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ABSTRACT

Initially proposed as a global standard for rapid species identification, DNA barcodes (cytochrome c oxidase subunit I or COI in the mitochondrial genome) were determined to assess diversity and genetic distances among 25 red jungle fowls (Gallus gallus philipensis Hatchisuka) obtained from different mountain areas in 23 provinces of 12 islands in the Philippines. Results of the evolutionary analyses using Kimura two-parameter model in MEGA5 indicated existence of two main evolutionary clades, and effectiveness of DNA barcodes in identifying and differentiatiang red jungle fowls between and within clades. Genbank-accessed COI sequences of three subspecies of red jungle fowls (Gallus gallus gallus, Gallus gallus bankiva, Gallus gallus spadiceus) and three Gallus species (G. lafayettei, G. sonneratii, G. varius) were clustered in the intermediate zone between differentiated populations of Philippine red jungle fowls, but more recently diverged with those in Clade A.

Based on 627 positions from 25 COI sequences, average genetic distance among red jungle fowls was 0.254 units, demonstrating close resemblance within clade, but greater divergence between clades (d>1). Genetic divergence within Clade A (d=0.294) was higher than Clade B (d=0.215). Moreover, pooled pair-wise genetic distance was not significantly correlated (P>0.05) with geographical distances among red jungle fowls between and within clades.

Evolutionary analysis of the DNA barcodes of Philippine red jungle fowls provided important information on genetic variability and population structure useful to support decisions on agrobiodiversity conservation and research in upland areas.

Key words: *DNA barcodes, evolutionary analysis, Philippine red jungle fowls*

INTRODUCTION

The red jungle fowl is the ancestor of the domestic chicken, and, though scarcely recognized on an international level, they contribute significantly to household food security in Southeast Asia (*Shand 1997*). Red jungle fowls are omnivorous and consume a variety of items (e.g. grain, weed seeds, berries etc.), as well as numerous species of insects and invertebrates. They are found in the southern most parts of Southeast Asia to the islands of Sumatra, and Java to Bali, Sulawesi and the Philippines, Malay archipelago, northern and eastern India and Himalayan foothills of northern Pakistan.

The monophyletic theory that hypothesizes the red jungle fowl as the main progenitor of the domesticated chicken is supported by archaeological discoveries in the Indus Valley, and in Hebei Province, China as early as 5400 BC (*West and Zhou 1988*) and further bolstered by molecular evidence such as mitochondrial control region sequences (*Fumihito et al. 1994; Fumihito et al. 1996*) and nuclear microsatellite data from a range of chicken populations (*Hillel et al. 2003*). The continental population of the red jungle fowl subspecies (*Gallus gallus gallus*) in Southeast Asia was even suggested as the sole ancestor of all domestic chickens and might have originated from a single domestication event that occurred in Thailand and adjacent regions (*Fumihito et al. 1994; Fumihito et al. 1996*).

On the other hand, molecular evidence for hybridization between species in the genus *Gallus* raised the possibility that the other jungle fowl species were also progenitors of the domestic fowl (*Nishibori et al. 2005*), (i.e. polyphyletic theory). Other jungle fowls in the *Gallus* genera include *G. varius* Shaw 1798 (green jungle fowl) in some Indonesian islands, *G. lafayettei* (Ceylon jungle fowl) in Sri Lanka, and *G. sonneratii* Temminck 1813 (grey jungle fowl) in peninsular India. Some possible progenitors from several *Gallus* subspecies are: *G. g. gallus* Linnaeus 1758 in Thailand and Indo-China, *G. g. spadiceus* in Burma and Yunnan Province, China, *G. g. jabouillei* in Southern China and Vietnam, *G. g. murghi* in India, and *G. g. bankiva* in Java and its neighboring islands (Madura, Kangean, Bawean, Bali, Lombok, Sumbawa, Flores and Alor) in Indonesia (*Delacour 1977*; *Howard and Moore 1984*; *Crawford 1990*; *Crawford 1995*). Numerous studies using microsatellites, generally have shown that jungle fowl populations, and traditional unselected breeds are widely heterogeneous populations that may include a large portion of the total genetic diversity (*Rosenberg et al. 2001*; *Hillel et al. 2003*; *Granevitze et al. 2007*).

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In the Philippines, red jungle fowls, locally known as "labuyo" or the wild type chickens,(*Gallus gallus philipensis* Hatchisuka) are part of the important diversity in many mountain areas scattered in the archipelago. Their existence may reflect the mountain ecology's current status, and future opportunities for sustainable land use and rural development in upland communities. Except for the paper by *Masangkay et al.* (*2010*), there is meagre scientific information on the Philippine red jungle fowl.

In this study, DNA barcodes proposed by *Hebert et al.* (*2003*) as a tool for rapid specie identification were determined to assess genetic diversity and distance of red jungle fowls taken from different mountain areas in the Philippines. Philippine red jungle fowls were likewise compared with three subspecies of jungle fowls and three Gallus species whose COI sequences were derived from GenBank. The study also aimed to evaluate the correlation of pooled pair-wise genetic distance using DNA barcodes with estimated geographical distance and some morphometric measurements of Philippine red jungle fowls.

MATERIALS AND METHODS

A total of 25 red jungle fowls captured from different mountain areas in 23 provinces and 12 islands in the Philippines were used to ascertain COI sequence divergences between geographical locations (**Figure 1**). Detailed information regarding the samples is presented in **Table 1**.

DNA sequences from the mitochondrial genome of three subspecies of the red jungle fowl (i.e. *Gallus gallus gallus*, *Gallus gallus bankiva*, *Gallus gallus spadiceus*) and 3 other jungle fowl species (i.e. *Gallus lafayettei*, *Gallus sonneratii*, *Gallus varius*) as reported by *Nishibori et al. 2005* (**Table 2**) were retrieved from the GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov.) and included for comparisons in the phylogenetic analysis. All new sequences have been deposited in GenBank under accession numbers JX178027 to JX178039, JX280464 to JX280472, and JX177994 to JX177996.

Laboratory analysis

Fresh blood samples extracted from the wing vein of

Table 1. Distribution of Philippine red jungle fowls (*Gallus gallus philipensis* Hatchisuka).

No.	Mountain Area	Municipality/ Province	Location Coordinates	Island	Clade
1	Mt. Sierra Madre	Lal-lo, Cagayan	18°11′ N 121°39' E	Luzon	B
2	Mt. Camandingan	Sarrat, Ilocos Norte	18°10′ N 120°39′ E	Luzon	A
3	Mt. Sierra Madre	Ilagan, Isabela	17°08′ N 122°08′ E	Luzon	A
4	Mt. Sierra Madre	Maddela, Quirino	16°21′ N 121°42′ E	Luzon	B
5	Mt. Natib	Orani, Bataan	14°48′ N 120°32′ E	Luzon	A
6	Mt. Daraitan	Tanay, Rizal	14°29′ N 121°17′ E	Luzon	B
7	Mt. Makiling	Los Baños, Laguna	14°10′ N 121°13′ E	Luzon	B
8	Mt. Guinatungan	Daet, Camarines Norte	14°07' N 122°57' E	Luzon	B
9	Mt. Taal	Taal, Batangas	14°00′ N 120°59′ E	Luzon	B
10	Mapolo Hill	Ibaan, Batangas	13°49′ N 121°08′ E	Luzon	B
11	Mt. Silungan	Virac, Catanduanes	13°34' N 124°13' E	Catanduanes	B
12	Mt. Agustin	Pasacao, Camarines Sur	13°31′ N 123°03′ E	Luzon	B
13	Mt. Mayon	Tabaco, Albay	13°21' N 123°44' E	Luzon	B
14	Mt. Halcon	Naujan, Oriental Mindoro	13°13' N 121°13' E	Mindoro	B
15	Mt. Castilla	Castilla, Sorsogon	12°56' N 123°52' E	Luzon	A
16	Mt. Bulusan	Bulusan, Sorsogon	12°45' N 124°08' E	Luzon	B
17	Mt. Pandan	Monreal, Masbate	12°39′ N 123°40′ E	Ticao	B
18	Mt. Supu	Ivisan, Capiz	11°31' N 122°41' E	Panay	B
19	Mt. Pangasugan	Baybay City, Leyte	10°41′ N 124°50′ E	Leyte	A
20	Mt. Kanlaon	San Carlos City, Negros Occidental	10°25′ N 123°23′ E	Negros	A
21	Mt. Bayugan	Roxas, Palawan	10°19' N 119°20' E	Palawan	A
22	Chocolate Hills	Batuan, Bohol	9°48′ N 124°08′ E	Bohol	A
23	Mt. Cambandilaan	Larena, Siquijor	9°13' N 123°37' E	Siquijor	A
24	Mt. Palomok	Titay, Zamboanga Sibugay	7°51' N 122°32' E	Mindanao	B
25	Mt. Tumatangis	Indanan, Sulu	6°00′ N 120°58′ E	Sulu	A

