Journal of Environmental Science and Management 19-2: 54-63 (December 2016) ISSN 0119-1144

Assessment of Genetic Diversity of Narra (*Pterocarpus indicus* Willd.) Populations From Various Seed Sources in the Philippines Using RAPD

ABSTRACT

Pterocarpus indicus Willd. (narra), a critically endangered group, is one of the priority species for conservation and reforestation in the Philippines due to its economic, industrial, and ecological importance. A range of 29 to 40 individual samples from each of six seed sources from the Philippines were tested for genetic diversity using 11 RAPD markers. A total of 134 loci were detected, 129 of which were polymorphic. The mean genetic diversity within population was found to be moderate at 0.3183, which could be attributed to the deciduous and outcrossing nature of narra. The genetic differentiation among populations (0.0575) and Wright's Fixation Index (0.1528) suggests nearness of the populations to each other and distance from fixation of alternative alleles in the populations. The genetic distance and cluster analysis did not conform to geographical distribution, but revealed the relationships and the possible origin/s of the individuals of the populations. The results of the study is useful in the selection of sources of good planting materials for the improvement of narra tree in the Philippines.

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Key words: *DNA, Genetic diversity, Polymorphism, Pterocarpus indicus Willd., RAPD*

INTRODUCTION

The improvement and rehabilitation of Philippine forests, which is considered as one of the most biologically diverse around the world representing about 5% of the world's total flora, was deemed top priority by the Department of Environment and Natural Resources (DENR) in support to the country's efforts to fight poverty and promote sustainable livelihood (*Ecosystems Research and Development Bureau 2012; Mangaoang et al. 2005, Israel and Lintag 2013*). The most recent effort of the government is the National Greening Program (NGP) which aims to plant 1.5 Billion seedlings in 1.5 Million hectares by 2016 (Exec. Order No.26, 2011; *Paje 2013*). Over a span of 20 years, however, DENR's goal in reforestation was not reached due to inefficiency in the implementation and monitoring of such programs (*Israel and Lintag 2013*).

One of the factors that caused delay and failure to reforestation in the Philippines is the lack of proper evaluation of individuals of known origins, which includes both morphological and molecular characterization, for their potential as sources of traits for breeding and Seed Production Areas (SPA) establishment. In order to ensure the genetic quality of forests while maintaining their genetic diversity, tree improvement is applied. This includes the establishment of SPAs in natural stands or plantations, establishment of Seedling Seed and Seedling Clonal Orchards (SSOs and SCOs), establishment of Seed Sources (SSs) provenance trials, progeny trials, clonal tests, and tree breeding (*ERDB 2012*). Finding sources of traits for exploitation, and evaluation of the initial diversity to conserve is important in the success of tree improvement.

Measuring genetic diversity aids the planning and management of resources and the success of tree breeding programs (*Lee et al. 2004; Finkeldey 2005; Brown 2008; Mondini et al. 2009; Aremu 2011; Balzarini et al. 2011*). It can help determine a population's rate of change with respect to diversity, genetic vulnerability, as well as assess relationships within and among populations (*Brown 2008*). Populations with high genetic variation are preferred as they are more genetically sustainable and adaptable (*Finkeldey 2005; Gregory et al. 2006*), while naturally outcrossing populations with low genetic variation may result to low seed yield, germination rate and survival (*Finkeldey 2005; Gregory et al. 2006; Muchugi 2008*).

Pterocarpus indicus Willd. (narra), a vulnerable (*RA 9147 2001; IUCN 2008*) and critically endangered group (*DAO 15 2004; DAO 1 2007*), was included in the priority species for conservation and reforestation in

the Philippines because of its limited potential to invade native plant communities, fast growth, adaptation to stress, ease in reproduction and industrial importance (*Thomson 2006; ERDB 2012*). It is a native species of the Philippines with a semi-deciduous habit. Besides the wood, narra is also notable for its medicinal (*Ragasa et al. 2006*) and ornamental uses as well as its nitrogen-fixing ability (*Joker 2000; Thomson 2006; Junanto et al. 2008; Orwa, et. al 2009; Lok 2011*). *Mangkoedihardio et al.* (*2008*) also showed the use of this tree in phytoremediation. Populations of narra with unknown genetic structures in the country are sometimes used as sources of study and planting materials (seeds and/or cuttings for progeny testing and clonal propagation). Without full knowledge of these populations, this practice could threaten the success of tree improvement. Knowledge of the extent of genetic diversity in selected narra populations may be used in determining the susceptibility of these narra populations to pests like the ambrosia beetles which are the causative agent of fusarium wilt (*Fusarium oxysporum*) (*Sanderson et al. 1997; Conde 2010*).

