METHODS OF SEX DETERMINATION IN DIOECIOUS ANGIOSPERMOUS PLANTS

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I. INTRODUCTION

Angiosperms are the most diverse group of land plants which provides fruits, food, seed for cultivation and material for hybridization purpose etc. Approximately 90% of all angiosperm species have hermaphrodite (bisexual) flowers and the remaining, one-tenth are either monoecious (male and female flowers are separate but grow in same plant) or dioecious (male and female flower grow on different plant). During evolutionary time the unisexual flowers have arisen from the bisexual ancestral through mutation either causing male sterility or causing female sterility resulting gynodioecious (having female flower on one plant and hermaphrodite flowers on the other plant of the same species) and androdioecious (having male flower on one plant and hermaphrodite flowers on the other plant of the same species) plants. But female-sterile mutation is rare in plants because female function is more valuable in nature (Charlesworth and Charlesworth, 1978). The comparative study on the evolution of dioecious plants support that they has evolved from an ancestral gynodioecious conditions, although there are few reports in which monoecy seems to be ancestral to dioecy.

Dioecy possess a barrier to self-fertilization, therefore plays a very important role in evolution. The exchange of genetic material between two different individuals leads to new combinations of genes which enable a new plant to easily adapt or survive to a changing environment (Milewicz & Sawicki, 2012). Plants which are fertilized with foreign pollen produce more abundant and healthier offspring than the individuals which are fertilized with own pollen (Malinowski, 1978). This correlation is clearly visible in many dioecious plants e.g. in *Cannabis sativa*, dioecy contributes to greater yield and produce offspring with more valuable fibre, therefore supports that crossbreeding leads to the production of better offspring (Mandolino *et al*., 1999).

II. PROLEMS WITH DIOECY

A dioecious plant species is different from other plant species by their reproductive habit, reproductive structures and their trait. These trait could be morphological and /or physiological differences having a genetic base (Dellaporta and Calderon- Urrea, 1993). In comparison to female plants very fewer male plants are required as pollen donor. For example, in sea buckthorn the ratio of male to female in cultivation should be 1:9 (Persson and Nybom, 1998). In *Dioscorea* spp., mostly grown for their edible tubers, females produce more tuber yield than from males (Akoroda *et al*., 1984). But in minority of dioecious plants, the male are agronomically superior to the female e.g. long pepper (*Piper longum*, cultivated in India for its medicinal properties; Banerjee *et al*., 1999), poplar (*Populus* spp., cultivated for ornamental or reforestation purpose near habitation; Tschaplinski and Taskan, 1994) and garden asparagus (*Asparagus officinalis*, cultivated for roots; Benson, 1982). It has also been reported that male of perennial plants have either larger growth rate or more biomass than female plants (*Acer negundo*; Jing and Coley, 1990) in

comparison to *Populus* female which produce more biomass in natural habitat (Khosla *et al.*, 1979).

So dioecious plants which are cultivated for fruit or seed, biomass and hybridization purposes, it is often difficult to identify females at early stage of growth (Irish and Nelson, 1999) e.g. kiwifruit (*Actinidia deliciosa*), hop (*Humulus lupulus*), date palm (*Phoenix dactylifera*), papaya (*Carica papaya*), pistachio (*Pistacia dactifera*), sea buckthorn (*Hippophae rhamnoides*) and cloudberry (*Rubus chamaemorus*) etc. because maleness and femaleness can only be determined at the oneset of flowering which takes about 5-10 years (depends upon plant species). Sex is the major problem in these plants and mechanisms of determination are not clearly understood (Westergard, 1958; Karlin and Lessard, 1987).

III. TECHNIQUES FOR GENDER DETERMINATION

Due to different ecological and economic importance of dioecious plants, it is necessary to identify the gender at juvenile stage (Irish and Nelson, 1989). Different techniques has been developed by workers for the identification of maleness or femaleness. These are based on chromosome identification and molecular markers. The markers used for gender determination purpose can be divided into morphological markers and genetic markers or molecular markers.

