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ISOZYME AND HEMOCYTE ANALYSES OF EUROPEAN HONEY BEE (*Apis mellifera* L.) POPULATIONS REARED IN AGRICULTURAL, FORESTED, AND INDUSTRIAL AREAS UNDER DIFFERENT MANAGEMENT SYSTEMS

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ABSTRACT

Cytological and other genetic differences among European honey bee (*Apis mellifera* L.) populations reared in agricultural (Calauan), forested (Forestry, Los Baños), and industrial (Canlubang) areas of Laguna Province, Philippines, under different management systems were determined by hemocyte and isozyme analyses. The diversity among bee samples was assessed before and after transport as the control and experimental groups, respectively. The total hemocyte counts before and after transport were not significantly different among managed honey bee colonies from the three areas, but significant differences were noted in the unmanaged colonies at six months after transport. The differential hemocyte counts in the control populations (before transport) were significantly different from the experimental group (after transport). The number of prohemocytes were notably increased after transport, especially in the unmanaged colonies from Canlubang. Isozyme analyses revealed that alkaline phosphatase, acid phosphatase, and malic enzyme were all polymorphic among honey bees from all sampled areas. The observed values for genetic distance suggested that all populations remained genetically similar despite the environmental variation caused by colony transport and the use of different rearing practices.

Key words: *Apis mellifera*, environmental effects, hemocyte count, isozyme analysis, population genetics

INTRODUCTION

The European honey bee (*Apis mellifera* L.) has been reared by humans for over 7,000 years (Parker et al., 2010). This foreign species was introduced into the Philippines as early as 1913 for apiculture or modern beekeeping (Cunanan-Deyto et al., 2012). Aside from honey, other bee products include beeswax, propolis, and royal jelly (Parker et al., 2010). The various honey-derived products are also known for their medicinal value. Given the slow and gradual evolution of apiculture, novel techniques have been developed for rearing bees to further improve the industry.

To date, beekeeping is common in certain agricultural and non-industrial areas. Industrial areas and certain agricultural areas like rice farms are deemed not suitable for beekeeping because of the possible dangers these environments may pose to honey bees. These areas are said to be highly exposed to chemicals, which have been reported to affect honey production. If a study however, can show that there is no lethal effect on the honey bees and they have adapted ways by which they can survive in the three different areas, through their hemocytes and isozymes, then beekeeping can be done even in such areas. Honey bees can adapt to their environment, making colony transport feasible.

Hemocytes have been used in studying genetics of honey bees because the number of hemocytes can indicate cellular immunity (Negri et al., 2016). The different hemocyte types were previously described by Woodring (1985). The smallest hemocytes (6–13 μm) are the round prohemocytes (PRs), which have ribosomes and mitochondria but have a sparse endoplasmic reticulum and few Golgi bodies. The dark staining of the PR cytoplasm usually obscures the nucleus, which occupies 7-80% of the cell. PRs occur in all stages of development and eventually differentiate into plasmocytes (PLs). PLs are pleiomorphic amoeboid cells with a characteristic spindle shape and a ruffled leading edge when creeping. This type of hemocyte has a well-developed Golgi apparatus and ER, as well as lysosomes and free ribosomes. The PL nucleus is often stained red-purple, whereas its cytoplasm is pale to dark blue. Generally, PLs are the predominant hemocytes, which may eventually differentiate into granulocytes (GRs). GRs have an oval spindle shape and contain a variable number of small to medium-sized granules. This type of hemocyte is rich in Golgi bodies, ER, lysosomes, and free ribosomes. The granules in GRs stain bright orange, red, or pink, with a light blue cytoplasm. Other known hemocyte types include the spherulocytes and coagulocytes.

The enzymes alkaline phosphatase (ALPH), acid phosphatase (ACPH), and malic enzyme (ME) are known isozymes that have been used in bee genetic diversity studies (Aseo & Laude, 1992; Sheppard & Berlocher, 1988). Genetic diversity among bee populations in the Philippines has been traditionally assessed by hemocyte and isozyme analyses because these methods are cost-effective and simple.

The present study aimed to determine the genetic effects of transporting and rearing *A. mellifera* L. in agricultural, forested, and industrial areas. The genetic diversity of the selected local populations was determined using cytological and biochemical metrics.

