Journal of Nature Studies 14 (1): 1-11
ISSN: 1655-3179

Genetic Diversity of *Vitex negundo* L. (Lagundi) in the Philippines using Inter-simple Sequence Repeat (ISRR) Marker Analysis

Nonnatus S. Bautista[1*], Anna Francesca Cubos[2] and Maria Catherine Lim[2]
[1]Plant Biology Division, Institute of Biological Sciences,
University of the Philippines Los Baños, College, Laguna 4031 Philippines
[2]Department of Biology, Ateneo de Manila University,
Katipunan Ave., Loyola Heights, Quezon City1108, Philippines
*Corresponding author: nonniebautista@gmail.com

ABSTRACT – *Vitex negundo* L. is now gaining popularity because of its use in cough medicines. It is listed as one of the ten medicinal plants being promoted by the Department of Health due to its anti-inflammatory properties. In order to improve breeding methods for the cultivation of lagundi, knowledge about its genetic diversity is needed since this could distinguish possible cultivars of the plant. This preliminary study was done using a molecular marker analysis on lagundi from fifteen different sites in the Philippines. Based on inter-simple sequence repeat (ISSR) markers, there is usually a common locus or pattern of bands that is present in all samples from the fifteen geographic locations that can be attributed to the fact that all samples belong to one species. Moreover, the samples also exhibited a great number of polymorphisms that suggests the genetic variation found within the species. It is then possible to say that although *V. negundo* is considered to have only one species, it can be further classified into several morphotypes based on the several clusters as revealed by the dendrogram. There was no correlation between the sample clusters and their geographic locations, since the groupings were not consistent with their locations. Nonetheless, results of this study in the establishment of genetic diversity patterns of *V. negundo* in the Philippines can provide baseline information for future research that could finally lead to the identification of lagundi cultivars that yield a high quality of active compound for medicinal purposes.

Keywords: Vitex negundo, ISSR markers, genetic diversity

INTRODUCTION

Lagundi, *Vitex negundo* L., is one of the ten medicinal plants being promoted by the Department of Health due to its antitussive and anti-inflammatory properties. It is a member of the family Verbenaceae, alongside other economically and medically important plants such as alagaw (*Premna odorata*), lemon verbena (*Lippia citriodorata*) and common vervain or "mosquito plant" (*Verbena officinalis*). It is an erect, branched shrub with five-foliate, opposite-arranged leaves (Fig. 1). It is indigenous to China, India and Malay Peninsula. However, it is known to be widely distributed in the Philippines, growing in low to medium altitudes and even in thickets and waste places. The leaves, bark, roots, and seeds of *V. negundo* have long been used in traditional medicine to heal wounds, cleanse

To cite this paper: Bautista, N., Cubos A.F. and Lim, M.C. 2015. Genetic Diversity of *Vitex negundo* L. (Lagundi) in the Philippines using Inter-simple Sequence Repeat (ISRR) Marker Analysis.
Journal of Nature Studies. 14 (1): 1-11

ulcers, prevent insect bites, treat snake bites, and relieve rheumatism. Its oil has also been used to relieve sores and sinuses. Recent studies have proven its antiseptic, antitussive, as well as its anti-inflammatory properties. It has been found to have antibacterial effects against *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*. Other studies have also shown its efficacy in reducing coughing and relaxing the bronchial smooth muscles (Galvez Tan 2010). These properties make it an effective drug for coughs and bronchopulmonary disorders such as brochial asthma and acute bronchitis.