A. Morphological identification

Morphology deals with the study of forms and features of different plant organs like stem, leaf, flower, height etc. that can be studied visually. Depending upon the number of genes that control the morphological characters of any organism (plant/animal), they can be divided into quantitative and qualitative traits. Both characters are controlled by the mutual effect of genes of plant and the environment in which plant grow. For the determination of inter and intra specific variability, different classical strategies (comparative morphology, anatomy, physiology, cytology and embryology) have been used in genetic analysis during early period of research. The morphological characters have been successfully and traditionally used to check the diversities and characterization of various plant species population e.g. hybrids of poplar (*Populus deltoids*, *Populus trichocarpa*; Ridge *et al*., 1986), *H. rhamnoides* (Li *et al*., 2006), Persian walnut (*Juglan regia*; Arzani *et al*., 2008) and wild rose species (Riaz *et al.*, 2011) etc. But all these traits, used for the identification purposes are time consuming and easily affected by the changed environment. This method can only be used at maturity and not applicable at juvenile stage.

B. Chromosomal identification

The cell is the basic structural, functional and biological unit of all living organism. Each cell contain genetic material in the form of DNA (Deoxyribose nucleic acids) in the nucleus. According to double helix model (Watson and Crick, 1950), DNA is a nucleic acid consist of two nucleotide strands coiled around each other to form a double helix, contain hereditary information in the form of codons or bases which transferred from parent to offspring during gametogenesis.

Chromosomes (coloured body) are observed first time by Karl Negi (1842) in the form of rod like structure in plants. In the nucleus, DNA molecule is packaged into thread-like structures called chromatin (Walter Fleming, 1878). DNA is then again condensed, tightly coiled many times around histones proteins and called chromosomes. During cell division chromosomes becomes more tightly packed and become visible under the microscope after staining with specific dyes (acetocarmine and feulgen).

Somatic cells of diploid organisms contain two set of chromosomes (2n) whereas gametic cells are always haploids (half number of chromosome, n). In diploid cells, autosomes always appear in pairs and their number remain same whereas number of allosome pair differ from one another and thereby determine sex. For example, in human diploid number of chromosomes are 46 out of which 22 pairs are autosomes and one pair is allosome. The allosome pair consists of two X chromosomes in females (homogametic) or one X and one Y

chromosome in males (heterogametic) which represent the sex of any organism.

Like animals, plants also contain diploid set of chromosomes e.g. *Arabidopsis thaliana* (2n= 10), Rye (2n= 14), maize (2n=20). The number of chromosomes is constant for a particular species, therefore are of great importance is determination of phylogeny and taxonomy of the species. But in plants variation is found in the number of chromosomes i.e. they could be triploids (3n), tetraploids (4n), hexaploids (6n) e.g. Durum wheat which is a tetraploids (4n) contain 28 number of chromosomes, cultivated tobacco which is tetraploid contain 48 number of chromosome etc. In animals variation in chromosomes number creates abnormalities but in plants these variation are helpful and increase the quality of trait e.g. size, flowers, seeds and productivity etc.

In dioecious organisms, the presence of allosomes (X/Y chromosome) used for the identification of gender. For this purpose, dividing cell of plant part (root tip, pollen grains mostly used) is taken, arrested at the metaphase stage of mitosis and meiosis by colchicine treatment, stained by specific dye and then observed under the microscope. Karyogram is prepared, chromosome numbers are counted and presence of X and Y chromosome is checked. Earlier, this method was mostly applied in animals but later it took popularity in plants.