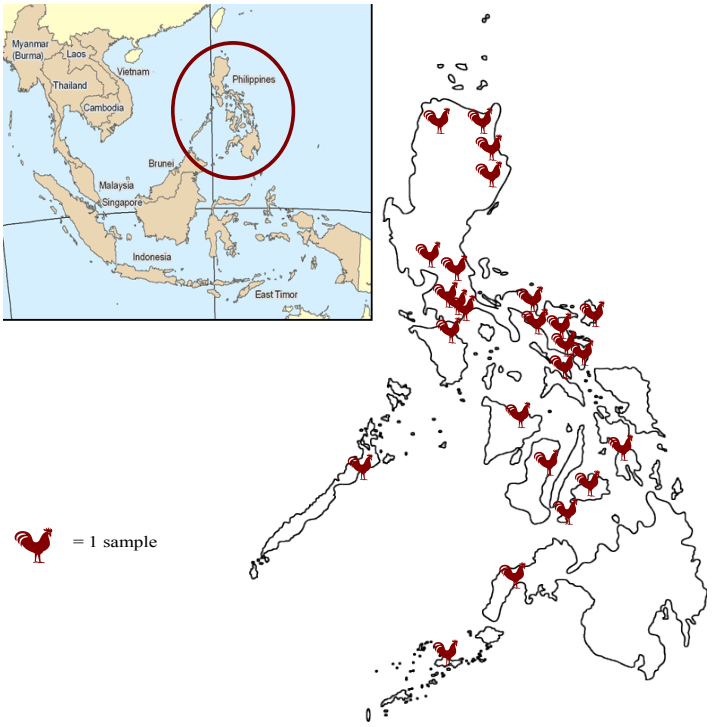

Figure 1. Location map for Philippine red jungle fowls with DNA barcodes.

male live specimens were placed in NucleoSave blood storage cards (Machery-Nagel, USA) and allowed to dry for 3 days under room temperature. Laboratory protocols for DNA extraction, purification, elution, and amplification methods using DNA barcoding procedures described by *Hebert et al.* (*2004*) were developed for poultry (birds) specimens at the Animal Biotechnology Laboratory, Animal and Dairy Sciences Cluster, College of Agriculture, University of the Philippines Los Baños.

DNA extraction, purification, and elution

Using a Harris 1.2 mm micropunch, at least 30 discs from each dried NucleoSave card or sample were collected then placed in labelled microcentrifuge tubes. Sample discs were washed with 200 µL of FTA Purification Reagent (Whatman Inc., USA) for four to five (4-5) times and rinsed with 200 µl sterile molecular biology grade water. Sample discs were the dried in a laminar hood overnight. Six dried sample discs were transferred in a sterile PCR tube then added with 55 µL sterile nanopure water. DNA was eluted by incubation at high temperature specifically at 90 °C for 10 minutes using Veriti 96 Well Thermal Cycler (Applied Biosystems). Eluted DNA was stored at -20 °C for further use.

DNA amplification

The COI gene was amplified using primers BirdF1 (5' TTCTCCAACCACAAAGACA TTGGCAC 3' and BirdR1 (5' ACGTGGGAGATAATTCCAAATCCTG 3') from *Hebert et al.* (*2004*). The 20-µL PCR reaction mix included 13.44 µL sterile ultrapure water, 2.0 µL of 10X buffer, 1.0 µL of $MgCl_2$, 0.8 units of Taq polymerase, 0.4 µL (0.2 mM) of each forward and reverse primer, and 2.0 µL of DNA template. The optimized PCR amplification program was composed of three min at 94 °C followed by five cycles of 40 sec at 94 °C, 30 sec at 56 °C and 45 sec at 72 °C, followed by another 30 cycles of 40 sec at 94 °C, 30 sec at 58° C, and 45 sec at 72 °C, and finally seven min at 72 °C.

PCR products were visualized in a 1.0 % agarose gel with ethidium bromide. Post stained gels were viewed using Molecular Imager® Gel DocTM XR System (Bio-Rad, USA). PCR products were purified using GF-1 PCR Clean Up Kit (Vivantis, Malaysia). The DNA amplification regime was repeated four (4) times for each sample specimen. The final PCR product for each sample specimen (about 30 to 50 µL final volume) was obtained from pooled amplicons of all PCR reactions (replicates).

DNA sequencing

PCR products were sent to Macrogen Inc., Seoul, Korea for unidirectional sequencing using appropriate forward primer, and analyzed using 3730L DNA analyzer (AB, USA) and BigDye (AB, USA). At least 30 µL each of the PCR product with a concentration of 100 ng μL^{-1} and the PCR primer with a concentration of 10 picomoles per µl was required. All COI sequences were then translated into illustrative DNA barcodes and recorded in the local DNA barcode library (*Oliva and Regado 2011*).

Table 2. Jungle fowls species and subspecies with mitochondrial DNA sequences taken from Genbank, NCBI (Source: *Nishibori et al. 2005*).

No.	Common name	Scientific name	Genbank accession number	Place and year of sampling
1	Red jungle fowl	Gallus gallus gallus	AP003322	Bali, Indonesia (1990)
2	Red jungle fowl	Gallus gallus bankiva	AP003323	Vientianne, Lao PDR (1998)
3	Red jungle fowl	Gallus gallus spadiceus	AP003321	Tama Zoological Park, Tokyo, Japan (1999)
4	Ceylon jungle fowl	Gallus lafayettei	AP003325	Delhi National Park, New Delhi, India (1995)
5	Grey jungle fowl	Gallus sonneratii	AP006746	Bali, Indonesia (1990)
6	Green jungle fowl	Gallus varius	AP003324	Bali, Indonesia (1990)

Evolutionary analyses of COI sequences

Evolutionary analyses were conducted in MEGA5 (*Tamura et al. 2011*). The COI sequences were initially aligned using ClustalW (*Thompson, Higgins and Gibson 1994*), (http://www.ebi.ac.uk/clustalw/). Sequence divergence, defined as the number of nucleotide substitutions (i.e. transition and/or transversion) or differences occurring between two COI sequences, was calculated using the Kimura two-parameter or K2P model (*Kimura 1980*). Standard error estimate(s) were obtained by a bootstrap procedure (1000 replicates) according to *Nei and Kumar* (*2000*).

The Neighbour-Joining (NJ) method was used to infer the evolutionary history of Philippine red jungle fowls. It was used to examine the nearest-neighbour distance or the minimum genetic distance between a sample and its closest relative, using DNA barcode polymorphisms. The NJ method was chosen since it is faster and most appropriate in recovering intra-species phylogeny when sequence divergences are low (*Hebert et al. 2004*). An NJ tree of K2P distances was subsequently created to provide a graphical representation of the pattern of divergences among red jungle fowl specimens (*Saitou and Nei 1987*). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (*Felsenstein 1985*). Phylogenetic clades of the lineages (main matrilineal components) that are discernable from the NJ tree were used to create and compare groups within the Philippine red jungle fowl subspecies. Member specimens in each clade were suspected to have descended from a common evolutionary ancestor.

Using the K2P method (*Kimura 1980*), the average distance (in d units) between a pair of sequences was measured as the number of base substitutions per site with their variances estimated by a bootstrap approach. Between (or within) group mean distance was estimated as the average evolutionary divergence over sequence pairs between (or within) clades.

Finally, correlation and regression analysis were used to test the association of pooled pair-wise genetic distance using DNA barcodes with estimated geographical distance between wild red jungle fowls. The distance parameters were compared between clades using ordinary least squares procedures. The approximate geographical distance (i.e. great-circle distance or shortest distance over the earth's surface) between latitude and longitude points was calculated using the 'haversine' formula (http://www.movable-type.co.uk/scripts/latlong.html).

RESULTS AND DISCUSSION

Phylogenetic tree for Philippine red jungle fowls

Figure 2 shows the phylogenetic tree representing the pattern of divergences in DNA barcodes of red jungle fowls found in different mountain areas of the Philippines. Two distinct genetic clades were detected from the NJ tree. Clade A was represented by 10 specimens from seven islands while Clade B included 15 samples from six islands. The existence of two main evolutionary clades suggests that Clade A samples might have originated from Mt. Sierra Madre, Isabela province while Clade B specimens could have descended from Mt. Naujan in the province of Oriental Mindoro. It is also noted that two specimens from the province of Sorsogon in the island of Luzon have diverged into different clades, suggesting no clear geographic structuring in the red jungle fowl populations. On the other hand, the wide divergence between the two clades implies that Philippine red jungle fowls represent more than one taxon (i.e. different subspecies) or perhaps a result of misidentification during sampling, although this is not very likely.