Molecular markers are important nowadays in genetic diversity studies because of their ease, rapidity and reliability in producing results (*Finkeldey 2005; Ranade 2006; Bhat et al. 2010*). Studies on genetic diversity of related species of narra employed various molecular markers including Amplified Fragment Length Polymorphism (AFLP), Inter-Simple Sequence Repeats (ISSRs), Random Amplified Polymorphic DNA (RAPD), and chloroplast and nuclear microsatellites. *Rivera-Ocasio et al.* (*2002*), utilized AFLP markers to determine the genetic diversity of a broadly distributed wetland tropical tree, *Pterocarpus officinalis* (Jacq.), from eight neotropical populations. In 2009, genetic diversity and geneflow of *Pterocarpus officinalis* (Jacq.) were assessed using three chloroplast and six nuclear microsatellite markers (*Muller et al. 2009*). Another study used ISSRs and RAPD to study the genetic diversity of Dalbergia species, a leguminous tree species in the Fabaceae family (*Andrianoelina et al. 2006; Phong et al. 2011*) . In 2012, Amri and Mamboya, used random RAPD markers to assess the genetic diversity in *Pterocarpus angolensis* DC. collected from six natural populations in eastern part of Tanzania.

This study was designed to assess the genetic diversity within and among populations of *P. indicus* from seed sources located in Ilocos Sur, Cebu, Iloilo, Marinduque, Nueva Vizcaya, and Quezon using RAPD markers.

MATERIALS AND METHODS

Place of Implementation

This study was conducted at the Ecosystems Research and Development Bureau Forest Molecular Laboratory, College, Laguna Philippines from July 2012 to July 2013.

Sample Collection and DNA Extraction

Leaf samples were collected from a range of 29-40 narra individuals each from Ilocos (CAN), Cebu (CD), Iloilo (ID), Marinduque (MAR), Nueva Vizcaya (NVN), and Quezon (QD) populations (**Figure 1**). These seed sources were considered as candidates for seed production areas by DENR. Healthy and young leaves were processed and placed in a biofreezer (-80°C) prior to the analysis.

DNA extraction was performed using the modified Narra extraction protocol method as described in *Delos Reyes et al.* (*2013*). The extracted DNA was visualized using agarose gel electrophoresis (AGE) and quantified using spectrophotometry and subsequent normalization (1ng/uL) was done.

DNA Amplification

Twenty RAPD primers were screened for the ability to amplify DNA from three representative samples from each of the six Narra populations. Primers with positive results were used to amplify all of the Narra samples (**Table 1**). To overcome the problem of reproducibility, amplification was performed thrice using Bio-Rad and Applied Biosystems thermal cyclers and only bands present in all amplifications were scored.

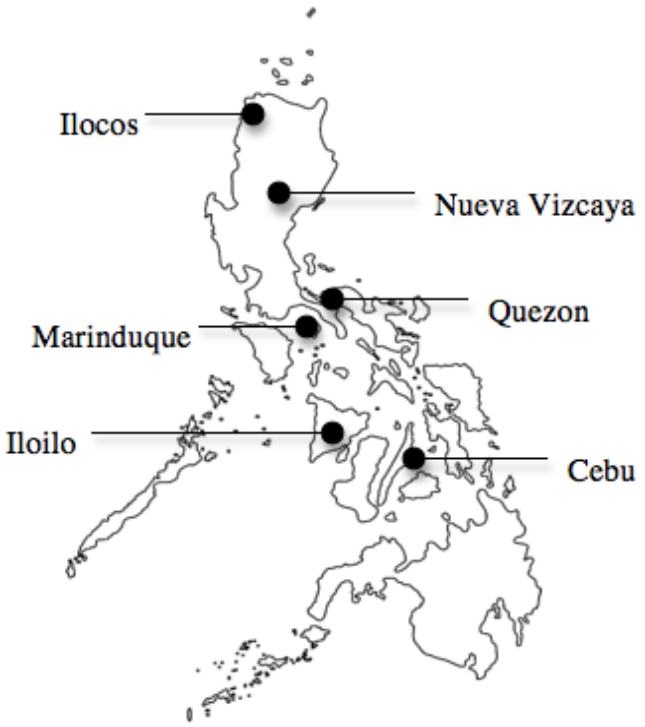

Figure 1. Collection sites of narra tree samples in the Philippines.