Like animals, sex chromosomes (allosomes or X/Y) have been successfully identified in dioecious *Rumex*, *Cannabis*, *Humulus*, and *Silene* (Parker, 1990). Hermophrodic sex chromosomes (male is heterogametic and female is homogametic) are identified in *Rumex acetosa* and *Silene dioica* (Blackburn, 1923), *Melandrium album*, *M. rubrum* (Blackburn, 1923), *Rumex nivalis* (Stehlik and Barrett, 2005) etc. Whereas, heterogametic female has also been reported in Fragaria (Dellaporta and Calderon-Urrea, 1993), *Myristica fragrans* (Flach, 1966). In *P. trichocarpa*, female genotype is heterogametic and gender is determined by using ZW system (Rottenberg *et al*., 2000; Markussen *et al*., 2007; Yin *et al*., 2008; Gaudet *et al*., 2008; Pakull *et al*., 2009, 2011 and Paolucci *et al*., 2010) like birds. According to Pakull *et al*. (2011) both ZZ/ZW (female heterogamy) and XX/XY (male heterogamy) gender determining systems could be present in some members of *Populus* (Tuskan *et al*., 2012). In *Rumex acetosa* (Ainsworth 2000), *Phoenix dactylifera* (Siljak-Yakovlev *et al*., 1996) and the three members of family Cannabiadaceae *i.e. Humulus lupulus* (Shephard, 1999a), *H. japonicas* and *Cannabis sativa* (Jacobsen, 1957; Parker, 1990), sex is determined by X/autosome ratio and doesn't depends on Y chromosome. While in some plant species, such as *Rumex hestatulus* (Parker and Clark, 1991), sex is determined by both mechanisms: an active Y chromosome and the ratio of X chromosomes to autosomes.

But the presence of sex chromosome is somewhat problematic because in most of cases sex chromosomes are not much different from autosomes (*Spinacia oleracea*, *Asparagus officinalis*; Michalic, 2009) whereas, in *Actinidia deliciosa* var. *deliciosa* size of sex chromosome is too small (Shirkot *et al*., 2002), therefore difficult for identification. Whereas, some dioecious plants possess labile sex system which include androdioecy (separate male and hermaphrodite individuals), gynodioecy (separate female and hermaphrodite individual) and subdioecy (flowers not clearly male or female) (Milewicz & Sawicki 2012) like insects e.g. *M. annua*, *Populus lasiocarpa* etc. Sex reversible system is also observed by some authors. Plants such as *Carica papaya* (Storey, 1953), *Pistacia vera* (Hormaza, 1994), *Ecballium elaterium* (Ainsworth, 2000), *Asparagus officinalis* (Gao *et al*., 2007) sex is found to be controlled by single gene mechanism whereas, in *Mercurialis annua* (Louis, 1989) a multiple-loci system is responsible for sex distinction. So the identification of gender by chromosomes become difficult and different molecular techniques have been develop which later seems to be useful in sex identification in diecious plants.

C. Molecular Markers

In genetics, a molecular marker (identified as genetic marker) is a fragment of

DNA that is associated with a certain location within the genome. That functional unit of DNA is called gene which transcribed to form mRNA and then translate to produce specific proteins. So this fragment of DNA and the protein or metabolite produce by it, are studied as molecular markers. These include macromolecules and secondary metabolites (chemical substances/ constituents present in plants parts/organ /cells e.g. alkaloids, saponins, glycosides, tannins, flavonoids and steroids). Macromolecules are DNA and proteins (isozymes or allozymes). They are mostly used for species identification, genetic variation of intra and inter population, comparison between wild and hatchery populations and now used for gender identification.

Molecular methods provides valuable tool for good and easy identification of gender at any stage of growth and development. Various works has been done with the help of molecular markers for the sex determination in many dioecious plants. The Applications of Molecular Markers has been given in Genetics and Breeding of Hemp (Mandolino and Ranalli, 2002).