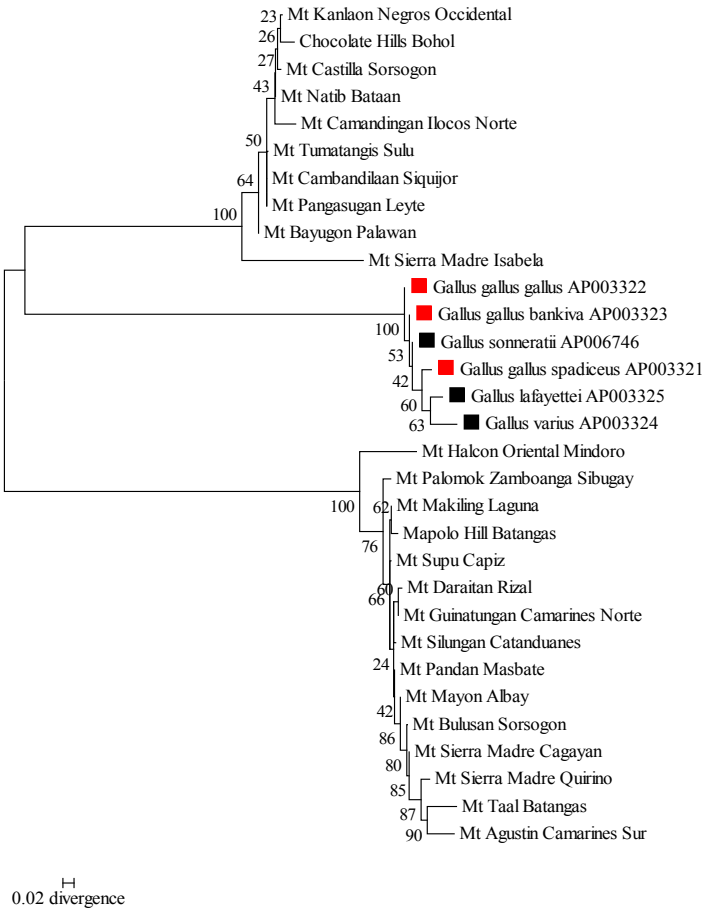

Figure 2. Neighbour-Joining tree with bootstrap support showing the evolutionary relationships of Philippine red jungle fowls, subspecies of red jungle fowls (■), and other Gallus species (■) (N=31 COI sequences; 552 positions).

Genetic diversity of Philippine red jungle fowls

Based on 627 positions from 25 COI sequences, the average diversity within a clade is 25.4 %. However, because of the high diversity between clades equivalent to 36.62 %, the overall genetic diversity increased to 62.0 % (**Table 3**), suggesting a significant reservoir of genetic diversity within red jungle fowls found in different mountain areas of the Philippines.

The high genetic diversity of the sequences of COI gene in red jungle fowls were comparable to genetic diversity in DNA barcodes among standard chicken breeds (71.4 %), among Philippine native chicken strains (51.0 %), and among game fowl lines or fighting cocks (38.3 %); and substantially higher than among commercial hybrid chickens (2.5 %) as reported by *Bondoc and Santiago* (*2012*).

More importantly, the high genetic variation (i.e. greater than 2 % as proposed by *Hebert et al.* (*2003*) further attested to the discriminatory power of COI barcodes in identifying subspecies of red jungle fowls found in the Philippines . It also warrants deeper understanding of the genetic relationships within clades. Alternatively, a sequence threshold of 10 times the average intra-specific variation could be used to identify those cases where a current specimen might represent more than one taxon (*Hebert et al. 2004*).

Genetic distances and clustering of Philippine red jungle fowls

The overall average genetic distance of DNA barcodes among the Philippine red jungle fowls was 0.254 units (**Table 4**), with higher genetic divergence within Clade A (d=0.294) than within Clade B (d=0.215). Red jungle fowls in Clade A were all distantly related to those in Clade B (i.e. d is greater than one). Specific pair-wise genetic distances between the red jungle fowl specimens are summarized in **Table 5** and **6**, respectively. The illustrative DNA barcodes for Philippine red jungle fowls from different clades are given in **Figure 3**. It should be noted that all 25 Philippine red jungle fowl samples had different DNA barcodes, except for samples taken from Leyte and Siquijor provinces which exhibited very similar COI sequences. The islands of Leyte and Siquijor are geographically separated by the island of Bohol.

Table 4. Estimates of evolutionary divergence* in COI sequences (in d units) in Philippine red jungle fowls belonging to different clades.

	Clade A	**Clade B**
Clade A	0.294 ± 0.023	0.748 ± 0.070
Clade B	1.002 ± 0.086	0.215 ± 0.017

* Value in diagonals is within clade mean distance; Off-diagonal value in the lower-left corner is between clade difference; Off-diagonal value in the upper-right corner is net between clade mean distances

Samples in Clade A were closely related to each other (i.e. d = 0 to 0.053) excluding the red jungle fowl from Mt. Sierra Madre, Ilagan, Isabela whose genetic distance to other members of the clade ranged from 0.232 to 0.250. In Clade B, members were also closely related to each other (d = 0.010 to 0.136) excludingthe red jungle fowl from Mt. Halcon, Naujan, Oriental Mindoro whose genetic distance to other members of the clade ranged from 0.154 to 0.209 .

Normally, genetic distance may be more indicative of the genetic variation partitioning among various levels of geographic structure, rather than of absolute taxonomic relationships as currently understood (e.g., *Patton 1985*). In a study of red jungle fowls from 745 museum specimens, *Peterson and Brisbin* (*1999*) claimed that most wild populations have been contaminated genetically by introgression of genes from domestic or feral chickens. However, in the evolutionary analysis in this study, the close genetic distance estimates within a clade, may be due to evolution out of a common ancestor, or hybridization among wild jungle fowls in adjacent geographical locations. Migration due to threats of forest degradation and human settlements in upland communities would most likely be the cause for the recent divergence within the clade. Dense human populations thus may have contributed more in making the genetic integrity of the Philippine red jungle fowl uncertain.

Further analysis of red jungle fowls obtained from different mountain areas in the island of Luzon showed a high average genetic distance with each another (i.e. d = 0.557 ± 0.047) but slightly lower than red jungle fowls from eleven other islands (i.e. d = 0.656 ± 0.057). The mean genetic difference between the two groups was also high (i.e. d = 0.645 ± 0.055).

Table 3. Mean diversity for Philippine red jungle fowls based on DNA barcodes.

Diversity measures	**Number of nucleotide sequences**	**N positions**	**Diversity (Percentage)**	
			Mean	**Standard Error**
Within population			25.40	1.96
Interpopulation			36.62	3.31
Entire population	25	627	62.05	5.27
Coefficient of differentiation			59.01	0.78

Table 5. Pair-wise distances (d units) between Philippine red jungle fowls in Clade A.

	2	3	5	15	19	20	21	22	23
2									
3	0.250								
5	0.038	0.235							
15	0.039	0.237	0.011						
19	0.048	0.240	0.013	0.024					
20	0.038	0.237	0.003	0.011	0.010				
21	0.053	0.247	0.026	0.034	0.013	0.029			
22	0.053	0.232	0.029	0.031	0.038	0.026	0.040		
23	0.048	0.240	0.013	0.024	0.000	0.016	0.013	0.038	
25	0.049	0.242	0.015	0.026	0.002	0.018	0.011	0.039	0.002

Table 6. Pair-wise distances (d units) between Philippine red jungle fowls in Clade B.

	1	4	6	7	8	9	10	11	12	13	14	16	17	18
1														
4	0.014													
6	0.031	0.036												
7	0.036	0.041	0.013											
8	0.026	0.031	0.005	0.010										
9	0.056	0.044	0.079	0.083	0.070									
10	0.046	0.051	0.019	0.013	0.019	0.093								
11	0.038	0.043	0.015	0.018	0.011	0.084	0.028							
12	0.093	0.084	0.119	0.125	0.113	0.086	0.136	0.120						
13	0.023	0.026	0.021	0.024	0.018	0.061	0.034	0.026	0.104					
14	0.176	0.173	0.154	0.154	0.154	0.200	0.157	0.166	0.209	0.161				
16	0.016	0.018	0.023	0.026	0.019	0.055	0.036	0.020	0.097	0.011	0.160			
17	0.031	0.033	0.011	0.015	0.008	0.070	0.024	0.013	0.112	0.016	0.15	0.018		
18	0.038	0.039	0.014	0.013	0.011	0.081	0.026	0.016	0.123	0.023	0.156	0.024	0.010	
24	0.048	0.060	0.034	0.038	0.038	0.101	0.034	0.048	0.136	0.046	0.140	0.051	0.044	0.043