Table 1. List of RAPD primers used to amplify *P. indicus* Willd. collected from Ilocos, Cebu, Iloilo, Marinduque, Nueva Vizcaya and Quezon.

Primer	Sequence (5'-3')	References
OPA03	AGTCAGCCAC	*Acharya et al. 2004*
OPA04	AATCGGGCTG	*Acharya et al. 2004*
OPB04	GGACTGGAGT	*Alzate-Marin 2009*
OPB09	TGGGGGACTC	*Alzate-Marin 2009*
OPB10	CTGCTGGGAC	*Alzate-Marin 2009*
OPC05	GATGACCGCC	*Lakshimi 2008*
OPD18	GAGAGCCAAC	*Josiah et al. 2008*
OPD20	ACCCGGTCAC	*Acharya et al. 2004*
OPN05	ACTGAACGCC	*Acharya et al. 2004*
OPN06	GAGACGCACA	*Acharya et al. 2004*
OPN10	ACAACTGGGG	*Acharya et al. 2004*

A modification of the protocol devised by *Sreekumar et al.* (*2006*) was utilized for the amplification of specific DNA loci. A 25uL reaction mixture was put in each PCR well containing the following components: 2.0 uL DNA, 1 x PCR buffer with 1.5 mM $MgCl_2$ (KAPA Biosystems), 1 µM RAPD primer, 0.2 mM dNTP's (KAPA Biosystems), and 0.2 uL (1.5 U) Taq Polymerase (KAPA Biosystems). The DNA amplification was performed using Bio-Rad and Applied Biosystems Veriti thermal cyclers under conditions adapted from *Sreekumar et al.* (*2006*). The amplified products were visualized through gel electrophoresis (1.8% agarose) using SYBR Safe gel stain at 180V for 1.5 hours. The gels were viewed using BioRad EZ gel imager and analysed through ImageLab software.

The resulting bands were scored as follows: 1- band present; 0-band absent. A binary matrix was generated for all populations. This was then converted into formats required for genetic diversity programs namely: AFLP Surv, DARWin and PHYLIP using GenAlEx 6 (Genetic Analysis in Excel).

Data Analysis

AFLP Surv (*Vekemans et al. 2002*), which uses Bayesian estimation using non-uniform prior distribution of allele frequencies, was used to compute genetic variation. The allelic frequencies were estimated first by computing the frequency null alleles at each locus. The genetic diversity, which includes expected heterozygosity (H_j), percent polymorphic loci (PLP), genetic differentiation (H_b) and Wright's fixation index (F_{st}), were then computed.

Genetic Diversity Within Population

Percent polymorphic loci (P) is the number of polymorphic loci is the number of polymorphic loci (n_{pj}) relative to the total number of loci (n_{total}) (*IPGRI and Cornell University 2003*).

$$P = n_{pj}/n_{total}$$

Expected heterozygosity (H_j), which is the probability that any two alleles, chosen at random from a population, are different from each other (*IPGRI and Cornell University, 2003*), was computed using the formula:

$$\hat{H}_j = \frac{1}{L}\sum_{i=1}^{L}\hat{H}_j(i).$$

where L is the total observed loci and i as locus (*Lynch and Milligan 1994*). From this, the mean gene diversity within populations (H_w) was computed. The formula used was:

$$\hat{H}_W = \frac{1}{n}\sum_{j=1}^{n}\hat{H}_j.$$

where n is the total number of samples, j is the locus and H_j is the expected heterozygosity.

Genetic Diversity Between Population

The genetic differentiation (H_b) was computed by averaging all distinct pairs of populations using the formula:

$$\hat{H}_B = \frac{2}{n(n-1)}\sum_{j<k}\hat{H}_{jk}.$$

where n is the total number of samples, j and k are two distinct populations (*Lynch and Milligan 1994*).

