ISOLATION OF TETRANUCLEOTIDE LOCI USING 5'-ANCHORED PCR IN PINEAPPLES (*Ananas comosus* var. *comosus*)

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DECLARATION

I declare that this dissertation is the result of my own independent work and original writing, otherwise stated.

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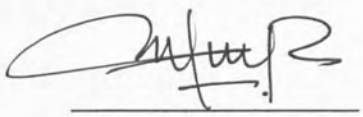
   (DR. VIJAY KUMAR) 

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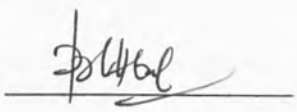
   (DR ROZIAH HAJI KAMBUL) 

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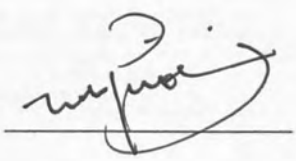
   (MISS TEOH PEIK LIN) 

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ABSTRACT

This project aims to shed some light on the molecular information of the *Ananas comosus* var. *comosus* species, which is a commercial variety of pineapples. This is achieved by isolating tetranucleotide microsatellites, a type of molecular marker which is very useful for a wide range of molecular studies. Therefore, the research objectives were to extract DNA from the pineapple, isolate the microsatellites using 5'-anchored PCR technique with a degenerate primer, PCT6 5'-KKBNVSS$(GATA)_6$-3', submit the isolated microsatellite sequences to the GenBank and obtain Accession numbers for the sequences. The results were five recombinant clones, four successful DNA sequencing reactions, namely PCT 7, PCT 8, PCT 33 and PCT 39, and a total of seven pure tetranucleotide microsatellites isolated from the clones, namely $(GATA)_n$, $(CTAT)_n$, and $(TATC)_n$. Also isolated were one mononucleotide repeat, namely $(A)_{13}$ and ten cryptic simple regions found in between the 5' and 3' terminal end. The GenBank Accession numbers assigned were AY929614 for locus PCT617, AY929615 for locus PCT618, AY939828 for locus PCT6133 and AY939829 for locus PCT6139 respectively. In conclusion, 5'-anchored PCR technique is highly efficient protocol which saves time and cost, as microsatellites can be isolated without having to perform library enrichment and screening protocols.

ABSTRAK

Projek ini bertujuan memberi maklumat molekular tentang nenas spesies *Ananas comosus* var. *comosus*, sejenis spesies nenas yang komersial. Maklumat didapati melalui pemencilan mikrosatelit tetranukleotida, sejenis penanda molekul yang berguna di dalam bidang molekular genetik. Objektif kajian adalah pengekstrakan DNA daripada nenas, PCR menggunakan teknik '5'-anchored' dan primer degenerat PCT6 5'-KKBNVSS$(GATA)_6$-3', mendeposit jujukan mikrosatelit yang diperoleh ke GenBank, dan mendapatkan Nombor Aksesi untuk jujukan-jujukan tersebut. Hasil kajian ialah lima klon rekombinan dan empat daripadanya berjaya dijujukkan, iaitu PCT 7, PCT 8, PCT 33 dan PCT 39. Daripada klon-klon tersebut, tujuh mikrosatelit tetranukleotida berjaya dipencilkan, iaitu $(GATA)_n$, $(CTAT)_n$ dan $(TATC)_n$. Selain daripada itu, satu mikrosatelit mononukleotida $(A)_{13}$ serta sepuluh 'cryptic simple regions' juga telah dipencilkan. Nombor Aksesi GenBank telah diberikan kepada klon-klon tersebut, iaitu AY929614 bagi lokus PCT 617, AY929615 untuk lokus PCT 618, AY939828 kepada lokus PCT 6133 dan yang terakhir ialah AY929829 bagi lokus PCT 6139. Sebagai kesimpulan, PCR menggunakan teknik '5'-anchored' merupakan protokol yang sangat efisyen dalam menjimatkan masa dan kos, kerana mikrosatelit boleh dipencilkan tanpa perlu menjalankan protokol 'library enrichment' dan penyaringan.