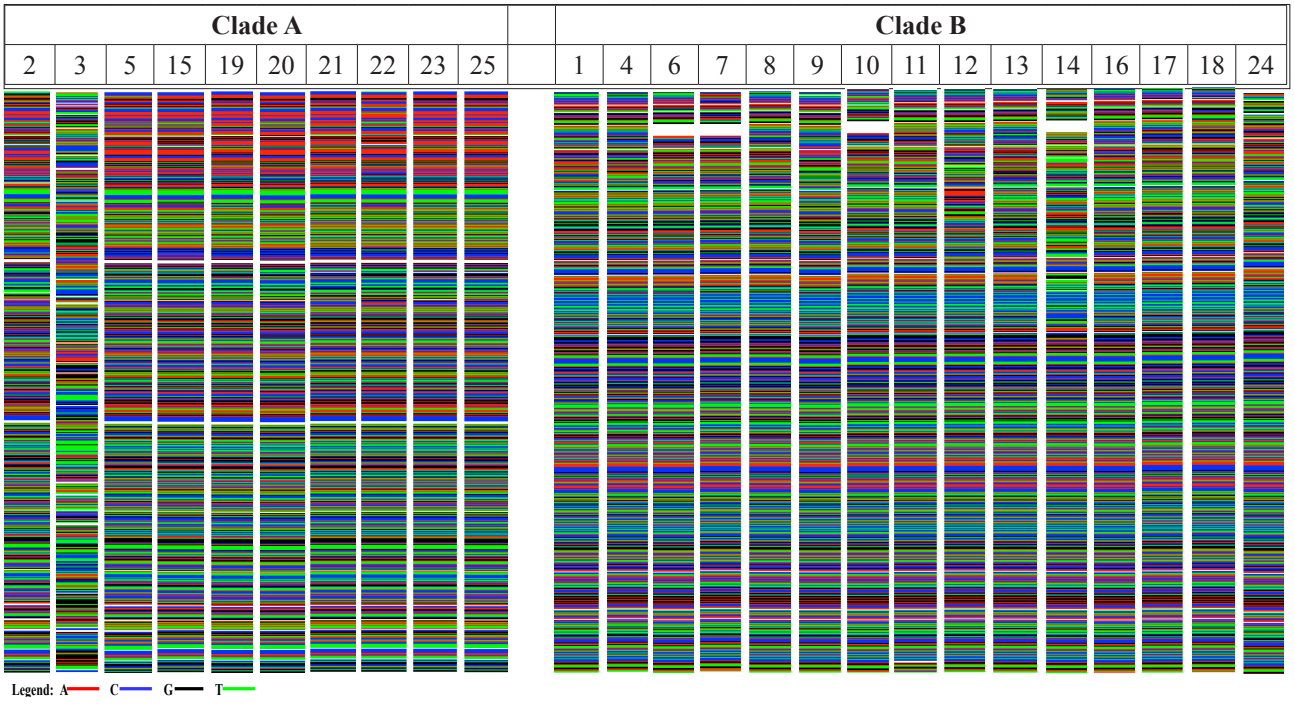

Figure 3. Illustrative DNA barcodes of Philippine red jungle fowls from different clades.

Comparisons among Philippine red jungle fowls and other jungle fowl species and *Gallus gallus* subspecies

The Genbank-accessed COI sequences of three subspecies of red jungle fowls and three *Gallus* species were clustered in the intermediate zone between the differentiated populations of Philippine red jungle fowls, but more recently diverged with members in Clade A (**Figure 4**). In contrast, *Liu et al.* (*2006*) analyzed highly divergent mitochondrial DNA (hypervariable segment I) data which fit into two main clades of 1) continental red jungle fowl subspecies *G. g. spadiceus* and *G. g. jabouillei* from Burma and China, and domestic chicken samples from Europe, India, Indonesia, Malaysia, and Middle East - Iran, Azerbaijan, and Turkmenistan, also named as the continental clade and; 2) *G. g. bankiva* samples from Java, Indonesia known asthe island clade. While the phylogeny analysis based on the DNA barcodes is not sufficient to conclude the phylogenetic positions of the jungle fowls in the genus, inter-species hybridizations between *G. varius* and *G. gallus*, and between *G. varius* and *G. lafayettei*, and divergence within subspecies are suggested in this study, creating conflicts with the current taxonomic classification of the subspecies (e.g., *Nishibori et al. 2005*; *Liu et al. 2006*).

The high bootstrap support for clade nodes suggested that NJ analysis of COI barcode sequences will be widely effective (e.g., *Ward et al. 2005*; *Hajibabaei et al. 2006*) between *Gallus* species and between subspecies. The high bootstrap support values (i.e. greater than 98 %) for specimens from the two distinct clades would also entail the need for their further taxonomic scrutiny (e.g., *Hebert et al. 2004*).

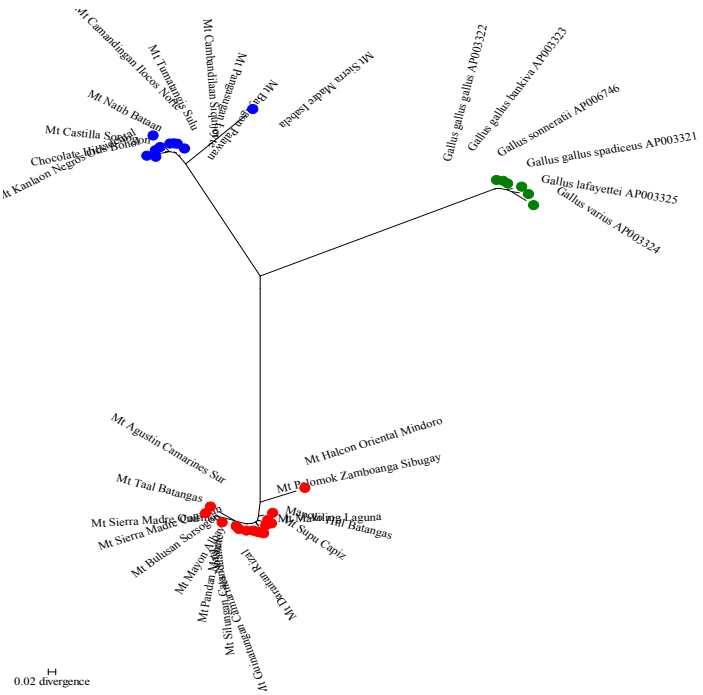

Figure 4. Unrooted NJ tree based on K2P distances of 25 Philippine red jungle fowls and six Genbank-derived *Gallus* species and subspecies of red jungle fowls showing the clear separation of three clades (i.e. Clade A = (●), Clade B = (●), and Clade C derived from Genbank = (●).

A low average genetic distance (i.e. d = 0.011 ± 0.004) was estimated among other jungle fowl species and *Gallus gallus* subspecies that were accessed through the Genbank. The Genbank accessions were distantly related (i.e. d > 1) to all Philippine red jungle fowl samples.

Comparison of clades in terms of genetic and geographical distances and some morphometric traits

The average genetic distance among samples in Clade A (d=0.061) was higher than among specimens in Clade B (d=0.027) (**Table 7**). The area coverage (average geographical distance) was however wider among samples in Clade A (562.3 km) than in Clade B (331.2 km).

There was no significant linear relationship between pooled pair-wise genetic distance and geographical distances among the red jungle fowls between and within clades, i.e. r=0 (P>0.05). **Figure 5** presents the regression of genetic distance on geographical distance in the different clades of Philippine red jungle fowls. Phenotypically, red jungle fowls in Clade A have significantly longer neck (P<0.05) and shank (P<0.01) than those in Clade B (**Table 7**). No significant differences (P>0.05) were however found in live weight and linear measurements of the wings, breast, and beak of red jungle fowls in the different clades.

Practical applications of DNA barcodes from Philippine red jungle fowls to agrobiodiversity conservation and research in upland areas

Red jungle fowls in the Philippines are still captured using indigenous bird traps mostly in mountain areas where there is minimal or no anthropogenic activity, only during the dry months. Their number and distribution as noted by upland farmers and hunters alike, seem to be declining and becoming rare especially with more people encroachment of the few remaining forest reserves nationwide. A few farmers were also successful in captive breeding of the red jungle fowl using an artificial incubator intended for domestic chickens (e.g., *Mr. Josenieto Bihis 2011, personal communication*). Like other *Gallus* species, the red jungle fowl is evaluated as "Least Concern" (i.e. population is suspected to be stable in the absence of evidence for any declines or substantial threats) in the current International Union for Conservation of Nature (IUCN) Red List. Threats to their declining number may include habitat loss and degradation, over-hunting for food, agricultural encroachment, overgrazing and fires, illegal trade, poaching, and interbreeding with domestic chickens (*del Hoyo, Elliott and Sargatal 1994*).

Table 7. Comparison of geographical and genetic distances between clades of Philippine red jungle fowls.

	Clade A	Clade B	Overall Mean*
Number of specimens	10.0	15.0	12.5
Average genetic distance, d units	0.061 ± 0.005 [a]	0.027 ± 0.009 [b]	0.073 ± 0.119
Average geographical distance, km	331.2 ± 21.9 [b]	562.3 ± 43.2 [a]	395.7 ± 256.9
Live weight, kg	0.79 ± 0.06 [a]	0.80 ± 0.05 a	0.80 ± 0.17
Linear measurements, cm			
Wing	27.9 ± 0.7 [a]	28.1 ± 0.5 [a]	27.9 ± 2.0
Neck	8.4 ± 0.7 [a]	6.1 ± 0.5 [b]	7.0 ± 2.3
Breast	14.3 ± 0.6 [a]	14.3 ± 0.5 [a]	14.3 ± 2.7
Shank	7.1 ± 0.2 [a]	6.2 ± 0.2 [b]	6.5 ± 0.8
Beak	2.9 ± 0.2 [a]	3.1 ± 0.1 [a]	3.0 ± 0.5

Note: Least square means and standard errors in the same row with different letter superscripts are significantly different from another (P<0.05).
* Overall mean does not include comparisons of distances between samples belonging to different clades.

