2.0 LITERATURE REVIEW

2.1 Introduction

Among the most popular and widely available aquarium plants are the Cryptocorynes. There are more than 60 species and only half of the species are suitable for aquarium. In nature, Cryptocorynes are found in different habitat including shallow rivers, fast-flowing rivers, marsh areas, bogs and swampy area. Some of the Cryptocorynes are either amphibious bog plants or true aquatic plants, but all can adapt to fully submerge in aquarium condition (Peter, 2005).

C. x*willisii* is one of the most popular aquarium plants. In the aquarium, the plant can grow up to 20-30 cm in height. The species was considered as ideal for display in the background area of an aquarium. It will spread beautifully in the aquarium when the condition is optimum.

The substrate for planting it in an aquarium is the plain washed gravel with moderate to bright lighting at pH value is 6.8-7.2. The water hardness required is 3-8ºdH and water temperature of about 20-26ºC**.**

2.2 Distribution

Cryptocoryne belongs to the Family Araceae. The genus *Cryptocoryne* is known as the water trumpet and native plants to tropical Asia. The distribution spreads from India (in the west) to Philippines (in the east) and to Malaysia, Indonesia and Papua New Guinea (in the south) (Othman *et al*, 2009).

According to Rataj (1977), 12 species of Cryptocoryne were found in Sri Lanka but only 9 species were recorded by Bastmeijer (1997) which were *C. bogneri, C. becketii, C. nevillii, C. parva, C. thwaitesii, C. undulate, C. walkeri, C. wendtii* and *C.* x*willisii.*

Reported by Rataj (1977) there are 15 species from the genus were endemic to Peninsular Malaysia but Jacobsen (1985) found only 8 species in Peninsular Malaysia and 11 species in Sarawak. The species found in Peninsular Malaysia were *C. affinis, C. elliptica, C.minima, C. cordata, C. schulzei, C. nurii, C. griffithii* and *C. purpurea* and the species found in Sarawak were *C. bullosa, C.keei, C. striolata, C. auriculata, C. zonata, C. grabowskii, C. longicauda, C. cordata, C.minima, C.lingua* and *C.ferruginea.*

In this study *C.* x*willisii* Reitz was chosen due to availability of the plants for further study. The synonyms for the species are *C. nevillii* and *C. lucens* De Wit (Othman *et al*, 2009), *C. pseudo-beckettii* (Wit, 1964), *C. axelrodii* Rataj and C. *undulata* Wendt (Rataj, 1977).

2.3 Morphological Description *C. xwillisii*

Plate 2.3.1. *Cryptocoryne* x*willisii*

The morphology descriptions of *C.* x*willisii* were as follows: the lengths of the leaves from petiole to the blades were 20-30 cm, the blades were smooth on both side, shapes of the blades were ovate with 2.0-2.7 cm broad and 6.8-12.0 cm long.

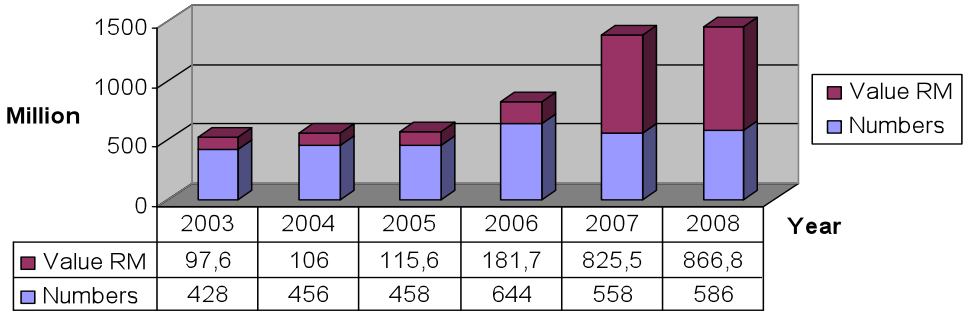
The upper surface of the leaves were shinning dark green and lower surface of the leaves were lighter green and the venation were noticeable with brown colour. The shoots acuminate from the base. The rhizomes are creeping, thick and normally developing runners.