Wright's fixation index (F_{st}) was computed using total gene diversity (H_t), which is the sum of gene diversity within populations (*IPGRI and Cornell University 2003*), and H_b. The formula is as follows:

$$\hat{F}_{ST} = \frac{\hat{H}_B}{\hat{H}_T} \times \left(1 + \frac{\hat{H}_B \mathrm{Var}(\hat{H}_W) - \hat{H}_W \mathrm{Var}(\hat{H}_B) + (\hat{H}_B - \hat{H}_W)\mathrm{Cov}(\hat{H}_B, \hat{H}_W)}{\hat{H}_B \hat{H}_T^2}\right)^{-1}.$$

where $Var(H_w)$ is the variance of the estimate of gene diversity within populations and $Cov(H_b,H_w)$ is the covariance of H_b and H_t (*Lynch and Milligan 1994*).

A matrix generated by AFLP Surv (*Vekemans et al.,2002*) and served as the in file for the PHYLIP software (*Felsenstein 2009*) to create a dendrogram. A higher resolution dendrogram which showed the relationships of all individuals in all populations was created using DARWin 5.0 (*Perrier et al. 2003; Perrier and Jacquemound-Collet 2006*). Bootstrap analysis with 10,000 iterations was performed in both softwares.

RESULTS AND DISCUSSION

The genetic diversity within and among populations of 29-40 tree samples of *P. indicus* Willd. with a planting interval of 10 m, from Ilocos (CAN), Cebu (CD), Iloilo (ID), Marinduque (MAR), Nueva Vizcaya (NVN) and Quezon (QD) were determined using RAPD markers. Out of 20 RAPD primers screened for ability to amplify segments in the Narra genome, 11 showed positive results (**Table 2**). The banding patterns are obtained using two of the primers (**Figure 2**).

Genetic Diversity within Populations

The proportion of polymorphic loci (PLP) of the narra populations tested for genetic diversity ranges from 75.4%