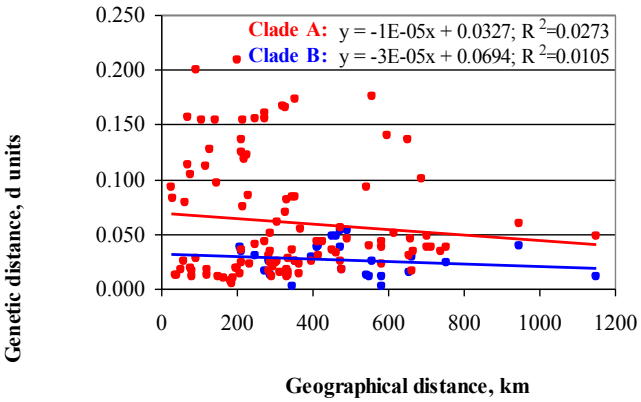

Figure 5. Regression of genetic distance on geographical distance between Philippine red jungle fowls in the different clades.

With DNA barcoding, Philippine red jungle fowls can be identified quickly and inexpensively using a single gene as the basis for a global bioidentification system (e.g., *Hebert et al. 2003*). Results of this study also add to the body of knowledge of genetic variability, and population structure of the Philippine red jungle fowl populations. Specifically, DNA barcodes of Philippine red jungle fowls may provide information on their genetic integrity and past history, and detect introgressions of genetic variation from *Gallus* species and subspecies. They could also be useful in supporting conservation and research of the Philippine red jungle fowls. For example, DNA barcoding could be used in producing evidence to prosecute smugglers and poachers of Philippine red jungle fowls since it can distinguish rare and threatened red jungle fowls from domestic poultry meat.

These wild populations may also be used in crossbreeding programs designed to create new genetic stocks with improved adaptability and productivity in smallholder and subsistence production systems. Future research studies involving DNA barcodes should thus be able to generate information on genetic diversity of disease resistance in these populations. Such may be used for developing the foundation population for selecting breeding stocks for subsistence native chicken production in upland communities with enhanced ability to resist an infectious disease outbreak.

More importantly, the high level of genetic divergence in COI sequences of red jungle fowls can serve as indirect indicators of the major causes of degradation of ecological diversity of mountain. DNA barcodes may thus be used to describe and predict the animals' responses to anthropogenic activities, climate change, pollution, and invasive competitors. DNA barcoding may also help assess opportunities for agrobiodiversity conservation and research leading to sustainable land use and rural development in upland communities.

CONCLUSIONS AND RECOMMENDATION

This study demonstrated that DNA barcodes can be effective in identifying and differentiating Philippine red jungle fowls between and within clades. Pooled pair-wise genetic distance was not correlated with estimated geographical distances among red jungle fowls between and within clades. Phenotypically however, red jungle fowls in Clade A have longer neck and shank than those in Clade B. No significant differences were found in live weight and length of the wings, breast, and beak of red jungle fowls in the different clades.

Additional sampling of DNA barcodes of red jungle fowls in other mountain areas and islands in the country is recommended to bring greater reliability to their identification. It should be noted that cataloguing Philippine red jungle fowls with standardized gene region in a national DNA library, could not compete with the GenBank since the latter aims for comprehensive coverage of genomic diversity. As the most probable wild progenitor of the domestic chicken worldwide, more ecological studies and local programs are further recommended and should be highly justified to monitor, protect, and conserve the wild

ancestor species, since Philippine red jungle fowls are valuable national treasures reflecting our rich natural genetic resources, culture, and heritage as a people.

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Evaluating Patterns of Fish Assemblage Changes from Different-Aged Reforested Mangroves in Lingayen Gulf

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ABSTRACT

Fish assemblages in planted mangroves of different ages in northwestern Lingayen Gulf, northwestern Philippines, composed of: seven-year (Tondol, Anda), nine-year (Pangapisan, Alaminos), 11-yr (Imbo, Anda), 12-yr (Pilar, Bolinao), and 19-yr stands (Bangrin, Bani) were investigated. A modified local triangular trap net was deployed ~1 m from the edge of the plantation of each site at low tide for three days (before, during, and after spring tide) in December 2008 and February 2009. Fish samples were collected the following day, measured, then weighed in the laboratory. Fish species were categorized based on trophic level and habitat preferences. A total of 593 individuals belonging to 50 species from 22 families were recorded. There were no apparent trends in terms of fish abundance, fish biomass, and trophic categories with age of mangrove stands. In terms of habitat preference, mangrove-associated species dominated the mature plantation (> 12 yr) while reef-associated species were mostly found in younger stands (< 12 yr). The fish assemblages have 43 % similarity between seven-year and nine-year plantation, and 35 % similarity between 11-year and 12-yr plantation. In contrast, the 19-yr old plantation was clearly separated from the younger plantations, indicating a possible shift of fish assemblage with age of mangrove stands.

Key words: *mangroves, planting, fish assemblage, triangular trap net, trajectory pattern, Lingayen Gulf*

INTRODUCTION

Mangrove forests perform several important ecological and socio-economic functions. They serve as habitat for various marine and terrestrial organisms, produce organic detritus, protect shoreline, and provide forest and fishery products (*White and Cruz-Trinidad 1998*). Mangroves are also considered as one of the most degraded coastal ecosystems in the country. The Philippines used to have about 450,000 ha of mangroves in 1918 but due to natural and anthropogenic stresses, mangrove cover shrunk to 288,000 ha in 1970 and was drastically reduced to only 256,185 ha in 2000 (*Long and Giri 2011*). At least sixty percent of mangrove loss can be attributed to conversion to aquaculture ponds particularly during the 1970s (*Primavera 2005*).

Similarly, mangroves in Pangasinan (west Lingayen Gulf, NW Philippines) are severely degraded. From an estimated area of 990 ha in 1978, only 400 ha in 2002 remains (*MSI 2002*). To address mangroves loss, mangrove restoration programs were implemented. Around 136 ha of planted mangroves were established in the municipalities of Bolinao, Anda, Bani, and Alaminos. This planting strategy, which has been implemented for almost two decades, aims to restore forest cover and ecological functioning of mangroves (*Salmo III et al. 2007*).

There have been interests on how planted mangroves contribute in fisheries production. Mangroves are known to attract fish because of the habitat complexity, food and refuge they provide (*Huxham et al. 2004*). Robertson and *Duke* (*1987*) proposed that mangroves are very important nursery habitat for commercially important fish species. *Ronnback et al.* (*1999*) further proved that mangroves are extensively used as habitat by various fish species.

However, planted mangroves offer a unique case. Being monospecific and with oftentimes stunted growth (*Samson and Rollon 2008*), it reduces habitat complexity and detritus production that may diminish their attractiveness as fish habitat (*Salmo III 2011*). The planted mangroves have to undergo developmental stage before it reaches a forest state comparable with that of a mature mangrove. Unfortunately, studies that compare the performance of natural and planted mangroves in enhancing fish assemblages are limited.

Recently, however, there has been an increase in the number of studies that examine fish assemblages in planted mangroves, comparing them to natural or mature mangrove stands. These studies have contrasting findings. For example, *Huxham et al.* (*2004*) compared the fish assemblages between vegetated mangroves and unvegetated sites in Gazi Bay, Kenya and results revealed significant difference in assemblage structure between the two sites. Species richness and abundance were found to be significantly higher at clear site than that of the vegetated site. In contrast, *Crona and Ronnback* (*2007*) showed no significant differences in juvenile fish recruits between planted and natural mangrove stands in Pagbilao, Philippines.

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Studies that evaluate the progress and impact of mangrove planting programs in enhancing fish assemblages are rarely undertaken. Thus, this study conducted documentation and evaluation of fish assemblages in planted mangroves representing a gradient of ages from young to mature plantation. The researchers tested the hypothesis that the fish assemblage will change as mangrove stands mature. Such shift in pattern could be used as a possible indicator of restoration trajectory in restored mangroves.

MATERIALS AND METHODS

Site description

The study utilized the mono-specific mangrove plantations of the species *Rhizophora mucronata* in Lingayen Gulf (**Figure 1**). These plantations are of varying ages and sizes located in Tondol in Anda (7 yrs old, 12 ha; P7), Pangapisan in Alaminos (9 yrs old, 10 ha; P9), Imbo in Anda (11 yrs old, 8 ha; P11), Pilar in Bolinao (12 yrs old, 8 ha; P12) and Bangrin in Bani (19 yrs old, 20 ha; P19). The planted mangroves in Bangrin have another separate 20 ha block in the eastern side composed of several cohorts of unknown ages. For this site, the study was conducted in the pure 19-yr stand.

Sites in Anda, Alaminos and Bolinao facing Lingayen Gulf are exposed to coastal currents while Bani is in a more sheltered area found in Tambac Bay. The average depth of the study sites is about 2 m during high tide but is generally exposed at low tide particularly during September to February. There are two pronounced seasons: dry from the months of November to April (northeast monsoon) and wet from the months of May to October (southwest monsoon) with an average annual precipitation of at least 2,500 mm (*FAO 2001*).