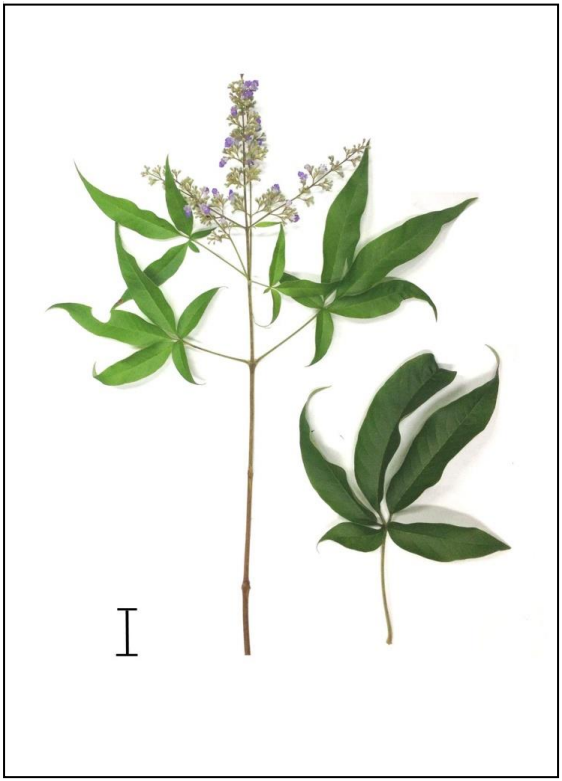

Fig. 1. Diagram of *Vitex negundo* L. (Lagundi) morphology. Scale is equivalent to 1 inch. Original photo taken by TJT Medina.

The therapeutic effects of lagundi can be attributed to the phytochemicals produced by the plants. Secondary metabolites like nishindaside, mussaenosidic acids, vitedoin, negundin, and vitexin are some important bioactive agents which impart a variety of medicinal uses to the plant (Basri et al. 2014). There is currently a continuous effort in extracting and isolating the different phytochemicals released by *V. negundo*. This has assisted in determining the different medicinal properties of the plant as well as in identifying the active compounds of lagundi. However, researchers have not yet utilized genetic tools in

determining variation among the different populations of lagundi in the Philippines. This hampers identification of possible lagundi cultivars for breeding improvement and germplasm management.

The geography of the Philippines supports great genetic variation among populations since its mountain ranges and the waters separating each of its islands act as barriers to gene flow. As a result, there is a possibility that genotypic variations can be found among the different populations of *V. negundo* in the Philippines. Analysis of the genetic diversity of lagundi can help in identifying cultivars of the plant. This can be used in plant breeding to produce better quality plants for use in medicine. The success of using ISSR markers in lagundi also suggests its possible use in the phylogenetic analysis of different *Vitex* species, as well as in the phylogenetic analysis of the whole *Vitex* genus. This gives a more accurate phylogenetic analysis than those based on morphological data alone.

To be able to detect variation on the genetic level, various DNA marker-assisted techniques can be used. One of these techniques is the inter-simple sequence repeat (ISSR) technique which applies the principle of simple sequence repeat (SSR)-anchored polymerase chain reaction (PCR) amplification. In this procedure, the designed primers can randomly amplify DNA fragments of the inter-repeat regions. It is simpler to use than SSR since prior knowledge of the target sequence is not required (Hu et al. 2008). It also produces higher frequency of polymorphisms as compared with random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) (Behera et al. 2007). These characteristics make ISSR markers favorable for studying genetic diversity.

In this preliminary study, the genetic diversity of *V. negundo* samples from fifteen different areas in the Philippines was examined using ten ISSR primers. The objectives of this study were to determine the genetic diversity among the different *V. negundo* samples collected from different parts of the Philippines using ISSR analysis; to identify if there are possible morphotypes of lagundi; and construct a phylogenetic tree describing the genetic and evolutionary relationships among the populations of *V. negundo* found in the Philippines.

MATERIALS AND METHOD

Plant materials and DNA extraction

Vitex negundo L. leaf samples were gathered from 15 sites in the Philippines, namely: Marikina City (1), Quezon City (1), La Union (1), Cavite (2), Batangas (2), Quezon (1), Oriental Mindoro (2), Leyte (1), Cebu (3), and Negros Occidental (1) (Fig. 2). Total genomic DNA was extracted using the protocol based on the study of Pirtilla et al (2001) on DNA extraction methods for medicinal and aromatic plants. The quality and concentrations of extracted samples were estimated and standardized using known concentrations of λ DNA by electrophoresis on 1% agarose gel.