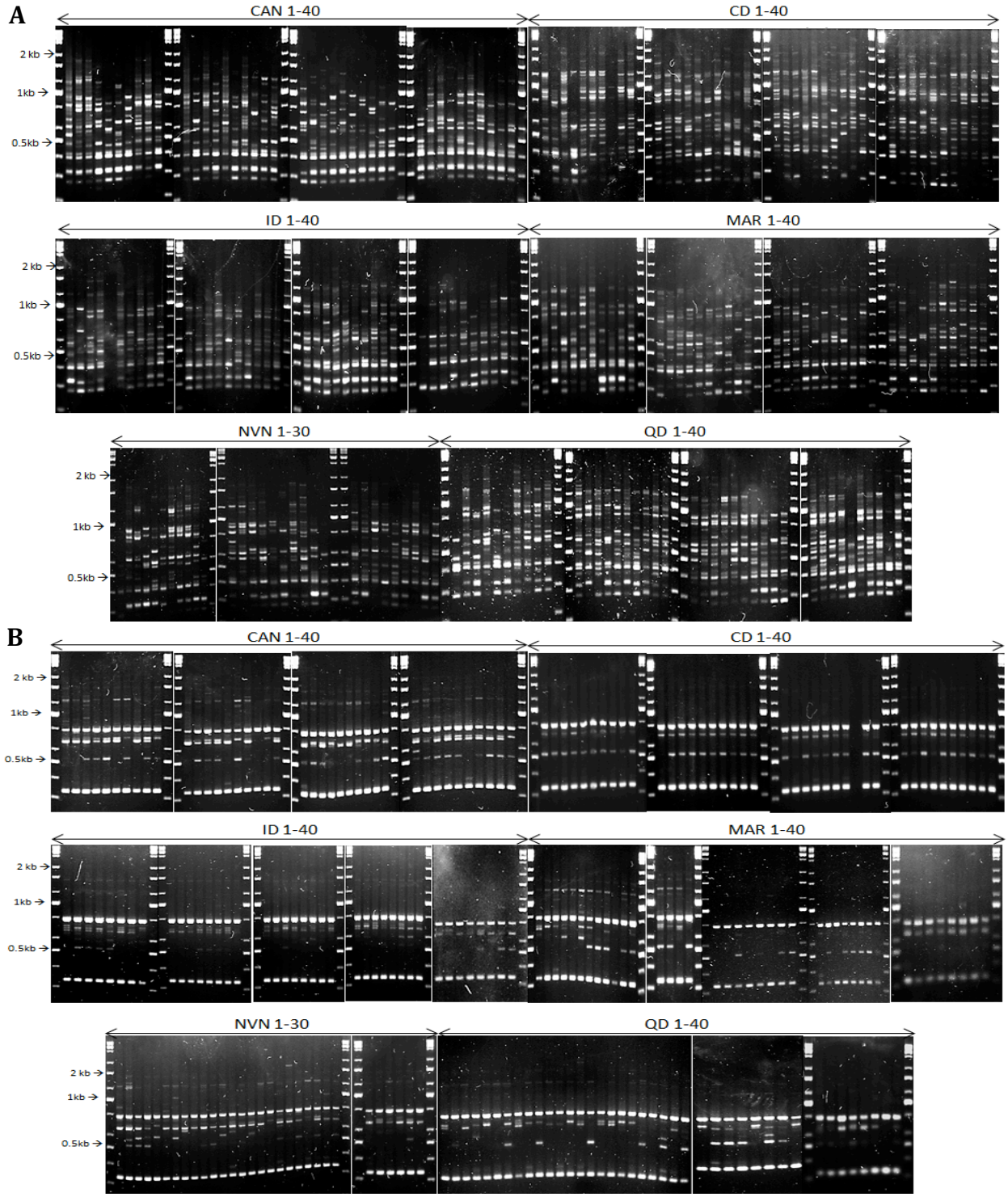

Figure 2. Electrophoretic images of Narra samples amplified using (A) OPB10 and (B)OPN05 RAPD primers run in 1.8% agarose at 180V for 100 minutes.

(MAR) to 96.3% (CAN) with a mean PLP af 87.2% across all loci tested (**Table 2**). These values are within the range and are higher than most of mean PLP values obtained from other organisms using RAPD markers: *Picea* spp. – 57-76% (*Narendula and Nkongolo 2012*); *Catharanthus roseus* – 70.3%-100% (*Lal et al. 2013*); *Achillea milefolium* – 87% (*Ebrahimi et al. 2012*); Syrian wheat varieties – 18.823% (*Saleh 2012*); *Pistacia* spp. - 56.05% (*Mohannad et al. 2006*); Sugarcane – 100% (*Ali et al. 2013*); *Gaeumannomyces graminis* var. *tritici* – 83.87%-100% (*Sadeghi et al. 2012*); *Vigna radiate* – 90% (*Datta et al. 2012*); Carthamus tinctorius L. – 65.53% (Panahi et al., 2013); *Vigan unguiculata* – 53.84% (*Girija et al. 2013*); *Lablab purpureus* – 57.69% (*Sanaullah et al. 2012*); *Hordeum vulgare* L. – 91.17% (*Giancarla et al. 2012*); *Artenisia capillaris* – 95% (*Zain Hasan et al. 2009*); Iranian green cumin – 86% (*Banghizadeh et al. 2013*); *Draba dorneri* – 78.94% (*Catana et al. 2013*).

The expected heterozygosities of the populations tested were between 0.25518 and 0.37378, wherein the upper limit is greater than the highest variation (*Alseis blackiana* – H_j=0.340) recorded for trees (*Finkeldey 2005*).

The mean H_j (0.31831) was also higher than some organisms surveyed for diversity using isozyme and molecular markers: Acacia mangium – 0.017 (*Moran et al. 1989*); *A. auriculiformis* – 0.081 (*Wickneswari and Norwati 1993*); *Pinus merkusii* – 0.058 (*Changtragoon and Finkeldey 1995*); *Plantago major* – 0.022 (*Zubair 2012*); *Picea* spp. – 0.27 (*Narendula and Nkongolo 2012*); *Tylophora rotundifolia* – 0.2643 (*Sebastian et al. 2010*). These values suggest that the narra populations tested have good levels of genetic diversity within each populations.

The observed levels of genetic diversity within each population for this study may be because of recombination. *P. indicus* is dioecious and was found to be predominantly outcrossing, which means that gene recombination is inevitable. Another probable reason of variation is genetic polymorphism. The populations used in this study were not identified as either natural stand or plantation type, so the presence of mixed seeds from different seed sources in a population is possible. The other two factors could not be confirmed since the uniformity of the environment was not assessed.