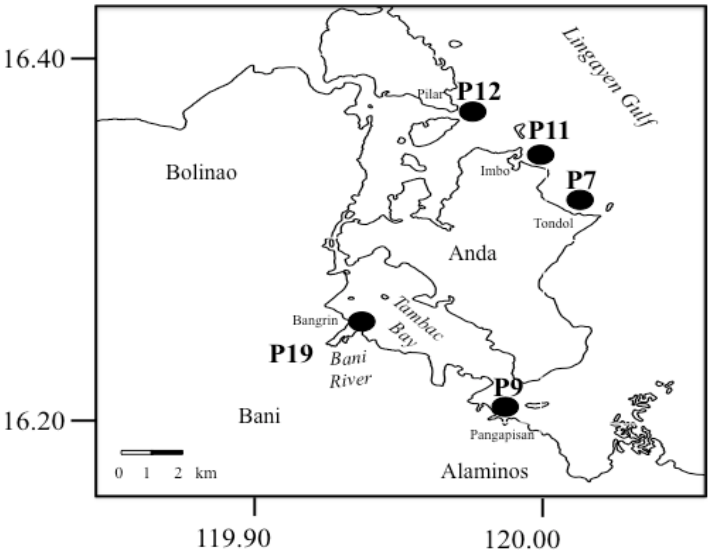

Figure 1. Location of mangrove plantations of different ages used in the study. The numbers indicate the ages of mangrove stands.

Experimental design

We used a space-for-time (SFT) substitution approach in inferring temporal trends from different aged sites to generate patterns in the trajectory of the restored system (*cf Pickett 1989*). Such approach has been used in similar studies in restoration ecology where optimal sampling design (i.e. presence of experimental controls and age replicates within one site) may not be possible (*cf Michener 1997*). Thus, the ages of the planted sites were used as a temporal point in the restoration trajectory of mangroves.

Field sampling

Fish sampling were carried out during spring tides in December 2008 and February 2009. Modified local triangular trap nets locally known as "baklad" were used to collect samples. The net has a 10-m wingspan on each side (area: 43.3 m^2) with a three-m pocket connected at the cod end.

All nets had a stretched mesh size of 2 mm. The trap net was assumed to catch fish that came in during high tide and trapped as tide recedes. One trap net was deployed at each site ~1 m from the edge of the plantation at low tide for three days (before, during, and after spring tide). Fish samples were collected the following day during low tide, early in the morning from the pocket of the net. All collected individuals were sorted from other catch (e.g. crustaceans, mollusks) and then identified to species level using *Kuiter and Debelius* (*2006*) and *Allen et al.* (*2003*). The collected fish samples were measured and weighed within the same sampling day in the Bolinao Marine Laboratory (Marine Science Institute of the University of the Philippines). Data on trophic category, habitat preference and juvenile size for each species were obtained from FISHBASE (*Froese and Pauly 2004*).

Data analysis

The fish assemblage was analyzed using a non-parametric approach. Relative abundance and relative biomass were computed for each species that were determined as the count and weight of a species divided by the total abundance and total biomass per site, respectively. Data for the two sampling periods were pooled since no temporal differences were observed (Analysis of Similarity test). Trophic categories and habitat preferences of all fish species per site were analyzed through frequency analysis. Species diversity (H') was calculated using the Shannon-Weiner index. A similarity matrix was constructed using Bray-Curtis index on standardized, fourth root-transformed biomass data. Cluster analysis was performed from this similarity matrix. Discriminating species was obtained using a similarity percentage procedure with a cut off of 90 % per site (*SIMPER; Clarke and Warwick 2001*). All multivariate

analyses were implemented in PRIMER 6 (*Clarke and Gorley 2006*).

RESULTS

Fish species composition

A total of 593 fish individuals belonging to 50 species from 23 families were collected (**Table 1**). All collected samples were identified as juveniles except for *Plotosus lineatus* and *Upeneus guttatus* from P11 that were identified as adult. Species richness and diversity index exhibited high variability across sites. Species richness was highest in P9 (24 species) and lowest in the oldest plantation (P19; 14 species). Diversity index was highest in P12 (2.34) and lowest in P19 (1.56).

Fish abundance and biomass

Fish abundance and biomass highly varied across sites (as represented by high standard deviation) and did not show clear pattern with age of the mangrove stands (**Table 1**). Highest fish biomass was observed in P9 (74 ± 1.33 g m^{-2} d^{-1}) followed by P19 (56.3 ± 1.78 g m^{-2} d^{-1}), P7 (32.6 ± 0.60 g m^{-2} d^{-1}), P12 (28.6 ± 0.52 g m^{-2} d^{-1}) and P11 (6 ± 0.06 g m^{-2} d^{-1}). The youngest plantation obtained the highest fish abundance (428 ± 7). The dominant species (both by abundance and by biomass) are from the families Ambassidae, Apogonidae, Atherinidae, Gobiidae, Hemirhamphidae and Tetraodontidae.

Different fish species dominated in different mangrove stands. *Hyporhamphus dussumieri* was the most abundant species in P7 (28.6 %), P9 (37.4 %) and P12 (25.6 %). In P11, *Arothron manilensis* was the most abundant species (16.7 %) followed closely by *P. lineatus* (11.1 %), *Siganus fuscescens* (11.1 %) and *Sphyraena barracuda* (11.1 %). In P19, *Atherinomorous lacunosus* has the highest relative abundance (46.2 %). Almost similar patterns were observed in relative biomass wherein *H. dussumieri* dominated in P9 (30.9 %) and P12 (27.6 %) while *A. lacunosus* prevailed in P19 (60.4 %). The species *A. manilensis* and *Conger* sp. have the highest relative biomass in P7 (23.2 %) and P11 (34.3 %).

Trophic category

The trophic categories of recorded fish species included carnivores, detritivores, herbivores and omnivores (**Figure 2**). A general pattern of changes in trophic categories with age of mangrove stands can be inferred. All mangrove stands have high proportion of carnivores but with varying amount. The youngest plantation has high proportion of carnivore (74.00 ± 14.60 %) and herbivore species (25.00 ± 14.67 %). Other plantations of intermediate age (P9, P11 and P12) were also dominated by carnivores (50-60 %) but showed a mixture of omnivores and detritivores as well (range: 1-25 %). Carnivorous species dominated in all sites but was most dominant in the oldest stand (P19; 96.00 ± 1.53 %).

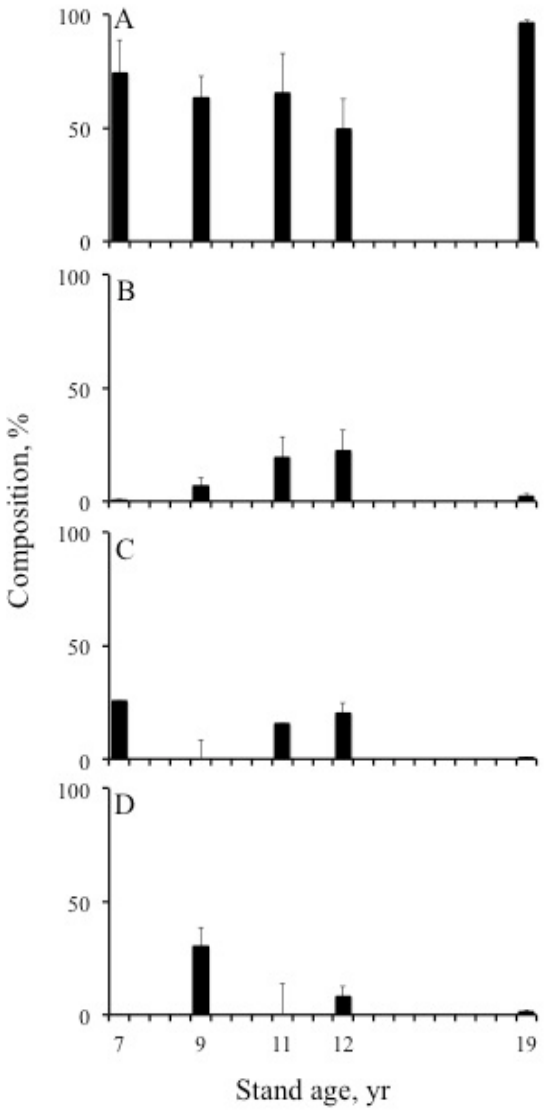

Figure 2. Trophic composition (A: Carnivore; B: Detritivore; C: Herbivore; and D: Omnivore) of fish species from different mangrove stands. The carnivore species have high proportion (at least 50 %) in all sites but have varying dominance with mangrove stand ages. Other trophic categories have minimal contribution (< 30 %).

Habitat preference

The habitat preference of recorded fish species varied across plantation ages (**Figure 3**). More than half of the fish collected in P7 and P19 are mangrove-associated species. In P9 and P12, there are more reef-associated species than the mangrove-associated species. Reef-associated species had the highest proportion in the youngest plantation (P7; 47.00 ± 33 %). In contrast, the mangrove-associated species dominated in the oldest plantation (P19; 60.00 ± 43 %).

Table 1. Fish species with relative density (%) and relative biomass (%) recorded from each mangrove stand. There were no apparent trends on species abundance and biomass with stand age. However, certain species appear to be more abundant in younger stands (e.g. *Hyporhamphus dussumieri*) and there are species that are more dominant in older stands (e.g. *Atherinomorus lacunosus*).