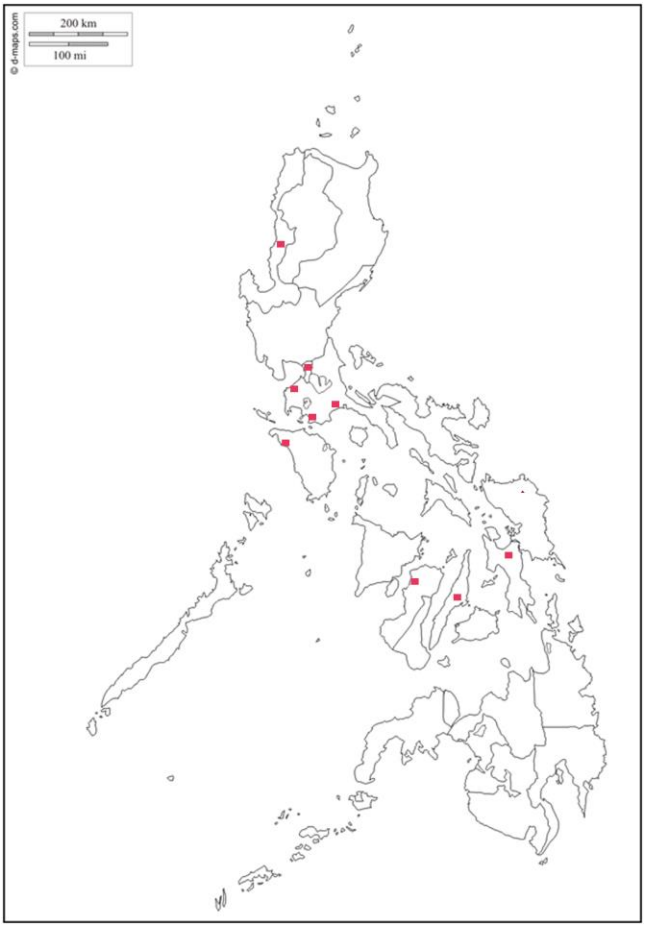

Fig. 2. Map of the Philippines showing the sampling sites for the *V. negundo* used in the study as represented by red squares (■). Map source: d-maps.com.

Molecular markers analysis

Ten of the twenty-two primers used for *V. rotundifolia* (Hu et al 2008) were used in the study (Table 1). The bases for the choice of primers were the clarity and reproducibility of the fragments used in the previous study. These primers were designed based on the UBC Primer Set No. 9 (Biotechnology Laboratory, University of British Columbia), Zhou et al 2001, Bornet and Branchard (2001), and Ross et al 2002, respectively. The rationale behind using the ten primers was the high percentage of polymorphic fragments produced, the number of DNA scored and the relatively close annealing temperature of some of the primers for efficiency use of the thermocycler.

The PCR machine was programmed for "an initial period of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50-65°C (or specifically depending on the type of primer used), and 1 min at 72°C and 10 min at 72°C for the final extension." These reactions were carried out in a total volume of 10 µL mixture containing 1X Vi Buffer A (w/o $MgCl_2$), 2 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTP, 0.2 µmol/L of ISSR primer, 1:20 dilutions of genomic DNA and 1 unit of Taq polymerase. Amplified products were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. It was photographed under UV light using GeneSnap from Syngene where two replications were accomplished to confirm its accuracy and reproducibility.

Evaluation of Molecular Marker Data

Method of measurement for the genetic relationships among the plant samples was done by scoring each polymorphic band as "1" for its presence and "0" for the absence of the bands. Genetic diversity within and among samples of a specific population can also be estimated by calculating effective allele number and percentage of polymorphic bands. All calculations were done using the free software program POPGENE Version 1.32. The similarity coefficients taken from the data analysis are then used to construct a dendrogram.which is based on Nei's unbiased measured of genetic identity and genetic distance.

RESULTS

All 10 selected ISSR primers successfully produced a total of 88 DNA fragments. Each primer generated products in the range of 5 to 15 bands with Primer 3 giving the highest number of bands while Primers 4, 5, 7, 9, 10 having 100% polymorphic bands are shown in Table 1. Of the 88 DNA fragments that were scored, 67 of which were considered polymorphic loci which is 76.4% of the total.

The dendrogram (Fig. 3) illustrated the genetic relationship among the 15 *V. negundo* samples from the different parts of the Philippines. It was shown that there was diversity in the samples taken from Luzon and Visayas islands. Supposedly, each of the samples from the same geographical location (island-based) must be grouped together but it was not reflected from the dendogram. It showed that the three samples from Cebu (Visayas) were grouped separately but in the same cluster as Quezon City, Marikina City and Cavite, all of which from Luzon island. The two samples from Oriental Mindoro province were from separate cluster, one is same group as samples from Cavite and the other one from samples from Quezon province. Moreover, two samples from Batangas were also in different groupings. Likewise, samples from the provinces of La Union, Negros Occidental and Quezon provinces were also in separate groupings.

Table1. Selected inter-simple sequence repeat (ISSR) primers used for DNA amplification of *Vitex negundo* individuals from 15 locations in the Philippines, with information on experimental conditions, number of fragments per prime, number of polymorphic loci and percentage of polymorphic fragments.