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LIST OF SYMBOLS, UNITS, AND ABBREVIATIONS

mg	milligram
µg	microgram
ng	nanogram
ml	milliliter
ds	double-stranded
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
A	adenine
T	thymine
G	guanine
C	cytosine
bp	base pair
°C	degrees Celsius
M	molar
mM	millimolar
U	units
’	prime
var.	variety
1X	one-time
rpm	revolutions per minute
λ	lambda
β	beta

CHAPTER 1

INTRODUCTION

The pineapple is the leading edible member of the family *Bromeliaceae*. There is about 2,000 species in this family. It is botanically known as *Ananas comosus* var *comosus*. The fruit has different names according to local dialect; *pina* by Spanish-speaking people, *abacaxi* by the Portuguese, *ananas* by the Dutch and French and the people of former French and Dutch colonies; *nanas* in southern Asia and the East Indies. In China, it is *po-lo-mah;* sometimes in Jamaica, sweet pine; in Guatemala often merely p*ine* (Morton, 1987).

In year 2003, approximately 80 countries around the world harvest a total of 32 million pounds of pineapples each year, more than double the average produced during the 1970s. Many of these producing countries have little presence in the world market as most of their production is intended for domestic consumption. Nearly three-quarters of world supplies are produced in Thailand, the Philippines, Brazil, China, India, Costa Rica, Nigeria, Kenya, Mexico, and Indonesia. Among these top ten producers, Costa Rica, Indonesia, the Philippines, Thailand, and Kenya has a significant proportion of their production geared towards international markets. Their combined exports of fresh, canned, and juice pineapple products comprise far more than half of world export supplies. (Economic Research Service, USDA, 2003)

Microsatellites are simple sequence repeats (SSRs) of one to six nucleotides. They appear to be present everywhere in higher organisms, although the frequency of microsatellites varies between species. Because of their abundance and inherent potential for variation, these simple sequence repeats have become a valuable source of genetic markers (Temnykh *et al.*, 2001). Other characteristics of microsatellites are highly polymorphic, evenly distributed across the genome, co-dominant, and easily genotyped. These features make them the marker of choice for a number of genetic studies (Yue *et al.*, 2003). SSRs have recently become important genetic markers in cereals, including wheat and barley (Holton, 2001).

Currently, very little research has been done on pineapples. There is an increasing need for more information on this species, since pineapples are a crop plant that is an important commodity for a lot of countries in the world, as highlighted above. Knowledge on its diversity is needed to develop new breeding lines. This project aims to provide more knowledge and understanding of this species, especially at its molecular level by using microsatellites as molecular markers to study the pineapples. However, microsatellite isolation using the conventional methodology is a difficult process. Therefore, this project utilized a much easier protocol to isolate microsatellites which is using 5'-anchored PCR. This is a more convenient method of isolating microsatellites compared to other protocols as it consistently anchors PCR primers at 5' ends of microsatellites, thus amplifying two close and inverted SSRs and the region between them.

The results obtained can be used to determine the polymorphism of this variety of pineapples, as well as investigation of its homology with other varieties or species. The data acquired can also be applied for further study in order to find ways to make this species disease-resistant, or to find ways to make its cultivation possible in cold climates or temperatures. This will hopefully boost its production and cultivation in countries that rely on pineapple export as its income.

There are several objectives of this research. The first was to successfully extract DNA from pineapples of the local varieties, such as Madu and Paun. The method of extraction that was used followed a rapid salting-out method, with some modifications made accordingly.

Second objective was to isolate DNA microsatellites from pineapples using a degenerate primer. The primer that was used is PCT6 with the sequence of 5'-KKBNVSS$(GATA)_6$-3', specially designed to be utilized with the 5'-anchored PCR method.