In general, all populations exhibited good levels of variation. However, it is notable that the geographically isolated natural stand of Marinduque population showed the lowest genetic diversity. The isolation of this population may be caused by inbreeding over time. Isolation makes a naturally outcrossing population suffer loss of genetic variation, fixation of deleterious alleles, and reduced viability leading to extinction. This can only be confirmed when the origin of the individuals of the Marinduque population becomes available.

Table 2. Intra-population genetic data of *P. indicus* Willd. populations collected from Ilocos (CAN), Cebu (CD), Iloilo (ID), Marinduque (MAR), Nueva Vizcaya (NVN), and Quezon (QD) computed using AFLPsurv.

Population	n[1]	No. of loci	No. of polymorphic loci	PLP[2]	H_j[3]
CAN	40	134	129	96.3	0.37378
CD	39	134	126	94.0	0.35534
ID	39	134	112	83.6	0.30303
MAR	39	134	101	75.4	0.25518
NVN	29	134	121	90.3	0.31163
QD	39	134	112	83.6	0.31090
MEAN				87.2	0.31831

[1]number of samples
[2]proportion of polymorphic loci
[3]expected heterozygosity

Genetic Diversity Among Populations

The degree of differentiation (H_b) among populations (0.0575) measures the difference between the mean diversity within populations and diversity of the populations as a whole (*Nei 1973*), hence indicating the average variation among populations (**Table 3**).

Based on Wright's (1965) guidelines, the computed F_{st} value (0.1528) suggests a weak correlation of randomly chosen alleles from the populations, indicating high variability among populations. This also implies that the genetic compositions of the six populations are near each other and are far from being fixed with alternative alleles (*Balloux and Lugon-Moulin 2002*).

The Marinduque-Iloilo-Quezon cluster has a strong bootstrap support (92.99%) indicating that these populations have genetic similarities across all the loci tested. The Nueva Vizcaya population separated from the other five

Table 3. Genetic diversity within and among *P. indicus* Willd. populations computed using AFLPsurv software@.

H_t[1]	H_w[2]	H_b[3]	F_{st}
0.3758	0.3183	0.0575	0.1528
S.E.	0.017094	0.000000	0.030065
Var	0.000292	0.000000	0.000904

[1]total gene diversity
[2]mean gene diversity within populations
[3]genetic differentiation among populations
@Lynch and Milligan method

with a bootstrap support of 58.15% (**Figure 3**).

The strongly supported clustering of the Quezon, Marinduque, and Iloilo populations may be attributed to common seed sources of the first two. Iloilo was found to have different seed sources. As confirmed by the Ecosystems Research Development Bureuau (ERDB), seeds of the Quezon and Marinduque populations came from Bicol and Romblon while the seeds of the Iloilo population came from Los Banos and regions 5, 6, and 10. With a bootstrap support value of 70.55% for the Marinduque–Iloilo cluster, the latter may also have similarity with the Marinduque and Quezon populations and probably share some of the seed sources. It may be inferred that the parental trees of Bicol and Romblon populations possibly came from Los Banos and regions 5,6 and 10, or vice versa. These regions of origin may also have close genetic structures. It is also suggested that the Marinduque, Iloilo, Quezon, Bicol, Romblon, and Los Banos populations may share some traits and is therefore an opportunity for further studies.

Information on seed origins of the other three populations is not recorded. However, from the clustering information gathered, Nueva Vizcaya has a different genetic structure from the other five and may be due to genetic isolation or a totally different seed source. The Ilocos and Cebu populations might have shared some of the seed sources of the other three.

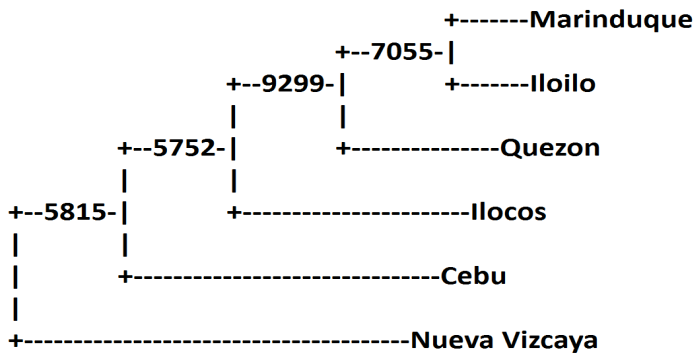

Figure 3. Consensus tree of *P. indicus* Willd. populations sampled from Nueva Vizcaya (NVN), Iloilo (ID), Marinduque (MAR), Quezon (QD), Ilocos (CAN) and Cebu (CD) generated from PHYLIP software with 10,000 bootstrap iterations.