MATERIALS AND METHODS

Sample Collection

Before the actual experiment, all honey bee colonies were established for a month at the Institute of Biological Sciences of the University of the Philippines at Los Baños (UPLB). Bee samples were collected as controls before the colonies were transported to three different locations, all in Laguna Province, for rearing: Calauan represented an agricultural area, the Makiling Forest Reserve (hereinafter referred to as "Forestry" or "Forestry Campus") represented a forested area, and Canlubang represented an industrial area. In each location, the colonies were divided into managed and unmanaged colonies (Figure 1).

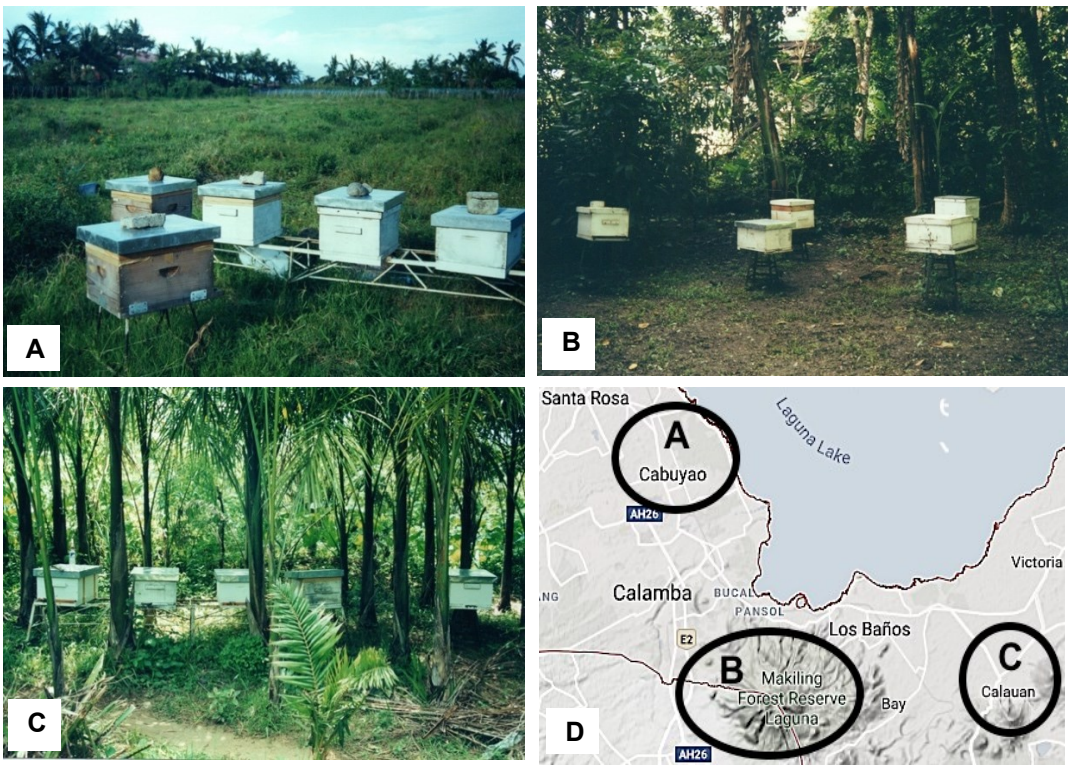

Figure 1. Bee colonies in the **a.** Calauan, **b.** Forestry, Los Baños, and **c.** Canlubang experimental stations and **d.** their relative locations on a topographical map of Laguna Province.

Before colony transport, 40 *A. mellifera* workers were collected from each sampling location. For the hemocyte analysis, 10 bees from each colony were placed in vials containing 5% formaldehyde. The remaining bee samples were immediately placed on ice for the isozyme analysis. A corresponding sample of 40 bees was collected from each location at six months after transport to determine the cytological and morphological effects of various environments on *A. mellifera* based on hemocyte and isozyme analysis, respectively.

During the study, the occurrence of a tropical storm during the experiment severely disrupted the colonies at the Forestry site, and no samples could be obtained after six months.