Family	Species	Relative density, %					Relative biomass, %				
		P7	P9	P11	P12	P19	P7	P9	P11	P12	P19
Ambassidae	*Ambassis* sp.		21.20		4.70	2.40		14.73		1.15	0.84
Antennaridae	*Histrio histrio*	1.00					1.17				
Apogonidae	*Apogon fraenatus*	14.30			5.80		14.09			8.80	
Atherinidae	*Atherinomorus lacunosus*	9.20	0.50		2.30	46.20	9.18	0.96		2.75	60.42
	Atherina sp.	7.10					9.63				
Blenniidae	*Blenny* sp. *1*					1.80					1.82
	Blenny sp. *2*				3.50					4.14	
Chanidae	*Chanos chanos*				7.00					9.18	
Clupeidae	*Clupeidae* sp.		0.50					0.25			
Clupeidae	*Conger cinereus*	1.00					7.99				
	Conger sp. *1*	1.00	0.50	5.60			6.01	7.65	34.33		
	Conger sp. *2*	1.00	0.90	5.60			4.23	10.94	5.40		5.08
	Conger sp. *3*				2.30	0.60				8.45	
Ephippidae	*Platax orbicularis*		0.90					0.20			
Gerreidae	*Gerres oblongus*		9.90					2.89			
	Gerres sp. *1*				2.30					0.07	
	Gerres sp. *2*		7.20	5.60					1.82	0.17	
Gobiidae	*Exyrias puntang*	2.00			1.20		0.77			5.25	
	Goby sp. *1*		6.80					2.99			
	Goby sp. *2*	1.00				3.00	0.07				4.62
	Goby sp. *3*				8.10	16.00				2.92	6.25
	Goby sp. *4*					0.60					0.93
	Goby sp. *5*		0.50	5.60	20.90	18.30		0.25	0.13	6.37	7.88
	Goby sp. *6*					0.60					0.56
	Oplopomus caninoides		4.10			4.10		2.23			1.17
	Yongeichthys criniger			5.60	5.80			2.61	2.23		
Hemirhamphidae	*Hyporhamphus dussumieri*	28.60	37.40		25.60	0.60	15.00	30.89		27.62	0.19
Lethrinidae	*Lethrinus harak*	2.00					2.17				
	Lethrinus sp.	1.00					0.02				
Lutjanidae	*Lutjanus fulviflamna*			5.60					1.29		
Mugilidae	*Valamugil* sp.				2.30					2.71	
Mullidae	*Upeneus guttatus*	1.00		5.60					6.09		
	Upeneus tragula		0.50				1.47	0.01			
Platycephalidae	*Cymbacephalus beauforti*		0.90					0.71			
	Platycephalus sp. *1*					0.60					4.10
	Platycephalus sp. *2*		0.50					1.57			
	Platycephalus sp. *3*					1.20					2.00
	Platycephalus sp. *4*		0.50			3.60		0.76			4.10
Plotosidae	*Plotosus lineatus*			11.10					12.56		
Siganidae	*Siganus fuscescens*	7.10	3.20	11.10	5.80		0.30	0.35	5.27	7.79	
	Siganus guttatus		0.50	5.60				8.20	1.67		0.05
	Siganus sp.		0.50				0.02	0.01			
	Siganus virgatus	1.00	0.01					0.01			
Soleidae	*Synaptura marginata*		1.80					8.46			
Sphyraenidae	*Sphyraena barracuda*			11.10					18.56		
Terapontidae	*Pelates quadrilineatus*	1.00					0.98				
	Terapon jarbua		0.50					0.76			
Tetraodontidae	*Arothron hispidus*	1.00		5.60	2.30		3.67		9.30	10.57	
	Arothron manilensis	19.40	0.90	16.70			23.21	1.77	2.61		
	Chelonodon patoca		0.50					1.62			
Species richness, S		*18*	*24*	*13*	*15*	*14*					
Diversity index, H'		*2.3*	*2.3*	*1.0*	*2.3*	*1.6*					

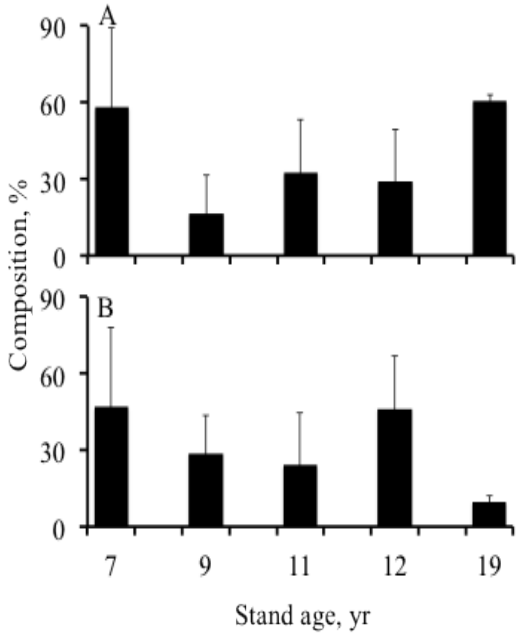

Figure 3. Habitat preference of collected fish species: (A) mangrove-associated; (B) reef-associated. Mangrove-associated species are more dominant in the youngest and oldest plantation, while reef-associated (B) species are more dominant in the intermediate-aged plantations.

Fish assemblages

The SIMPER Analysis identified the fish species that contributed most to the similarities and dissimilarities between and among mangrove stands (**Figure 4**). Two major clusters on fish assemblages can be inferred: the mature group (P19) and the young group (P7, P9, P11 and P12). The oldest plantation was clearly separated from the young plantations (56 % dissimilarity). Young plantations were further subdivided into two groups: P7 and P9 (43 % dissimilarity), and P11 and P12 (intermediate age stands; 35 % dissimilarity).

There were no consistent patterns in the similarities and dissimilarities of stand-discriminating fish species between and among mangrove stand ages. However, some general patterns can be inferred. The species *A. manilensis* and *A. fraenatus* occurred in all stands but have decreased dominance as mangrove stand age increased. The intermediate-aged stands have mixture of *H. dussumieri*, *Ambassis* sp., *Goby* sp., *S. fuscescens*, *S. marginata*, and *G. oblongus*. The species *A. lacunosus*, *Goby* sp. and *H. dussumieri* occurred in most sites but have increased dominance as stand age increased.

DISCUSSION

The study provide new and valuable information that could be used in assessing impacts of mangrove planting programs in terms of its relationship with fish assemblages. To the knowledge of the researchers, this is the first study that evaluates the differences in fish assemblages in planted mangrove stands of different ages in the country. Mangroves are known to attract fishes because of the structural complexity, refuge and food that it provides (*Robertson and Duke 1987; Parrish1989; Nagelkerken et al. 2008*). Planted mangroves are expected to provide the similar ecological function (*Salmo III et al. 2007*). The potential to increase fish abundance and biomass has been essentially one of the primary motivations in the proliferation of mangrove rehabilitation programs in the Philippines (*Salmo III and Duke 2010*). But studies that evaluate impacts of planted mangroves in enhancing fish assemblage are still rare, casting doubts whether these rehabilitation programs are really effective or not.

Fish species composition, abundance and biomass not correlated with mangrove age

The 50 fish species (from 23 families) we collected in Lingayen Gulf are higher than the fish species documented from Pagbilao mangroves (South Luzon; 37 species; *Ronnback et al. 1999*). Almost all collected fish species are at their juvenile stage consistent with several studies that suggest mangroves as an effective nursery grounds to many juvenile fish species (*Robertson and Duke 1987; Crona and Ronnback 2007; Bosire et al. 2008*). The dominant species (both by abundance and biomass) are from the families Ambassidae, Apogonidae, Atherinidae, Gobiidae, Hemirhamphidae and Tetraodontidae. These species are the typical species that inhabit tropical mangrove forests (see for example *Ronnback et al. 1999; Feutry et al. 2010*).

Across sites, the mean fish abundance and biomass are higher by at least five-folds from the reported fish catch in Pagbilao mangroves (*Ronnback et al. 1999*). The study of *Ronnback et al.* (*1999*) used stake net method in different mangrove species (with *Avicennia marina* and *Rhizophora apiculata* stands) and geographical settings (mostly located in coves). But contrary to what is expected in planted mangroves, our study showed no clear patterns in fish assemblage with age of the mangrove stands. In fact, the younger mangrove stands have higher fish species diversity, species richness, abundance and biomass than the oldest stands.

Carnivorous and mangrove-associated species dominate in the oldest mangrove stands

The trophic categories of caught fish species varied in young and intermediate-aged mangrove stands. The trophic compositions in young and intermediate-age stands (< 12 yrs) are a mixture of detritivores, omnivores, herbivores and carnivores. But in the most mature stands, carnivores dominate the species composition. Conversely, carnivorous species exhibits low abundance in young mangrove stands, which was similarly observed in Pagbilao, Quezon (*Ronnback et al. 1999*).