C.A. Isozymes (Biochemical) markers

Isozymes were first reported by Markert and Moller in 1959. These are enzymes that differ in amino acid sequence but catalyse the same chemical reaction whereas, allozymes represent enzymes from different alleles of the same gene. They are codominant, quick and reliable markers system for the identification of genotypes and cultivars and are suitable biochemical markers or finger printers for the estimation of all population genetics parameters. They can be stained by selective staining methods which results in a small number of specific bands. The general forms of protein data as isozymes and allozymes are generated by using gel electrophoresis. Enzyme electrophoresis was mostly used for the identification of clones, hybrids and cultivar but now playing useful role for the gender determination in dioecious plants since last few decades. In this technique, an objective biochemical trait is determined by a subsequent visualization or by separating the isozyme and/or allozymes of different electrical charges in a gel. That objective biochemical trait refers to the genotype of the plant.

Isozyme polymorphism is widespread in many plants and has been used for cultivar and sex identification in many horticultural plant and tree species (Sharma *et al*., 2010). Isozyme variation has also been used in genetic studies in many economically useful plantation species such as teak (*Tectona grandis*), Caribbean pine (*Pinus caribbea*) and rubber (*Hevea brassiliensis*) etc. Isozyme technique has been successfully used for clonal identification of poplars and genetic diversity of *Populus tremuloides*. Two enzyme systems, Peroxidase and esterase are successfully used in gender determination in *H. rrhamnoides* (Sharma *et al*., 2010), *P. ciliata* (Kumari *et al*., 2014). About 90 isozymes systems have been used for plants, with isozyme loci being mapped in several loci. It has been observed that Peroxidase induces auxin modulation of morphogenesis and has an indirect role in sex determining mechanisms in many monoceious and dioecious plants and makes an important contribution to sex expression (Sharma *et al*., 2010). But the use of isozyme system for gender determination is limited due to its some disadvantages (*i.e.* affected by environmental conditions, their expression varies from tissue to tissue, plant phenological stages and post transcriptional modification) but still they are used for gender determination.

C.B. DNA markers

DNA marker is a term used to refer to a specific DNA variation between individuals that has been found to be associated with a certain characteristic (e.g., increased tenderness). These different DNA or genetic variants are known as alleles. Among all the molecular markers, DNA markers are found to be more suitable and ubiquitous to most of living organisms. They are distributed all over the genome, can be detect at any stage of development and are not influenced by season and environment. Various types of molecular markers which have been used to evaluate DNA polymorphism are generally classified as hybridization based markers (RFLP) and

polymerase chain reaction based markers (RAPD, AFLP, VNTR, SSR, ISSR etc.)

RFLP (Restriction fragment length polymorphism) is a technique that identify the variations in DNA sequences which are homologous. In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting *restriction fragments* are separated according to their lengths by gel electrophoresis. It is mostly used in construction of genome mapping, identification of genetic disorders, determination of disease risk and in paternity testing. This technique has been used as marker for gender determination in only some species e.g. in *Asparagus officinalis* (Biffi *et al*., 1995) and *Rumex acetosa* (Ruiz *et al*,. 1994) due to its many limitations (requires a large amount of sample DNA, radioactive probes, DNA fragmentation, hybridization, and take up to a month to completion), therefore replaced by other PCR based techniques like RAPD, AFLP etc.

The PCR (Polymerase Chain Reaction) technique was invented during the mid-1980's and introduction of thermostable DNA polymerase has revolutionized many molecular biological techniques with modifications of the original procedure designed to suit a range of needs. One such variation generates a specific class of molecular markers termed as Random Amplified Polymorphic DNA (RAPD). RAPD (Random Amplified Polymorphic DNA) is a PCR based molecular technique, amplify a set of DNA fragment distributed randomly throughout the genome using single short oligonucleotide primers. It is most popular and commonly used, environment friendly technique (Milewicz and Sawicki, 2013), involves non-radioactive assay and simple experimental setup requiring only a thermal cycler (PCR) and an electrophoresis assembly. It does not require any species specific libraries and this method is quick, cheap can be visualized on agarose gel and relatively economical. It is a cheap, rapid technique require small amount of DNA and seems to be better technique than RFLP.