Hemocyte Analysis

The legs of each bee were cut with fine scissors and placed on a glass slide. The dismembered legs were squashed with a bent needle, and a drop of formaldehyde was added to extract the hemolymph and fix the hemocytes. After the resulting debris was removed, each smear was air-dried and stained with a small amount of Wright's stain. A cover slip was placed on top of each slide, and the preparation destained as needed by adding 45% acetic acid on the sides of the cover slip. The slides were temporarily sealed with paraffin.

The observed hemocytes were identified according to the classification by Woodring (1985). The total hemocyte count (THC) and differential hemocyte count (DHC) were determined for each individual. The DHC is based on the number of PRs and GRs. The average THC and DHC values for each colony were also obtained. ANOVA was performed to test for significant differences in THCs among different areas and between hemocyte types.

Isozyme Analysis

Individual bees were placed in pre-chilled Eppendorf™ tubes and macerated in 200 µL homogenizing buffer (0.01 µL 2-mercaptoethanol in 50 mL Tris-His buffer) with a stirring rod. Crude enzyme extracts were obtained from the homogenized sample of each bee. The extracts were electrophoresed on Mupid-2-Mini gels with 13% starch in a continuous Tris-Histidine buffer (pH 8.0) system. The samples for acid phosphatase (ACPH) and alkaline phosphatase (ALPH) genotyping were run at 50 V for the first two h and at 100 V for the succeeding hours. For esterase (EST) and malic enzyme (ME), a similar set-up was used at 100 V for three h or until bands appeared or until the tracking dye (0.05% bromophenol blue) had almost reached the end of the gel.

The number of bands and distance traveled (in mm) were scored for each well to determine the relative mobility (Rf) value as described by Laude & Carpena (1979). The chi-squared test at $\alpha = 0.05$ was used to test whether the genotype frequencies were in Hardy–Weinberg equilibrium. Yate's correction and combination of classes were employed to adjust the results of the chi-squared test (Haviland, 1990).

The intrapopulation genetic variation was estimated from the proportion of polymorphic loci (*P*), the average number of alleles per locus (*A*), and the mean heterozygosity (H_e) as described by Laude et al. (2006). Gene and genotypic frequencies were computed for each presumptive locus to determine the genetic distance (*D*) as described by Nei (1972). The Welch Two Sample t-test was performed in RStudio (http://www.rstudio.com/) to evaluate for significant differences in the abovementioned parameters between populations under different management strategies before and after transport (RStudio Team, 2016).

The evolutionary history of the bee populations were inferred using the neighbor-joining (NJ) method (Tamura et al., 2004). Phylogenetic analysis based on the isozyme genotypes was done using Molecular Evolutionary Genetics Analysis (version 7; MEGA7) software (Kumar et al., 2016).

RESULTS AND DISCUSSION

Changes in Hemocyte Counts

The number (frequency) of PRs and GRs among bee samples from the *A. mellifera* colonies before transport and after six months (Figure 2) are listed in Table 1. For all locations, GRs were present in all colonies before and after transport. In contrast, PRs were only present in colonies prior to transport to Calauan. After six months, PRs were observed in all managed populations and only in the unmanaged population of Canlubang. The frequencies of the two hemocyte types in all colonies were significantly different before and after they were transported.

The PR frequency in the managed colonies at six months after transport was similar among the three locations. However, the unmanaged colonies in Canlubang showed a marked increase in prohemocytes from zero to 162 after six months. PRs are hemocytes that transform to PLs, while PLs transform into GRs to form other types of hemocytes. Differences between the environmental conditions of the forested area where the bees were collected (UPLB) and the industrial area where they were reared (Canlubang) may have induced prohemocyte production.

No significant differences in the THCs of the different areas were observed after six months of rearing, except in the unmanaged populations. This trend implies that the location generally has no effect on the total hemocyte count. The number of PRs were observed to increase after rearing in the three different areas. Therefore, the granulocyte frequency probably had a significant decline to balance the effect on the increased PR levels.