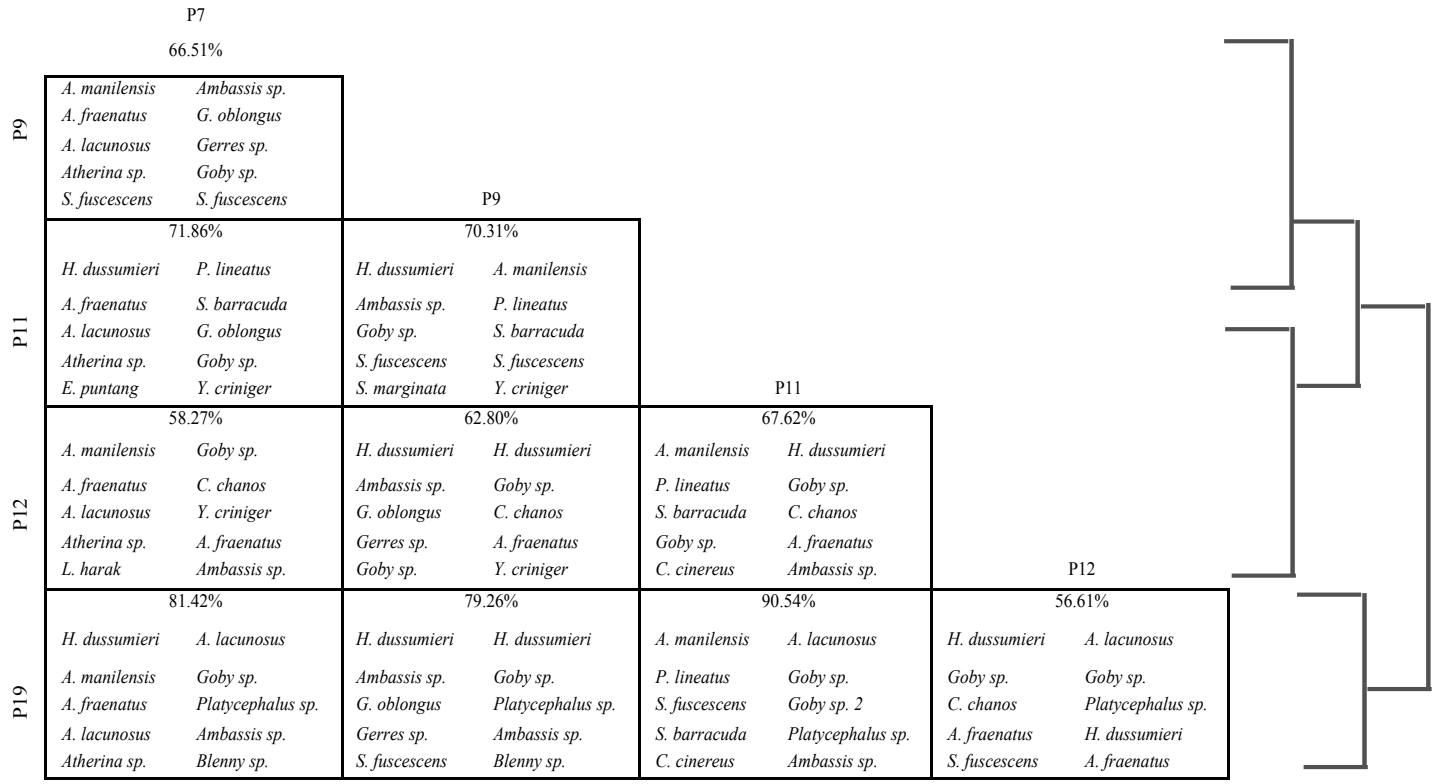

Figure 4. Summary results of SIMPER and cluster analyses showing the fish species that contributed most strongly to the similarities (left) and dissimilarities (right) between and among mangrove stand ages. Species are listed in order of their contribution of similarities and dissimilarities. The percentages indicate the dissimilarities between the two compared mangrove stands.

The researchers suspected that resident species (those species that are known to be mangrove-dwellers) would tend to become more abundant with age of mangrove stands. Among resident species, the longer-lived species (mostly the carnivores) would tend to be more dominant in the older stands. Carnivorous species are known to be long-lived species (*P. Aliño, pers. comm.*). It could be possible that carnivorous species prefer mature mangroves due to higher availability of food as compared to young, developing mangrove stands.

There was no consistent pattern on habitat preference of fish with age of mangrove stands. Mangrove-associated species dominates in both the youngest and the most mature plantation while reef-associated species have relatively higher dominance in the younger plantations. However, younger mangrove stands seem to attract more generalist species (i.e. species that are not exclusively mangrove dependent) but tend to have more mangrove-associated species in mature mangroves. This pattern probably indicates that certain fish will dominate as mangroves grow and develop. Many of these species might show ontogenetic shifts in habitat preference as they grow, probably as a response to increasing availability of food and complexity of forest structure.

Different fish species may use mangrove as a nursery ground at different stages of their life cycles. For example, the catadromous species barramundi, *Lates calcalifer* (Bloch), migrate from inland freshwaters to estuaries and mangroves during spawning (*Russell and Rimmer, 2004*). There are also some fish species that complete their entire life cycle in estuaries near mangroves (e.g., members of the Gobiidae and Atherinidae). Certain fish species such as *Mugil cephalus*, *Sillago* spp. and *Platycephalus* spp. spawn offshore. Their eggs are then carried by currents, and eventually, their post-larval or early juvenile stages settle in estuaries and mangroves (*Manson et al. 2005*).

Alternatively, the habitat preference of the caught fish species can be explained by localized site differences (i.e. proximity to reef and riverine systems) where particular trophic group of species naturally thrive. Notably, mangrove stands with nearby reefs (< 1 km in 9-, 11- and 12-yr stands) obtained more reef-associated species while site located in a bay (at least > 2 km from reef; 19-yr stands) has more riverine-associated species.

Fish assemblage shifts with age of mangrove stands but is weakly correlated

While the study did not find consistent patterns in changes in fish species composition, abundance, and biomass with age of mangrove stands, the cluster and SIMPER analyses however indicated general groupings of mangrove stands (**Figure 4**). Although weakly evident, a possible shift

in fish assemblages with age of mangrove stands can be inferred. It is suspected though that such shift in fish assemblages is not mainly related to the age nor the presence of the mangrove stands per se but rather with other inherent localized environmental factors. The proximity of the reef to the young and intermediate-age stands (9-, 11- and 12-yr stands) probably influenced the composition of reef-associated catch. Similarly, the 12-yr and 19-yr stands that are located near an estuary, obtained more mangrove-associated species.

In the Philippines, most planted mangroves are monospecific and have stunted growth (*Samson and Rollon 2008; Salmo III and Duke 2010*). Thus, habitat complexity is reduced as compared to natural mangrove stands. It is also possible that since planted mangroves can resemble the vegetation and soil characteristics of natural stands only after 25 yrs (*Salmo III 2011*), it may probably need the same amount of time for planted mangroves to effectively perform its ecological function as fish nursery.

There are contrasting views on the relationship, or lack thereof, between fish and mangroves (*Nagelkerken and van der Velde 2004*). The dependency of fish on mangroves is questioned (see *Blaber 2007* for example) citing that fish only use the seaward fringe of mangroves (*Halliday and Young 1996*) to forage or seek refuge from predation for a limited time (i.e. during high tide; *Lewis and Gillmore 2007; Lugendo et al., 2007*). Unlike crustaceans and shrimps that have stronger dependence on organic detritus produced by mangroves, fishes are considered transient species and may only be partially dependent on mangroves (*Halliday and Young 1996*). Fish species can migrate to adjacent ecosystems like coral reefs and seagrass beds for shelter and food. In addition, *Mumby et al.* (*2004*) proved that mangroves play an important role as an intermediate nursery habitat to increase the survivorship of young fish.

Inherent site-specific geographic and environmental conditions (e.g. proximity to reef or estuary, salinity, elevation, among others) possibly influence the availability of fish on mangroves (*Nagelkerken et al. 2008; Salmo III 2011*). In addition, tidal inundation is one of the known factors that affect the length of stay of fish in mangroves (*Ellis and Bell 2008*). While we acknowledged the role of environmental factors on fish assemblages in mangroves, these factors are beyond the scope of this study. We suggest that future studies that will investigate the impacts of mangrove rehabilitation programs should incorporate the contribution of environmental parameters on fish assemblage. In addition, the effects of the design of the trap nets used (e.g. fish activity or mobility, soak time) are some of the important factors that need to be considered.

CONCLUSION AND RECOMMENDATIONS

There were no consistent patterns in terms of fish species diversity, abundance and biomass with age of mangrove stands contrary to what is expected. Younger mangrove stands have higher fish species diversity, species richness, abundance and biomass than the more mature stands. In terms of trophic category and habitat preferences, there was higher dominance of carnivorous and mangrove-associated species in mature stands. Fish species may show ontogenetic changes as they grow, thus, a shift in their diet and habitat preferences can be expected. This could be inferred as a shift in fish assemblage with age of the mangrove stands. However, fish assemblages in mangroves may not necessarily be solely influenced by the age or presence of mangrove stands but rather can be attributed to some localized environmental factors, e.g. proximity to reef or estuary, salinity, elevation, tidal height, etc. Long-term studies focused both on temporal (month or season of sampling) and spatial (more replicate fish traps) aspects are necessary to document trends on changes in fish assemblage and if such can serve as a possible indicator of restoration trajectory in planted mangroves.

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