ISSR Primer	Sequence	Annealing temperature (°C)	No. DNA fragments scored	No.of polymorphic loci	Percentage of polymorphic fragments
1	TGT GTG TGT GTG TGT GC	51	12	8	66.67%
2	GAA GAAGAAGAAGAAGAA	44	13	5	38.45%
3	GGA GAG GAG AGG AGA	44	15	10	66.67%
4	GACA GACAGACAGACA	40	6	6	100%
5	TGT GTG TGT GTG TGT GRC	53	5	5	100%
6	ACA CAC ACA CAC ACA CYG	52	5	4	80%
7	GAG AGA GAG AGA GAG AYG	44	11	11	100%
8	GAG AGA GAG AGA GAG AT	38	6	3	50%
9	CTC TCT CTC TCT CTC TT	39	8	8	100%
10	CAC ACA CAC ACA CAC ART	49	7	7	100%

Fig. 3. Dendrogram illustrating the genetic relationship between the 15 samples of *Vitex negundo L.* based on Nei's Unbiased Measures of Genetic Identity and Genetic distance.

DISCUSSION

This study was done to elucidate the genetic diversity of *V. negundo* found in the Philippines using the inter-simple sequence repeats. All 10 primers have produced bands that showed genetic variation between the samples gathered in the Philippines. This is supported by the data on Table 1 that revealed polymorphism among 15 *V. negundo* samples. Initially, it is predicted that samples taken from the same geographical location will be clustered on the same groups but the dendrogram (Fig 3.) has shown otherwise. Several factors (i.e. climate type, topography, and soil type) can be attributed to the diversity observed among the samples used in the study. Owing to the archipelagic nature of the Philippines, topographic as well as climate variations can be identified as factors that contributed to the diversification of the *V. negundo*. Most of the samples were taken from locations exhibiting the Type III climate which is characterized by not very pronounced maximum rain period, with a short dry season lasting from 1 to 3 months. This is a possible explanation for having samples from different geographical locations clustered in one group. In some cases, samples from location with a different type of climate (i.e. Type I or Type IV) such as the one from La Union (Type I) and Quezon (Type IV) were shown to be in separate cluster or groups.

There was a common locus or pattern of bands observed in the gel images of all the samples. This can be attributed to the fact that all samples belong to one species. The differences in the bands are caused by the genetic variation found within the species. Although *V. negundo* has only one species, it can be further classified into several morphotypes. This is based on the several clusters revealed by the dendrogram from the ISSR markers. Another possible reason for the formation of several clusters can be due to gene flow. There is no complete guaranty that the samples taken from each area naturally belong to the area. There are instances when Lagundi plants from other regions are brought to different places. This is due to its well-known medicinal value which increases the likelihood that they can come from other places. In a previous study conducted in China to find out whether the Great Wall acts as a physical barrier to gene flow, one of the plant species that were selected was *V. negundo*. It revealed that the Great Wall has indeed served as a physical barrier to gene flow between the two subpopulations of *V. negundo* that have been separated for more than 600 years (Su et al. 2003). Morever, the Yangtze River was also considered as another barrier to gene flow in *V. negundo*. It was suggested that the river is a barrier to seed dispersal (Zhang et al. 2007). These studies, however, were more focused on biodiversity conservation than on the improvement of plant varieties for use in drugs. Overall, in this preliminary study, the molecular marker analysis employed has revealed that the establishment of genetic diversity patterns of *V. negundo* in the Philippines and could provide vital information for future research regarding the identification of lagundi cultivars that can yield a high quality of active compounds for medicinal purposes.

RECOMMENDATION

Elucidating the genetic diversity of *V. negundo* is vital in the determination of the Lagundi morphotype that will contain the most active phytochemical responsible to its therapeutic effect. Thus, an increase in the samples size from different regions in the Philippines will give a better a better picture

of the overall genetic diversity of *V. negundo* L. More than one sample can be taken from the each population so that genetic diversity within the population can also be obtained and not just between populations. Samples from Mindanao should also be gathered for better representation. Moreover, other molecular marker analysis can also be employed together with morphological and ecological characterization to further elucidate the genetic diversity in *V. negundo* L.

ACKNOWLEDGMENT

The authors would like to thank the Biology Department of the Ateneo de Manila University and Prof. Julienne Therese T. Medina of the Plant Biology Division of the Institute of Biological Sciences, University of the Philippines Los Baños for the providing the photo of the Lagundi plant.