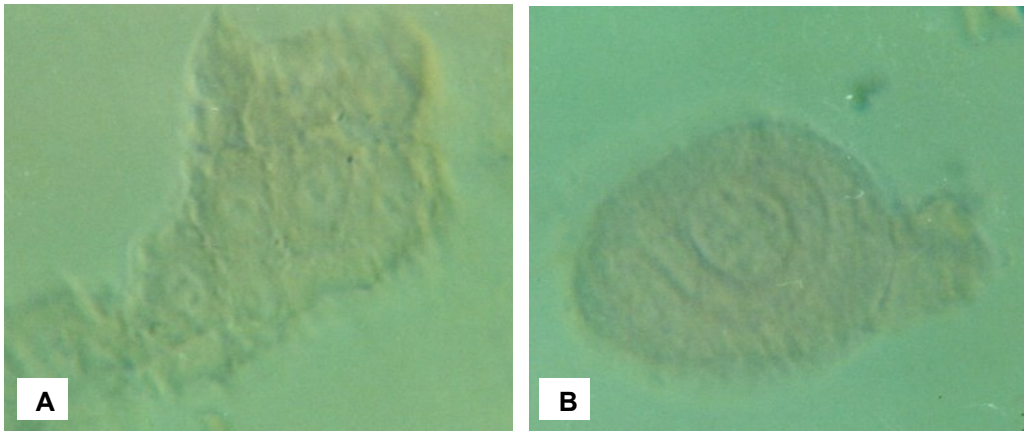

Figure 2. Representative photographs of **a.** prohemocytes and **b.** granulocytes at 400X magnification.

Based on the DHCs and THCs, the most significant changes occurred in the unmanaged colonies. This trend may be attributed to the presence of pathogens and other infecting particles while bees forage for food. Marked changes were specifically observed in the unmanaged colonies in Canlubang, where passers-by often disturb the colonies.

Isozyme Variation Among Populations

The ALPH, ACPH, and ME loci (see Figure 3 for representative photographs of starch gels) were all polymorphic in the Calauan, Forestry, and Canlubang populations of *A. mellifera.* These were for both managed and unmanaged colonies (see Table 2 for isozyme frequencies), and before and after transport. Nine presumptive loci were detected. ALPH had four presumptive isoloci: ALPH-1, ALPH-2, ALPH-3, and ALPH-4, whereas ACPH had three isoloci: ACPH-1, ACPH-2, and ACPH-3. Finally, ME only had two presumptive isoloci: ME-1 and ME-2. Three autosomal codominant alleles (designated as S, M, and F) were detected for ALPH-1, ACPH-1, ACPH-2, and ACPH-3; the remaining loci only had S and F alleles.

All ALPH isoloci had autosomal codominant S and F alleles, except for ALPH-1, which had S, M, and F alleles. Only ALPH-1 was polymorphic in all colonies before transport. The colonies transported to Calauan and Forestry remained polymorphic for ALPH-1 after six months but those transported to Canlubang were monomorphic. ALPH-2 was polymorphic in all colonies prior to transport but became monomorphic at six months after transport to Calauan. The colonies in Forestry were polymorphic for ALPH-2 at six months after transport. ALPH-3 was only present prior to transport to Calauan but became absent in the same colonies after six months. ALPH-3 was also monomorphic in colonies prior to transport to Canlubang. The managed colonies in Canlubang were monomorphic for ALPH-3 at six months after transport, whereas the unmanaged ones were polymorphic. ALPH-4 was only present in colonies before they were transported to Calauan and in the managed group of Canlubang after six months. ALPH-3 and ALPH-4 were absent in the Forestry populations.

ACPH-1, ACPH-2, and ACPH-3 all had the autosomal codominant S, M, and F alleles. ACPH-1 was polymorphic before transport to the three areas, and polymorphism was still detected after six months, Meanwhile, ACPH-1 was observed to be monomorphic in the colonies to be transported to and to be left unmanaged in Calauan and Forestry. After six months, the unmanaged Calauan colonies still exhibited monomorphism whereas the locus was polymorphic in the unmanaged colonies in Canlubang before and after transport. Before and after transport, ACPH-2 was polymorphic in the managed colonies in Calauan. ACPH-2 was not observed before colony transport to Forestry for both management systems. However, ACPH-2 was observed to be monomorphic in the managed colonies in Forestry after six months. ACPH-2 was polymorphic in managed colonies and monomorphic in unmanaged colonies before transport to Canlubang and six months thereafter. The polymorphic ACPH-3 was only present in the managed colonies in Canlubang.