The third objective of this study was to obtain serial accession number for the microsatellite sequences that were submitted. In order to achieve this, the microsatellite sequences were sent to GenBank using its BankIt tool and the accession numbers were assigned by GenBank staff upon receiving the sequences. These sequences were subsequently tested for homology using BLAST program, more specifically blastn to determine its nucleotide homology.

CHAPTER 2

LITERATURE REVIEW

2.1 PINEAPPLES

The pineapple plant is a terrestrial herb of 0.75 to 1.5 meters high with a spread of 0.9 to 1.2 meter; has a very short, stout stem and a rosette of waxy, strap like leaves which are pointed, 50 to 180 centimeters 1ong; usually needle tipped and generally bearing sharp, upcurved spines on the margins. The leaves may be all green or variously striped with red, yellow or ivory down the middle or near the margins. At blooming time, the stem elongates and enlarges near the apex and puts forth a head of small purple or red flowers, each accompanied by a single red, yellowish or green bract. The stem continues to grow and acquires at its apex a compact tuft of stiff, short leaves called the "crown" or "top". Occasionally a plant may bear two or three heads, or as many as 12 fused together, instead of the normal one (Morton, 1987).

In international trade, there are numerous pineapple cultivars of *Ananas comosus* var. *comosus*. The pineapples are grouped in four main classes: *Smooth Cayenne, Red Spanish, Queen*, and *Abacaxi*, with a lot of variation in the types within

each class (Morton, 1987). However, there is a fifth group or class comprising of *Motilona* or *Perolera,* which is commercially very important in South America (Australian Gene Technology Act, 2003).

Smooth Cayenne, *Cayenne*, or *Cayena Lisa* in Spanish (often known in India, Sri Lanka, Malaysia and Thailand as *Sarawak* or *Kew*) was selected and cultivated by Indians in Venezuela long ago and introduced from Cayenne (French Guyana) in 1820. The plant is almost free from spines, except for the needle at the leaf tip. The size is 1.8 to 4.5 kilograms. The physiological characteristics are cylindrical form, shallow eyes, orange rind, yellow flesh, low fiber, juiciness and rich mildly acid flavor. It has gained the most importance worldwide even though it is subject to disease and does not ship well. Mainly, it is prized for canning, having sufficient fiber for firm slices and cubes as well as excellent flavor.

The next type of cultivar is called *Red Spanish.* Despite the spininess of the plant, it still is the most popular among growers in the West Indies, Venezuela and Mexico. It is only suitable for canning. The fruit is quite round, orange-red externally, with deep eyes, and ranges from 1.36 to 2.7 kilograms. The flesh is pale-yellow, fibrous, with a large core, aromatic and flavorful. The fruit is hard when mature, breaks off easily and cleanly at the base in harvesting, and stands handling and transport well. It is highly resistant to fruit rot though subject to gummosis (Morton, 1987).

Abacaxi (also called *White Abacaxi of Pernambuco*, *Pernambuco*, *Eleuthera*, and *English*) is well known in Brazil, the Bahamas and Florida. The plant is spiny and disease-resistant. Leaves are bluish-green with red-purple tinge in the bud. The numerous suckers need thinning out. The fruit weighed one to five kilograms, is tall and straight-sided; sunburns even when erect. It is very fragrant. The flesh is white or very pale yellowish, of rich, sweet flavor, succulent and juicy with only a narrow vestige of a core. This is rated by many as the most delicious pineapple. It is too tender for commercial handling, and the yield is low. The fruit can be harvested without a knife, and breaks off easily for marketing fresh (Morton, 1987).