Another phylogram (**Figure 4**) was done to support the above analysis. Most of the branches, however, have low bootstrap supports which suggests that the tree was not highly favored.

The values of genetic distance and pairwise F_{st} ranged from 0.0411 - 0.1292 and 0.0954 - 0.2148, respectively (**Table 4**). The highest genetic distance and F_{st} are both between Iloilo and Nueva Vizcaya while the lowest are Iloilo and Marinduque. The values between Nueva Vizcaya from both Ilocos and Cebu are proximal. While Ilocos and Cebu are nearer each other than with Nueva Vizcaya. This may indicate near or common seed source(s) of Cebu and Ilocos populations. The separation of the Nueva Vizcaya population may indicate its different origin. The genetic distance and pairwise F_{st} for all populations were relatively near each other indicating proximate genetic structures that are far from fixation.

The results did not show any correlation between genetic structure and geographic location. Common origin(s) of seeds used as planting materials in the six populations studied and the reproductive characteristics of Narra are the most probable bases of the clustering and diversity observed in this study.

CONCLUSION AND RECOMMENDATIONS

The genetic diversity analysis showed that the six populations of *Pterocarpus indicus* Willd from Ilocos Sur, Cebu, Iloilo, Marinduque, Nueva Vizcaya and Quezon have good levels of genetic variation and can serve as good sources of potentially useful genes. Out of 134 loci detected, 129 were polymorphic suggesting efficiency of the chosen RAPD primers in detecting polymorphism. The mean gene diversity was 0.3183 which is not low possibly because narra is dioecious and naturally outcrossing. This indicates good survival and potential source of useful genes for

Table 4. Genetic distance (below the diagonal) and pairwise F_{st} (above the diagonal) between populations of *P. indicus* Willd. generated using the AFLP surv software in three sampling sites: Ilocos (CAN), Cebu (CD), Iloilo (ID), Marinduque (MAR), Nueva Vizcaya (NVN) and Quezon (QD).

Population	CAN	CD	ID	MAR	NVN	QD
CAN		0.1187	0.1123	0.1496	0.1781	0.1354
CD	0.0803		0.1834	0.2047	0.1796	0.1460
ID	0.0654	0.1159		0.0954	0.2148	0.0991
MAR	0.0803	0.1173	0.0411		0.1885	0.1228
NVN	0.1187	0.1154	0.1292	0.0955		0.1528
QD	0.0839	0.0887	0.0499	0.0507	0.0850	

*Nei's genetic distance (*Lynch and Milligan 1994*)