Since nineties, RAPD marker has been used for gender determination successfully in many economically and ecologically important dioecious plant species such as *Pistacia vera* (Hormaza *et al*., 1994), *Asparagus officinalis* (Jiang and Sink, 1997), *Distichlis spicata* (Eppley *et al*., 1998), *Piper longum* (Banerjee *et al*., 1999), *Actinidia deliciosa* var. *deliciosa* (Shirkot *et al*., 2002), *Encephalartos natalensis* (Prakash & Staden, 2006 ; Gao *et al*., 2007), *Trichosanthes dioica* (Kumar *et al*., 2008), *Salix perpurea* (Sulima *et al*., 2009), *Populus tremuloides* (Hou *et al*., 2009), *H. rhamnoides* L. (Sharma *et al*., 2010), and *M. dioica* (Baratakke *et al*., 2013) etc. But this method has also some disadvantages, like poor reproducibility.

AFLP (Amplified fragment length polymorphic DNA) is the combination of two techniques (RAPD and RFLP) and took importance in mid-1990. This technique used for gender determination in Asparagus *officinalis* (Spada *et al*., 1998), *Dioscoria tokoro* (Terauchi *et al*., 1999), *Poa arachifera* (Renganayaki *et al*., 2005) *Rumex nivalis* (Stchlik and Blattner, 2004) and *Vapaca kirkiana* (Mwase *et al*., 2007). The popularity of AFLP was decreased because of its high cost and time consuming analytical phase. Paran and Michelmore (1993) recommended that RAPD markers are converted to specific SCAR markers. RAPD-SCAR markers has been successfully used in *Asparagus officinalis (*Jiang and Sink, 1997; Gao *et al*., 2007), *Carica papaya (*Parasnis *et al*., 2000), *Piper longum* (Manoj *et al*., 2005), *Gingo biloba*, *M. dioica (*Baratakke *et al*., 2013) etc. or other economically important plant species.

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are repeating sequences of 2-5 base pairs of DNA. It is a type of Variable Number Tandem Repeat (VNTR). Microsatellites are typically co-dominant. It is a simple method with high reproducibility, overcome the limitations of RAPD and AFLP. Microsatellites have proved to be versatile molecular markers, particularly for population analysis, but they are not without limitations. Therefore, still it has been used in only few plants (approximately 17% analysed species) (Milewicz and Sawicki, 2013).

C.C. Secondary Metabolites

Recently secondary metabolites (phenols, alkaloids, terpenes, glycosides etc.) are also becoming the centre of research in gender determination purposes because all plants contain the secondary metabolites in their plant parts which are active against pest and diseases, have ability to interfere with the cellular signalling system, vital enzymes and can block the metabolic pathways (Winks, 1999). The analysis of secondary metabolites is restricted to those plants that produce a suitable range of metabolites. These metabolites can be used to see the variations between the members of a population or between two populations. According to Joshi *et al.* (1999) the metabolites used as markers should be ideally neutral to environment effects or management practices. Tuskan *et al.* (2012) analyse the metabolic profiles of male and female floral bud of *Populus* trees using a gas chromatography-mass Spectrometry and found gender-specific accumulations of phenolic glycosides. So they hypothesized that gender determination and resistance to and regulation of a floral pathogen are coevolved and both events triggered the emergence of a nascent sex chromosome. But the role of secondary metabolites in dioecious plants is not confirmed and work is still going on.

IV. CONCLUSION

A wide range of molecular markers linked to sex have been identified in many dioecious plants where sex chromosomes are either not identified (*Populus* spp., *Salix* spp.) or difficult to identify (*Actinidia* spp.). Molecular methods provides valuable tool for good and easy identification of gender at any stage of growth and development. Analysis of DNA can identify individual that differ genetically in most extreme cases by only a single base pair. The most modern molecular markers techniques like RAPD, AFLP, SSR and isozyme analysis are useful option for genotypic and cultivar identification but these have some limitations and only useful in few plants not all plants. Despite some efforts, there is a need to devise certain reliable and quick methods to select the required characters at juvenile stage in all dioecious plants with an aim to differentiate males and females.

V. REFFERENCES

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