Queen (also called Common Rough in Australia) is the leading cultivar in South Africa, Queensland and the Philippines. The plant is dwarf, compact, more cold-resistant and more disease-resistant then *Smooth Cayenne*. It matures its fruit early but suckers freely and needs thinning, and the yield is low. The fruit is conical, deep-yellow, with deep eyes; weighs 0.45 to 1.13 kilograms; is less fibrous than *Smooth Cayenne*, but more fragrant; it is juicy, of fine flavor with a small, tender core. It is sold fresh and keeps well. It is only fair for canning because of its shape which makes for much waste.

The pineapple is a tropical or near tropical plant limited (except in greenhouses) to low elevations between 30 degrees North and 25 degrees South. A temperature range of 18.33 to 45° C is most favorable. Prolonged cold retards growth, delays maturity and causes the fruit to be more acid. Altitude also has an important

effect on the flavor of the fruit. At higher elevations the fruit is too acid. The best soil for pineapple culture is a well-drained, sandy loam with a high content of organic matter and it should be friable for a depth of at least 60 centimeters, and pH should be within a range of 4.5 to 6.5. When unripe, the pineapple is not only inedible but also poisonous, irritating the throat and acting as a drastic purgative. Excessive consumption of pineapple cores has caused the formation of fiber balls (bezoars) in the digestive tract (Morton, 1987).

2.2 DNA-BASED MOLECULAR MARKERS

DNA markers are versatile tools used to study the comparative genetic differences or similarities both among and within species. This is because although significant DNA sequence information is available for several model species, very little, if any, DNA sequence information is available for most other species. Genomes are so large that most studies on DNA and amino acid sequencing can provide information on just a fraction of the genome. Thus, it is possible to use molecular markers selected systematically or at random to serve as samples that represent the entire genome. The degree of marker similarity will then represent the degree of genetic similarity (Fairbanks and Andersen, 1999).

The usefulness of molecular markers can be measured based on their polymorphic information content (PIC) (Botstein *et al.*, 1980). PIC refers to the value of a marker for detecting polymorphism in a population. PIC refers to the number of detectable alleles and the distribution of their frequencies, and equals one minus the

sum of the square of all allele frequencies. Thus, the greater the number of alleles, the greater the PIC; and for a given number of alleles, the more equal the allele frequencies, the greater the PIC. Therefore, comparison of PIC values can give researchers a rough idea of the power of the various marker types discussed (Cordes and Liu, 2004).

The ideal DNA markers will contain properties such as highly polymorphic, able to determine homozygous and heterozygous states of diploid organisms (co-dominant), frequent occurrence and dispersed evenly in the genome, selective neutral behavior of DNA sequences towards environment, easily available, fast assay, high reproducibility, and easy exchange of data between laboratories. It is difficult to find a molecular marker that fits all of the criteria. Depending on the type of study to be undertaken, a marker system that fulfills at least a few of the characteristics is to be identified before selecting the suitable marker for use according to the objective of a particular study (Gupta *et al.*, 2000). This means that there is no perfect or the best marker compared to the other markers, and the marker selection depends on the purpose and the desired findings of the experiment. However, it is to be noted here that microsatellites fulfill almost all of the criteria above and is suitable for use for a wide range of applications as will be described later on.

2.2.1 TYPE I DNA MARKERS

There are several types of DNA markers. These markers are classified into two categories, type I and type II. Type I are markers associated with genes of known function. They are becoming increasingly important in studies of genetic linkage and

quantitative trait loci (QTL) mapping. The advantages of type I are in studies of comparative genomics, genome evolution, candidate gene identification, and enhanced communication among laboratories. Type I markers also serve as a better channel for interspecific comparison and transfer of genomic information from a map-rich species into a relatively map-poor species, as compared to type II markers. This is because sequence conservation within genes are high and can be used as anchor-points for genomic segments to be compared among species. For comparative studies using type II markers (e.g. microsatellites), they can only depend on conservation of the flanking sequences used for the design of PCR primers (O'Brien, 1991).