2.4 Economic Importance Of *C. xwillisii*

There is a tremendous increase in demand for ornamental fish and aquatic plants from 2003 to 2008 as shown in Figure 2.4.1 where Malaysia exports 428 million (2003) to 586

million number (2008) numbers of ornamental fish and aquatic plants. Value of the export increased from RM 97.6 million in 2003 to RM 866.8 million (2008) (Department Fisheries Statistic 2003-2008).

High demands of the aquatic plants were mainly from the developed countries such as Japan, France, German, and Australia and also from developing countries like Singapore, Indonesia, Hong Kong, China and Brunei.



	2003	2004	2005	2006	2007	2008
Value RM	97,6	106	115,6	181,7	825,5	866,8
Numbers	428	456	458	644	558	586

Figure 2.4.1. Estimated production and export value in million Ringgit Malaysia of ornamental fish and aquatic plants from year 2003-2008

2.5 Conventional Propagation Methods Of Aquatic Plant

There is a large demand from the industry that requires mass production of aquatic plant within a short period of time. However through conventional method, the plants take up to six months to grow from cutting before reaching marketable size. Other disadvantages of conventional methods were the requirement of large planting area, predators or pest attack and non-uniform sizes of the plant. Alternatively, micro-

propagation offers a solution to overcome the problem of time, supply and quality (Kane *et. al*, 1999).

Conventional methods for propagating Cryptocoryne are planting the plantlets in wet soil or by hydroponic system. The plantlets were vegetative propagated from either tubers or runners and due to sterility; the plants do not produce any seeds. The numbers of propagules are small and not commercially viable for large-scale industry. The common pests for these plants are white fly and nematodes. White fly is the vector for plant diseases and nematodes will cause the plants to wilt.

2.6 Tissue Culture Of Aquatic Plants

Tissue culture is a method of propagation of plants by placement of small portion of tissue or plant parts in artificial environment in aseptic condition. The plant tissues were culture in nutrient medium for production of shoots and roots.

There are many advantages in adopting tissue culture technique for aquatic plants. Tissue culture can create a large number of clones from a single seed or explant. The time required is shortened and life cycle of seed development can be avoided. Rapid production is possible for species that have long generation time, low levels of seed production, or seeds that do not readily germinate. This technique can also overcome the seasonal restrictions for seed germination. It is also useful to multiply plants that produce less seeds, sterile plants and do not produce seeds or when the seeds cannot be stored.

It also enables the preservation of pollen and cell collections form of the propagated plants. The plants can also be selected for the desirable traits directly from the culture and therefore it will decrease the extra space required for field trials.

Tissue culture also helps to eliminate plant diseases through careful stock selection and sterile techniques. And it also allows for the international exchange of aseptic plant materials, thereby eliminating the need for quarantine.

Tissue culture has been developed for other aquatic plants such as *Anubias barteri* (Huang, 1994 and Wannada, 2005), *Aponogeton madagascariensis* (Nathakorn, 2005), *Cryptocoryne lucens* (Kane *et al*, 1990), *Cryptocoryne wenthii* (Kane *et al*, 1999), American Lotus *Nelumbo lutea* (Kane *et al*, 1988), *Nymphae* (Lakshmanan, 1994) and *Nymphoides indica* (Jenks *et al*, 2000). However, reports are limited since the micropropagation of these plants are developed for commercial purposes this remains as trade secret. Currently, there are no published reports for micropropagation of *C.* x*willisii.*

2.7 Mutation

In genetics, mutation is a process of changing the DNA sequence within gene or chromosome of an organism and the changing create a new character or trait that is not found in the parental type. Mutation can be defined as heritable changes in DNA sequence that is not derived from genetic segregation or recombination (Van Harten, 1998).

Mutations take place in the genes, it occur when one base is substituted for another in the sequence of bases that determine the genetic code, or when one or more bases are inserted or deleted from a gene (Hasting *et al*, 2009).