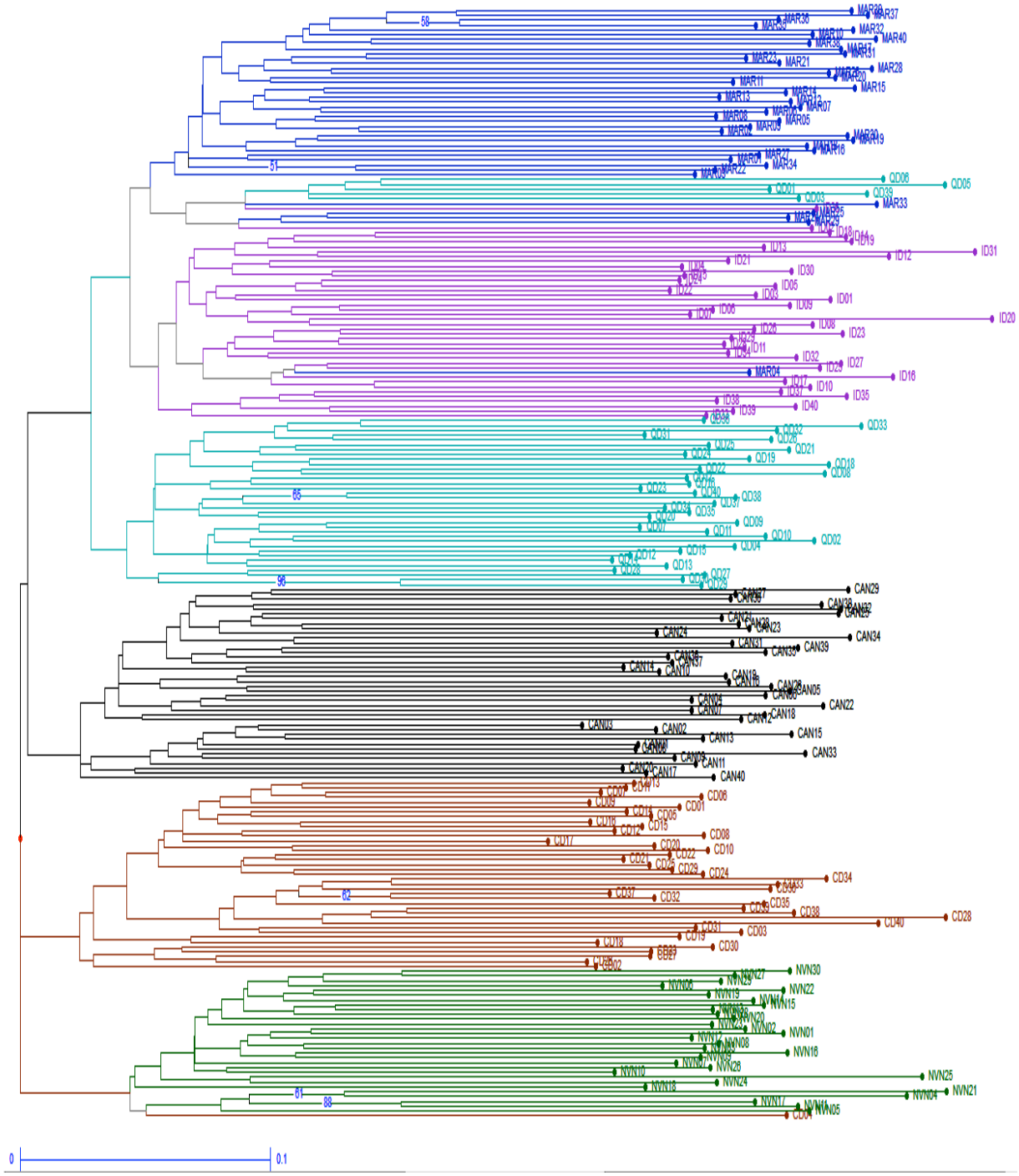

Figure 4. Phylogram (vertical heirarchical tree) for the gene frequency of *P. indicus* Willd. populations sampled from Ilocos (CAN), Cebu (CD), Iloilo (ID), Marinduque (MAR), Nueva Vizcaya (NVN), and Quezon (QD) generated using DARwin 5.0 software by Weighted Neighbour Joining using JACCARD coefficient. Numbers on the tree represent bootstrap analysis performed on the clusters.

future improvement of narra tree population. The H_b and F_{st} were close to zero at 0.0575 and 0.1528, respectively, which suggest the populations' nearness of allelic frequencies and distance from fixation.

The genetic distance and cluster analysis showed that there are close relationships among populations from Quezon, Marinduque and Iloilo, as well as between Cebu and Ilocos, suggesting the possibility of having common seed sources. Nueva Vizcaya grouped singly which may indicate different origin.

Genetic diversity is important to ensure long-term survival of existing narra tree populations. Development of markers for economically important traits for the improvement of narra tree populations as well as the use

of molecular characterization in the identification of Seed Production Areas (SPAs) is highly recommended.

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ACKNOWLEDGMENT

The authors are grateful to the ERDB management for the continuous support of project funds and acquisition of equipment for the ERDB Forest Molecular Laboratory. Sincere thanks is also extended to the following: Dr. Maria Genaleen Q. Diaz, Professor 4 and her staff, Mr. Wilson Aala, Jr., Genetics and Molecular Biology Division, Institute of Biological Sciences, College of Arts and Sciences, UPLB for sharing their time and expertise; Dr. Kenneth McNally (Head, Genetic Resources Center, International Rice Research Institute, College, Laguna) and staff, Mrs. Elizabeth Naredo and Ms. Sheila Quilloy for the accommodation and support; And to our Sovereign God the Father and Source of Everything, our highest praises, honor and thanks for the wisdom and success of this endeavour.