The first type of marker that falls under this category is allozymes as the proteins they encode has known function. Allozymes require prior molecular information before it can be used, investigates single locus with two to six allele numbers, has low levels of polymorphism, and its mode of inheritance is Mendelian and co-dominant. Its major applications are linkage mapping and population studies. A second type of type I marker is expressed sequence tags (ESTs) as they represent transcripts of genes. EST also requires prior molecular information. EST has the same mode of inheritance as allozymes, single locus investigation with two allele numbers, and also has low investigation.

It has a wider range of applications than allozymes, such as linkage mapping, physical mapping, and comparative mapping. Most RFLP markers are type I markers as they were identified during analysis of known genes. RFLP is one of the first types of DNA-based molecular marker. To utilize RFLP, prior molecular information is

required. RFLP has the same mode of inheritance and identifies the same type of locus as other type I markers. It has low polymorphism, and is used in linkage mapping.

2.2.2 TYPE II DNA MARKERS

Type II markers are referred to as anonymous genomic segments. These markers are considered to be non-coding and therefore selectively neutral. The usage of these markers in population genetic studies utilizes assumptions by Hardy-Weinberg equilibrium and selective neutrality of the markers used. They are also useful in aquaculture genetics for species, strain and hybrid identification, breeding studies, and recently, as markers linked to quantitative trait loci (Cordes and Liu, 2004).

There are several types of markers in this category, such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), both of which do not require prior molecular information. However, AFLP detects a higher level of polymorphism than RAPD. The former is mostly used in linkage mapping and population studies, while the latter is utilized in fingerprinting for population studies and hybrid identification.

Most microsatellites are type II markers unless they are associated with genes of known function. Microsatellites are also known as simple sequence repeats (SSR) and using microsatellites would require prior molecular information. Its mode of inheritance are Mendelian and co-dominant, it investigates single locus with multiple alleles, and has high levels of polymorphism. It is used mostly in linkage mapping,

REFERENCES

Archie, E.A., Moss, C.J., and Alberts, S.C., 2003. Characterization of tetranucleotide microsatellite loci in the African Savannah elephant (*Loxodonta africana africana*). *Molecular Ecology Notes.* **3**, 244-246.

Ashworth, V.E.T.M., Kobayashi, M.C., De La Cruz, M., and Clegg, M.T., 2004. Microsatellite markers in avocado (*Persea americana* Mill.) development of dinucleotide and trinucleotide markers. http://www.science-direct.com/science/html

Bae, Y., Kim, H., Myoah, P., Lee, J., Hwang, D., Hwang, Y., Ahn, C., and Kang, S., 2003. Characterization of microsatellite markers to diagnose ADPKD. *Molecular and Cellular Probes*. **18**, 155-159.

Ball, A.O., Leonard, S., and Chapman, R.W., 1998. Characterization of $(GT)_n$ microsatellites from native shrimp (*Penaeus setiferus*). *Molecular Ecology.* **7**, 1251-1253.

Barker, J.H.A., Beismann, H., Karp, A., and Speck, T., 1997. Genetic variation of three *Salix* taxa measured with AFLP technique along an ecological gradient and the effect of their biomechanical properties on dispersal [AP6.3]. *SpringerLink: The Society for Experimental Biology Annual Meeting 1997.* http://194.94.42.12/licensed_materials/00898/free/meeting/cant97/ap6.html.

Baydar, N.G., Baydar, H., and Debener, T., 2004. Analysis of genetic relationships among *Rosa damascena* plants grown in Turkey by using AFLP and microsatellite markers. http://www.sciencedirect.com/science.html.

Bhassu, S., Yusoff, K., Panandam, J.M., Embong, W.K., Oyyan, S., and Tan, S.G. 2003. The genetic structure of *Oreochromis spp.*(Tilapia) populations in Malaysia revealed by microsatellite DNA analysis. *Biochemical Genetics* **42**. 217-229.

Birnboim, H, 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods in Enzymology* **100,** 243 – 255.