STATEMENTS OF AUTHORSHIP

The senior author conceptualized the framework of this paper. The experimental part of the thesis was done by the second and third authors under the supervision of the senior author. All of the authors have contributed in the writing, but the final content, especially, the analysis and discussion of the results were done primarily by the first author.

REFERENCES

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and P Walter. 2008. Molecular Biology of the Cell. 5th ed. New York: Garland Science, Taylor & Francis Group, LLC.

Anand R, Sundaramorthi C, Saritha S and K Bhuvaneswari. 2008. Antibacterial Activity of Three Medicinal Plants: *Eucalyptus globulus*, *Aristolochia platas* and Vitex negundo Against Enteric Pathogens. The Icfai University Journal of Biotechnology, 2 (4): 77-81.

Ballard J and E Harvey. 2001. Protocol for the inter-simple sequence repeat (ISSR) approach. 8 February 2001. Protocols for some Useful PCR-based Molecular Systematic Approaches. 10 March 2010. http://oak.cats.ohiou.edu/~ballardh/molsyst/issrs.doc

Basri F, Sharma HP, Firdaus S, Jain P and A Ranjan. 2014. A Review of Ethnomedicinal Plant – *Vitex negundo* Linn. International Journal of Advanced Research (2) 3: 882-894.

Behera TK, Singh AK and J Staub. 2007. Comparative analysis of genetic diversity in Indian bitter gourd (*Momordica charantia* L.) using RAPD and ISSR markers for developing crop improvement strategies. Scientia Horticulturae, 115: 209-217.

Bornet B and M Branchard. 2001. Nonanchored Inter Simple Sequence Repeat (ISSR) Markers: Reproducible and Specific Tools for Genome Fingerprinting. Plant Molecular Biology Reporter (19): 209-215.

Boyer R. 2006. Concepts in Biochemistry 3rd ed. New Jersey: John Wiley & Sons, Inc.

Bramley G, Forest F and R de Kok. 2009 Troublesome tropical mints: re-examining generic limits of *Vitex* and other relations (Lamiaceae) in South East Asia. Taxon, 58 (2): 500-510.

Cantino PD, RM Harley and SJ Wagstaff. 1992. Genera of Labiatae: status and classification. In: Harley RM & T Reynolds (Eds.) Advances in Labiate Science. 511–522.

Curran JL. 1997. Human Linkage Mapping. In: Genome Mapping A Practical Approach. pp. 1-22. New York: Oxford University Press.

Dayrit FM. 1989. Phytochemical Studies on the Leaves of *Vitex negundo* L. (Lagundi). Philippines: Council for Health Research and Development. "Lagundi" Technical Report Series No. 6: p 67.

deKok R. 2008. The Genus *Vitex (Labiatae)* in the *Flora Malesiana* region, excluding New Guinea. Kew Bulletin, 63: 17-40.

Dharmasiri, MG, Jayakody JR, Galhena G, Liyange SS and WD Ratnasooriya. 2003. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitexnegundo*. J Ethnopharmacology 87 (2-3): 199-206.

Exeter Software (n.d.) NTSYSpc, Numerical Taxonomy System. Retrieved February 5, 2010, from http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html

Fang DQ, Rose ML and CT Federici. 1997. Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs, and inter-simple sequence repeat markers. Theoretical and applied genetics (95): 211-219.

Galvez Tan J. 2010. Herbals and Food Supplements. Online posting. 2010 Mar 1. Docstoc. 2010 Mar 8. http://www.docstoc.com/docs/27118391/Lagundi.

Gonzalez A, Wong A, Delgado-Salinas A, Papa R and P Gepts. 2005. Assessment of Inter Simple Sequence Repeat Markers to Differentiate Sympatric Wild and Domesticated Populations of Common Bean. Crop Science (45): 606-615.

Guo HB, Huang KY, Zhou TS, Wu QH, Zhang YJ and ZS Liang. 2009. DNA isolation, optimization of ISSR-PCR system and primers screening of *Scutellaria baicalensis*. Journal of Medicinal Plants Research, 3 (11): 898-901.

Gutierrez H. 1980. An Illustrated Manual of Philippine MateriaMedica, vol. I. Taguig: National Research Council of the Philippines, pp. 112-113.