Both ME-1 and ME-2 had autosomal codominant S and F alleles. ME-1 was polymorphic in all managed colonies before transport and after six months in the three areas, except for the colonies in Canlubang, which showed monomorphism for this locus after six months. The unmanaged colonies were consistently polymorphic for ME-1 in Calauan. In contrast, the unmanaged colonies to be transported to Canlubang and Forestry were monomorphic for ME -1 but demonstrated polymorphism after six months. ME-2 in the managed colonies in Calauan and Canlubang was polymorphic before transport but monomorphic after six months. The reverse was observed in the managed

colonies in Forestry, which shifted from monomorphism before transport to polymorphism six months after transport. All unmanaged colonies showed monomorphism at ME-2 before transport. After six months, only the unmanaged colonies in Canlubang remained monomorphic, whereas the unmanaged colonies in Calauan were polymorphic for ME-2.

Measures of Genetic Variation

Table 3 shows the proportion of the polymorphic loci (*P*), average number of alleles per locus (*A*), and mean heterozygosity (H_e) before and after six months in each group. There was no general pattern among values of *P* in managed and unmanaged colonies, with the exception of those in Calauan, whose values remained consistent. After six months, the value of *P* generally increased in both managed and unmanaged colonies. Meanwhile, the average number of alleles ranged from 1.33-2. Therefore, all the observed loci were consistently controlled by more than one allele.

Heterozygosity ranged from 0.0889 for the unmanaged colonies in Canlubang to 0.7506 for the unmanaged colonies transported to Calauan. The values of H_e decreased after six months in the managed colonies in Calauan and Forestry. In contrast, H_e increased in the managed colonies transported to Canlubang. A similar increase was observed in the unmanaged colonies transported to Calauan and Canlubang. The values of *P* and H_e decreased in the managed colonies at six months after transport to Calauan, whereas both values increased in the unmanaged colonies six months after transport to Canlubang.

Genetic Distances between Bee Colonies

The genetic distance values are summarized in Table 4 for managed populations and Table 5 for unmanaged populations. The respective optimal neighbor-joining trees are shown in Figure 4. Among the managed populations, the sum of branch lengths was 1.1242 before transport and 1.3598 six months after transport. Each tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The values for genetic distance were significantly different before and after rearing in the three different areas, thereby implying that colony transport may induce intrapopulation variation. Therefore, the genetic composition of the populations was conserved despite any possible variation caused by transporting them to the three different areas. However, the transport of colonies limits the possible genotypes that may be present in the new location, thereby accounting for the observed changes in genetic distance among populations.

Implications in Beekeeping

Using isozyme and hemocyte analysis, the present study determined the genetic effect of transporting and rearing *A. mellifera* L. in agricultural, forested, and industrial areas.

PRs and GRs were observed in the bee samples. GRs were present in all the *A. mellifera* populations of this study, whereas PRs were mostly observed in all

Table 1. Total and mean frequencies of prohemocytes (PR) and granulocytes (GR) in bees from managed and unmanaged colonies.

Hemo-cytes	Calauan				Forestry				Canlubang			
	Managed		Unmanaged		Managed		Unmanaged		Managed		Unmanaged	
	Before[a]	After[a]	Before[a]	After[b]	Before[a]	After[a]	Before	After	Before[a]	After[a]	Before[a]	After[b]
PR	10**	54	0*	0	0**	66	0*	n/a	0**	39	0*	162
GR	89**	133	82*	91	129**	46	135*	n/a	119**	123	104*	121
Total	99	187	82	91	129	112	135	n/a	119	162	104	283
Mean	49.5	93.5	41	45.5	64.5	56	67.5	n/a	59.5	0	52	141.5

'Before' and 'After' columns with different letter superscripts are significantly different. Values with * are significantly different between managed and unmanaged populations; values with ** have highly significant differences.

Table 2. Isozyme frequencies of managed and unmanaged bee colonies before transport and six months thereafter. S, M, and F are autosomal codominant alleles.