Bonhomme, F., Garcia de Leon, F.J., Canonne, M., Quillet, E., and Chatain, B., 1997. The application of microsatellite markers to breeding programmes in the sea bass, *Dicentrarchus labrax. Elsevier Science B.V.* **159**, 303-316.

Botstein, D., White, R.L., Skolnick, M., and Davis, R.W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Human Genetics* **32**, 314-331.

Brown, S.M., Hopkins, M.S., Mitchell S.E., Senior, M.L., Wang, T.Y., Duncan, R.R., Gonzalez-Candelas, F., and Kresovich, S. 1996. Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum [*Sorghum bicolour* (L.) Moench]. *Theoretical and Applied Genetics* **93**, 190-198.

Bryan, G.J., Collins, A.J., Stephenson, P., Orry, A., Smith, J.B., and Gale, M.D., 1997. Isolation and characterization of microsatellites from hexaploid bread wheat. *Theoretical and Applied Genetics* **94**, 557-563.

Caldeira, R.L., Vidigal, T.HDA, Simpson, A.J.G., and Carvalho, O.S., 2001. Genetic variability in Brazilian populations of *Biomphalaria straminea* complex detected by SSS-anchored PCR amplification. *Mem Ins Oswaldo Cruz* **96,** 535-544.

Chambers, G.K. and MacAvoy, E.S., 2000. Microsatellites: consensus and controversy. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **126**, 455-476.

Cho, Y.G., Ishii, T., Temnykh, S., Chen, X., Lipovich, L., McCouch, S.R., Park, W.D., Ayres, N., and Cartinhour, S., 2000. Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **100**, 713-722.

Cordes, J.F., and Liu, Z.J., 2004. DNA marker technologies and their applications in aquaculture genetics. *Elsevier B.V.* **238**, 1-37.

Dale, J.W. and Von Schantz, M., 2002. *From Genes to Genomes: Concepts and Applications of DNA Technology*. John Wiley and Sons Ltd.

Dallas, J.F. and Piertney, S.B., 1998. Microsatellite primers for the Eurasian otter. *Molecular Ecology* **7**, 1248-1251.

Eckert, K.A., and Yan, G., 2000. Mutational analyses of dinucleotide and tetranucleotide microsatellites in *Escherichia coli*: influence of sequence on expansion mutagenesis. *Nucleic Acids Research* **28**, 2831-2838.

Economic Research Service, USDA. 2003. *Fruit and Tree Nuts Outlook*. **FTS-307**, 12-17.

Ellegren, H., 2000. Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics* **16**, 552-558.

Epplen, C., Over, I., Lubjuhn, T., Epplen, J.T., and Sauer, K.P., 1998. Genetic distinction of scorpionflies (*Panorpa vulgaris*) by microsatellites. *Molecular Ecology* **7**, 1256-1258.

Fairbanks, D.J., and Andersen, W.R., 1999. *Genetics; The Continuity of Life.* Brooks / Cole Publishing Company, USA.

Fisher, P.J., Gardner, R.C., and Richardson, T.E., 1996. Single locus microsatelites isolated using 5' anchored PCR. *Nucleic Acids Research* **24**, 4369-4371.

Gupta, V.S., Joshi, S.P., and Ranjekar, P.K., 2000. *Molecular markers in plant genome analysis.* http://www.ias.ac.in/currsci/jul25/articles15.html.

Holton, T.A., 2001. Plant Genotyping by Analysis of Microsatellites. In: *Plant Genotyping: the DNA Fingerprinting of Plants.* CAB International, Australia, 15-27.

Hughes, C.R., Melland, R.R., and Beissinger, S.R., 1998. Polymorphic trinucleotide microsatellite loci for a neotropical parrot, the green-rumped parrotlet, *Forpus Passerinus*. *Molecular Ecology* 7, 1247-1248.

Invitrogen, 2003. *TOPO TA Cloning® Version Q Instruction Manual.* USA.