Hu Y, Zhang QY, Xin HL, Qin LP, Lu BR, Rahman K and HC Zheng. 2007. Association between chemical and genetic variation of *Vitex rotundifolia* populations from different locations in China: its implication for quality control of medicinal plants. Biomedical Chromatography, 21 (9): 967-975.

Jarne P and PJ Lagoda. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution , 424-429.

Khokra SL, Prakash O, Jain S, Aneja KR and Y.Dhingra. 2008. Essential Oil Composition and Antibacterial Studies of *Vitex negundo* Linn. Extracts. Indian Journal of Pharmaceutical Sciences, 70 (4): 522-526.

Kulkarni RR, Virkar AD and P D'Mello. 2008. Antioxidant and Antiinflammatory Activity of *Vitex negundo*. Indian Journal of Pharmaceutical Sciences, 70 (6): 838-840.

Lagurin L. 1999. Isolation, Identification, and Quantification of the Major Compound in the Polar Extract of the Leaves of *Vitex negundo* Linn. [thesis]. Quezon City: Ateneo de Manila University. p 6.

Lindqvist C, Scheen AC, Yoo MJ, Grey P, Oppenheimer D, Leebens-Mack J, Soltis D, Soltis P and V Albert. 2006. An expressed sequence tag (EST) library from developing fruits of an Hawaiian endemic mint (Stenogynerugosa, Lamiaceae): characterization and microsatellite markers. BMC Plant Biology 6:16.

Lynch M and Milligan BG. 1994. Analysis of population genetic structure with RAPD markers. Molecular Ecology (3): 91-99.

Mader E, Lukas B and Novak J. 2008.A Strategy to Setup Codominant Microsatellite Analysis for High-Resolution-Melting-Curve-Analysis (HRM). BMC Genetics (9): 69.

Maramba NC, Dans LF, De Leon D, Ramos SP, Del Rosario WA and HF Aquino. 1989. Clinical Trial of *Vitex negundo* Tablet as Antitussive. Philippines: Philippine Council for Health Research and Development. "Lagundi" Technical Report Series No. 6: p 25.

Nagaoka T and Y Ogihara. 1997.Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theoretical and applied genetics (94): 597-602.

Novak J, Lukas B, Bolzer K, Grausgruber-Gröger S and J Degenhardt. 2008. Identification and characterization of simple sequence repeat markers from a glandular *Origanum vulgare* expressed sequence tag. Molecular Ecology Resources, 8: 599-601.

Quisumbing E. 1978. Medicinal Plants of the Philippines. Quezon City: Katha Publishing Co., Inc., pp. 806-808.

Rodriguez RL and RC Tail. 1983. Recombinant DNA Techniques: An Introduction. Massachusetts: Addison-Wesley Publishing Company.

Su H, Qu L-J, He K, Zhang Z, Wang J, Chen Z and H Gu. 2003. The Great Wall of China: a physical barrier to gene flow? Heredity, 2003 (90): 212-219.

Saiki R, Gyllensten U and H Erlich. 1990.The polymerase chain reaction. In: Davis KE, editor. Genome analysis: a practical approach. Oxford: IRL Press. p 141-152.

Surzycki S. 2000. Basic techniques in molecular biology. Berlin-Heildelberg: Springer-Verlag.

Sylianco CV. 1989. Mutagenicity, Clastogenicity and Antimutagenicity of Expressions, Decoctions, Tablet and Syrup Preparations from "Lagundi" (*Vitex negundo* L.). Philippines: Philippine Council for Health Research and Development. "Lagundi" Technical Report Series No. 6: p 14.

Tsumura Y, Ohba K and SH Strauss. 1996. Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsugamenziesii*) and sugi (*Cryptomeria japonica*). Theoretical and applied genetics (92): 40-45.

Watson J and F Crick. 1953. A Structure for Deoxyribose Nucleic Acid. Nature , 171 (4356): 737-738, 964-967.

Wells RA. 1990. DNA fingerprinting. In: Davis KE, editor. Genome analysis: a practical approach. Oxford: IRL Press. p 153-170.

Zhang ZY, Zheng XM and S Ge. 2007. Population genetic structure of *Vitex negundo* (Verbenaceae) in Three-Gorge Area of the Yangtze River: The riverine barrier to seed dispersal in plants. Biochemical Systematics and and Ecology, 35 (8): 506-516.

Zietkiewicz E, Rafalski A and D Labuda. 1994.Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification. Genomics (20): 176-183).