LO-CUS	Location of Managed Colonies									Location of Unmanaged Colonies								
	Calauan			Forestry			Canlubang			Calauan			Forestry			Canlubang		
	S	M	F	S	M	F	S	M	F	S	M	F	S	M	F	S	M	F
Before Transport																		
ALPH-1	-	0.100	0.900	-	0.773	0.227	0.033	0.800	0.167	-	0.474	0.526	-	0.275	0.725	-	0.933	0.067
ALPH-2	0.750	-	0.250	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ALPH-3	1.000	-	-	-	-	-	-	-	1.000	0.143	-	0.857	-	-	-	-	-	1.000
ALPH-4	0.400	-	0.600	-	-	-	-	-	-	1.000	-	-	-	-	-	-	-	-
ACPH-1	-	0.222	0.778	-	0.500	0.500	-	0.071	0.929	-	-	1.000	-	-	1.000	0.286	-	0.714
ACPH-2	-	-	-	-	-	-	0.714	0.286	-	1.000	-	-	0.950	-	0.050	1.000	-	-
ACPH-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ME-1	0.750	-	0.250	0.667	-	0.333	0.286	-	0.714	0.385	-	0.615	-	-	1.000	1.000	-	-
ME-2	0.588	-	0.412	1.000	-	-	0.917	-	0.083	1.000	-	-	1.000	-	-	1.000	-	-
Six Months After Transport																		
ALPH-1	-	0.600	0.400	0.100	-	0.900	-	1.000	-	0.385	0.230	0.385	N/A	N/A	N/A	-	-	1.000
ALPH-2	1.000	-	-	-	-	-	0.158	-	0.842	-	-	-	N/A	N/A	N/A	0.333	-	0.667
ALPH-3	-	-	-	-	-	-	-	-	1.000	-	-	-	N/A	N/A	N/A	0.333	-	0.667
ALPH-4	-	-	-	-	-	-	0.750	-	0.250	-	-	-	N/A	N/A	N/A	-	-	-
ACPH-1	-	0.250	0.750	-	0.091	0.909	0.273	0.545	0.182	-	-	1.000	N/A	N/A	N/A	0.184	0.289	0.526
ACPH-2	0.781	0.219	-	-	1.000	-	0.444	0.556	-	0.947	0.053	-	N/A	N/A	N/A	-	-	-
ACPH-3	-	-	-	-	-	-	0.308	0.461	0.231	1.000	-	-	N/A	N/A	N/A	-	-	-
ME-1	0.727	-	0.273	0.188	-	0.812	-	-	1.000	1.000	-	-	N/A	N/A	N/A	0.267	-	0.733
ME-2	1.000	-	-	0.786	-	0.214	1.000	-	-	-	-	-	N/A	N/A	N/A	1.000	-	-

Table 3. Measures of genetic variation in managed and unmanaged populations of the European honey bee in Calauan, Forestry, and Canlubang. The following parameters were calculated: proportion of polymorphic loci (*P*), average number of alleles per locus (*A*), and mean heterozygosity (H_e).

Para-meter	Calauan				Forestry				Canlubang			
	Managed		Unmanaged		Managed		Unmanaged		Managed		Unmanaged	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
P	87.5	66.67	42.86	40.4	75	80	40	N/A	83.33	55.56	33.33	57.14
A	1.875	1.67	1.43	1.6	1.75	1.8	1.4	N/A	2	1.78	1.33	1.71
H_e	0.3417	0.2657	0.1739	0.7506	0.3238	0.1974	0.0988	N/A	0.2387	0.2632	0.0889	0.2694

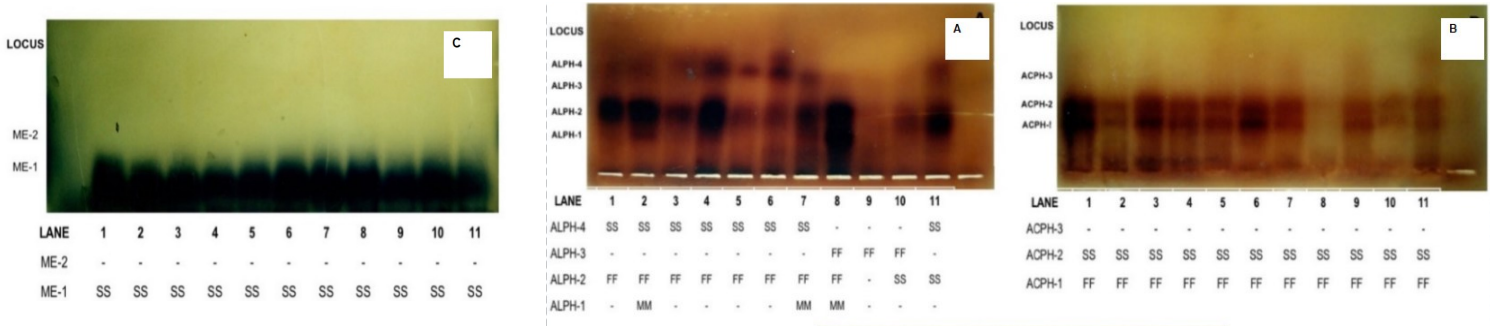

Figure 3. Representative gels of isozymes. **a.** alkaline phosphatase (ALPH). **b.** acid phosphatase (ACPH). **c.** malic enzyme (ME).