Javornik, B., and Jakše, J., 2001. High thoroughput isolation of microsatellites in Hop (*Humulus lupulus* L.). *Plant Molecular Biology Reporter* **19**. 217-226.

Jones, E., Dupal, M., Dumsday, J., Hughes, L., and Forster, J., 2002. An SSR-based genetic linkage map for perennial ryegrass (*Lolium perenne* L.). *Theoretical and Applied Genetics* **105**, 577-584.

Koblizkova, A., Dolezel, J. and Macas, J., 1998. Subtraction with 3' modified oligonucleotides eliminates amplification artefacts in DNA libraries enriched for microsatellites. *BioTechniques* **25**, 32–38.

Kumar, S.V., Tan, S.G., Quah, S.C., and Yusoff, K., 2002. Isolation and characterization of seven tetranucleotide microsatellite loci in mungbean, *Vigna radiata. Molecular Ecology Notes* **2**, 293-295.

Lagercrantz, U., Ellegren,H., and Andersson, L., 1993. The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research* **21**, 1111-1115.

Maguire, T.L., 2001. Producing and Exploiting Rich Microsatellite Libraries. In: *Plant Genotyping: the DNA Fingerprinting of Plants*. CAB International, Australia, 193-209.

Martin, J.P., Montaner, C., Casanova, J., Marti, C., Badia, D., Cabello, F., and Ortiz, J.M., 2004. Application of microsatellite markers for the characterization of 'Parraleta': an autochthonous Spanish grapevine cultivar. http://www.sciencedirect.com/science.html.

McCouch, S.R., Chen, X., Panaud, O., Temnykh, S., Xu, Y., Cho, Y.G., Huang, N., Ishii, T., and Blair, M., 1997. Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Molecular Biology* **35**, 89-99.

Miller, S.A, Dykes, D.D., and Polesky, H.F., 1988. Salting out procedure for DNA extraction. *Nucleic Acids Research* **1**, 1215.

Morton, J. 1987. Pineapple. 18–28. In: *Fruits of warm climates*. http://www.hort.purdue.edu/newcrop/morton/pineapple.html.

Njiokou, F., Bellec, C., Berrebi, P., Delay, B., and Jarne, P., 1993. Do self fertilization and genetic drift promote a very low genetic variability in the allotetraploid *Bulinus truncates* (Gastropoda: Planorbidae) populations? *Genetic Research* **62**, 89-100.

Nolan, D.V., Martin, S.A.M., Kelly, Y., Glennon, K., Palmer. R., Smith, T., McCormack, G.P., and Powell, R., 2000. Development of microsatellite PCR typing methodology for the sea louse, *Lepeophtheirus salmonis* (Krøyer). *Aquaculture Research* **31**, 815.

O'Brien, S.J., 1991. Molecular genome mapping: lessons and prospects. *Current Opinion in Genetic Development* **1**, 105-111.

Office of the Australian Gene Technology Regulator, 2003. *The Biology & Ecology of Pineapple (Ananas comosus var. comosus) in Australia.*

Paniego, N., Echaide, M., Muňoz, M., Fernández, L., Torales, S., Faccio, P., Fuxan, I., Carrera, M., Zandomeni, R., Suárez, E.Y., and Hopp, H.E., 2001. Microsatellite isolation and characterization in sunflower (*Helianthus annuus* L.), *Genome* **45**, 34-43.

Parker, P.G., Snow, A.A., Schug, M.D., Booton, G.C., and Fuerst, P.A., 1998. What molecules can tell us about populations: choosing and using a molecular marker. *Ecology* **79**, 361-382.

Paternity Experts, LCC, 2004. *STR Analysis.* http://www.paternityexperts.com/str.html.

Piertney, S.B., Marquiss, M., and Summers, R., 1998. Characterization of tetranucleotide microsatellite markers in the Scottish crossbill (*Loxia scotica). Molecular Ecology* **7**, 1261-1263.