Mutations may occur spontaneous or induced by various physical and chemical agents (mutagenic agents). Examples of chemical mutagens are acridine orange, proflavin, ethidium bromide, ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS), diethylsulfate (DES), and nitrosoguanidine (NTG). The chemicals that have been found

effective as mutagens are the alkylating agents (Van Harten, 1998). Some chemical mutagens resemble the bases found in normal DNA (base analog) (Freese, 1959), some alter the structure of existing bases (base-altering mutagen), insert the molecule in the helix between bases (intercalating agents) and other creating reactive compounds that damage the DNA structure (free radicals compounds) as Bertram J, 2000, Aminetzach *et al*, 2005 and Burrus and Waldor, 2004.

Mutations also occur by physical mutagen that is the radiation. Radiation is the process in which energetic particles or waves travel through a medium or space. There are two types of radiation, the ionizing and the non-ionizing radiation (Van Harten, 1998).

Non-ionizing radiation refer to the sufficient energy produce by the electromagnetic radiation that during passing through matter. The energy managed to change the rotational, vibration or electronic valence configurations of molecules and atoms. Non-ionizing radiation includes neutron radiation, electromagnetic radiation, visible light, infrared, microwave, radio waves, very low frequency (VLF), extremely low frequency (ELF), and thermal radiation (US EPA).

Ionizing radiations include X-rays, neutrons, cosmic rays, and radiation from radioactive materials, such as alpha particles, beta particles, and gamma rays. Ionizing radiation produce charged water molecules when passes through cellular tissue. These molecule break up into free radical that highly reactive and can alter molecules in the cell including the DNA molecules (US EPA).

Induction of mutations by using ionizing radiation started since beginning of the 20^{th} century and proven to be useful in plant breeding after 30 years. Radiation mutation breeding and isotope technique and combined with tissue culture, introduced new technique for induce genetic variation, improving selecting technology and shorten

breeding time (Novak and Brunner 1992). The combination of mutagenesis and *in vitro* culture (also called *in vitro* mutagenesis) has been found to make the induction and the selection of induced somatic mutations more effective (Jain and Swennen, 2004). Induced mutations have contributed significantly to plant improvement and more than 1393 mutant varieties have been developed through mutation breeding (Basiran, 1998).

Hence the combined use of mutation induction and *in vitro* technology is more efficient, because it speeds up the production of mutants as a result of an increased propagation rate and a greater number of generations per unit time and space (Morpurgo *et al*. 1997).

All these new methods can be used for plant breeding. Beside from the traditional plant breeding, new techniques are needed for further improving crop cultivars. Mutagenesis is the best way to obtain new variety in a short period of time. The U.S. Patent Classification System defines the mutant plant as the plant wherein a change in the structure of the genetic material has occurred and mutations result in heritable alterations in the genotype (Predieri, 2001). Types of mutation are point mutation, substitution, deletion, insertion and frame shift of the gene in the DNA.

2.7.1 Gamma Irradiation

Gamma irradiation is the main physical mutagen used to induce genetic variation. Novák *et al,* (1986), Espino *et al,* (1986) and Epp (1987) described the dose response of tissue-cultured shoot tips to gamma irradiation. Roux (1997) standardised the methodology to provide guidelines to mutation induction programmes in *Musa* spp.

Many examples related to different vegetative propagated species show that the mutation induction can be empowered by *in vitro* techniques. Studies on induced *in vitro* mutagenesis were done on aquatic plants *Anubias congensis* (Pakorn Tangpong *et al*, 2009) and also on crops to improve the varieties such as sugar cane (Patade *et al*, 2008), Basmati rice (*Oryza sativa* L.) (Saleem *et al,* 2005), banana (Mohan, 2004) and groundnut (Adu Dapaah, 2004).

Mutagen agent used in the study is gamma ray from radioactive cobalt (^{60}Co). Gamma ray is electromagnetic ray with higher penetration due to short wave length. Gamma ray can cause both type of mutation, gene mutation and chromosomes mutation. Gamma irradiation causes chromosomal rearrangements that may be related to multiple trait alterations (Fehr, 1993). There are three type of treatment (Basiran *et al*, 1998):

1) **Acute irradiation**: the radiation is delivered at a high rate in single exposure
2) **Chronic irradiation**: the radiation is given at a low rate during a prolong period of time
3) **Fractionated dosage**: irradiation is given in several interrupted sequences. Fricke's solution is usually used to measure absorbed doses of gamma irradiation.