Table 5. Genetic distance based on nine presumptive loci observed in unmanaged *A. mellifera* colonies before transport (below the diagonal) and six months thereafter (above the diagonal). A tropical storm during the experiment severely disrupted the colonies in Forestry. No samples could be obtained after six months.

	Calauan	Forestry	Canlubang
Calauan		N/A*	0.6115
Forestry	0.2834		N/A*
Canlubang	0.2288	0.2921	

Table 4. Genetic distance based on nine presumptive loci observed in managed *A. mellifera* colonies before transport (below the diagonal) and six months thereafter (above the diagonal).

	Calauan	Forestry	Canlubang
Calauan		0.2385	0.2899
Forestry	0.6756		0.7780
Canlubang	0.8028	0.2886	

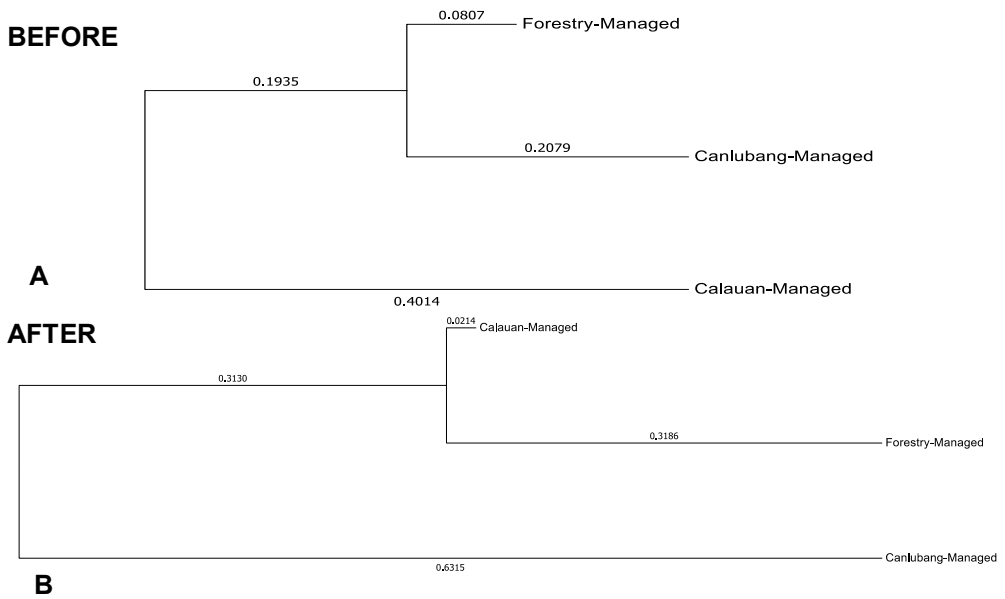

Figure 4. Neighbor-joining dendrograms constructed from the genetic distances based on isozyme analysis of managed colonies of *A. mellifera*. **a.** before transport. **b.** six months thereafter. Each tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

the managed colonies and the unmanaged Canlubang colony after six months. The observed DHC was significantly different between GRs and PRs before the colonies were transported to the three different areas. In contrast, the THCs were not significantly different among managed colonies of the different areas before and after transport, but the differences were significant in the unmanaged colonies at six months after transport.

The genotypes in all colonies before or after transport were consistently controlled by more than one allele as shown by the values of *A* (Table 3). Increased polymorphism after transport was only observed in the managed colonies in Calauan and the unmanaged colonies in Canlubang.

The genetic identity and genetic distance values from the present study showed that colony transport to the three different areas caused some variation between populations. However, these variations were not significant enough to make the colonies distinct. Therefore, rearing in different populations did not significantly influence the genetic variation between the subpopulations.

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