Pongsomboon, S., Whan, V., Moore, S.S., and Tassanakajon, A., 2000. Characterization of tri- and tetranucleotide microsatellites in the Black Tiger prawn (*Penaeus monodon*). *Science Asia* **26**, 1-8.

Powell,W., Machray, G.C., and Provan, J., 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* **1**, 215-222.

Ratnaparkhe, A.B., Tekeoglu, M., and Muehlbauer, F.J., 1998. Inter-simple-sequence-repeat (ISSR) polymorphisms are useful for finding markers associated with disease resistance gene clusters. *Theoretical and Applied Genetics* **97**, 515-519.

Raymond, M.M., Butler, J.M., David, V.A., and O'Brien, S.J., 2000. *The Meowplex: A New DNA Test using Tetranucleotide STR Markers for the Domestic Cat.* (pdf file)

Scott, K.D., 2001. Microsatellites Derived from ESTs, and their Comparison with those Derived by Other Methods. In: Henry, R.J., *Plant Genotyping: the DNA Fingerprinting of Plants*. CAB International, Australia, 225-237.

Seidman, L.A., and Moore, C.J., 2000. *Basic Laboratory Methods for Biotechnology; Textbook and Laboratory Reference.* Prentice Hall Inc., USA.

Soranzo, N., Provan, J., and Powell, W., 1998. Characterization of microsatellite loci in *Pinus sylvestris* L. *Molecular Ecology* 7, 1260-1261.

Steinkellner, H., Lexer, C., Turetschek, E., Glössl, J., Streiff, R., Kremer, A., Fluch, S., and Burg, K., 1997. The use of microsatellites to assess genetic variation in forest trees [AP6.5]. *SpringerLink: The Society for Experimental Biology Annual Meeting 1997.* http://194.94.42.12/licensed_materials/00898/free/meeting/cant97/ap6.html

Stewart, W.A., Piertney, S.B., and Dallas, J.F., 1998. Isolation and characterization of highly polymorphic microsatellites in the water vole, *Arvicola terresris*. *Molecular Ecology* 7, 1258-1259.

Sun, X., and Liang, L., 2003. A genetic linkage map of common carp (*Cyprinus Carpio* L.) and mapping of a locus associated with cold tolerance. *Elsevier B.V.* **238**, 165-172.

Temnykh, S., DeClerck, G., Lukashova, A., Lipovich, L., Cartinhour, S., and McCouch, S., 2001. Computational and Experimental Analysis of Microsatellites in Rice (*Oryza sativa* L.): Frequency, Length Variation, Transposon Associations, and Genetic Marker Potential. *Genome Research* **11**, 1441-1452.

Winnepenninckx, B., and Backeljau, T., 1998. Isolation and characterization of microsatellite markers in the periwinkle *Littorina striata* King & Broderip, (Mollusca, Gastropoda, Prosobranchia). *Molecular Ecology* **7**, 1253-1254.

Wu, K.S., Jones, R., Danneberger, L., and Scolnik, P.A., 1994. Detection of microsatellite polymorphisms without cloning. *Nucleic Acids Research* **22**, 3257-3258.

Young, N.D., 2000. Constructing a plant genetic linkage map with DNA markers.. In: Phillips, R.L. and Vasil, J.K. DNA-Based Markers in Plants. *Kluwer Academic Publishers* 31-47.

Yue, G.H., Ho, M.Y., Orban, L., and Komen, J., 2003. Microsatellites within genes and ESTs of common carp and their applicability in silver srucian carp. *Elsevier* **234**, 85-98.

Zhu, Y., Strassman, J.E., and Queller, D.C., 2000. Insertions, substitutions, and the origin of microsatellites. *Genetic Research Cambridge* **76**, 227-236.

Zietkiewicz, E., Rafalski, A. and Labuda, D., 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **20**, 176–183.