2.8 Molecular Markers

Molecular marker techniques are applied for the classification and genetic analysis of cultivars and related wild species (Novak and Brunner 1992). A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. Molecular markers may or may not correlate with phenotypic expression or trait. Advantages of molecular markers are they are stable and detectable in all tissue regardless of growth, differentiation and development (Agarwal *et al*, 2008).

Basic molecular marker techniques can be classified into two categories: (1) non-PCR based techniques and (2) PCR-based techniques. Non-PCR based technique is the Restriction fragment length polymorphism (RFLP) technique. The PCR-based techniques are further subdivided into two subcategories: (1) arbitrarily primed PCR-based techniques or sequence non-specific techniques and (2) sequence targeted PCR-based techniques.

The techniques under the arbitrarily primed PCR-based techniques are Random amplification of polymorphic DNA (RAPD) and Amplified fragment length polymorphism (AFLP). The techniques under sequence targeted PCR-based techniques are the microsatellite-based marker technique and single nucleotide polymorphism technique (SNPs).

Advance molecular marker techniques are the combination of several advantages of basic techniques and incorporated with modification in the methodology to gain better result of the genetic material. The advance techniques are chloroplast microsatellites, mitochondrial microsatellites, sequence characterized amplified regions (SCAR), cleaved amplified polymorphism sequences (CAPS), randomly amplified microsatellite

polymorphisms (RAMP), sequence-related amplified polymorphism (SRAP), target region amplification polymorphism (TRAP), single strand conformation polymorphism (SSCP), transposable elements-based molecular marker, retrotransposon-based molecular marker including (1) Inter-retrotransposon amplified polymorphism (IRAP) and REtrotransposon-microsatellite amplified polymorphism (REMAP), (2) sequence-specific amplification polymorphism (S-SAP), (3) Retrotransposon-based insertion polymorphism (RBIP).

IRAP markers were used to analyzing genome-wide discontinuities among closely related individuals (Hafez el al 2006). IRAP analysis was firstly described by Kalendar *et al* (1999). IRAP is based on the PCR amplification of genomic DNA fragments, which lie between two-retrotransposon insertion sites. Polymorphism is detected by the presence or absence of the PCR product. Lack of amplification indicates the absence of the retrotransposon at the particular locus. The technique was originally developed using the BARE-1 retrotransposon, which is present in the barley genome in numerous copies. About thirty bands were visualized by a single PCR reaction (Kalendar *et al,* 1999). IRAP markers were extremely polymorphic, which made them useful for evaluating intraspecific relationships, investigating linkage, evolution, determination of varieties and mutants and genetics diversity in plants (Dariusz, 2006).

The IRAP method was carried out using LTR primers derived from barley, *Hordeum vulgare* (Kalendar *et al*, 1999). The primer sequences of retrotransposons have varying degrees of phylogenetic resolution, allowing choice of marker system to match the task. IRAP and retrotrasposon-microsatellite amplified polymorphism (REMAP) (Kalendar *et al*, 1999) detect high levels of polymorphism without the need of DNA digestion, ligation or probe hybridization to generate marker data thus increasing the reliability and robustness of the assay.

Primers derived from retrotransposon have been used to study biodiversity and phylogeny in the genus *Brassica* (Tatout *et al,* 1999, Karine, 2004), apple (Kristiina, 2006), *Hordeum* (Kalendar *et al,*1999), *Spartina* (Baumel *et al,* 2002), *Pisum* (Pearce *et al*, 2000) and Banana (*Musa* sp.) (Teo *et al,* 2002, Teo *et al,* 2005).

This is the first attempt to produce molecular data bank for the selected endemic and exotic (Sri Lanka) *Cryptocoryne* species using Inter-Retrotransposon