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Full Length Research Paper

Determination of sex in *Zamia fischeri* Miq., an endangered gymnosperm

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Sex determination of *Zamia fischeri* Miq., one of the endangered cycads was done using peroxidase profiling, two-dimensional gel electrophoresis of proteins and RAPD-based approaches to find an easy and reliable molecular marker. Sex at pre-flowering stage could not be resolved by peroxidase isozymic profiling. Use of sex organ specific tissues as enzyme source resulted in marked differentiation in peroxidase profiles. Fifteen unique spots of relatively high molecular mass were noted in microspore derived protein (male) sample while twenty five unique spots ranging between 20 to 94 kD were identified in ovule derived protein (female) sample as resolved by two dimensional gel electrophoresis. DNA from leaf samples of contrasting sex resulted in four male and female specific RAPD derived DNA fragments (two each of both sexes) of which one male specific band showed homology with micro satellite sequence of *Araucaria angustifolia*; this can be used as a convenient molecular marker for sex determination of *Zamia fischeri* at pre-flowering stage.

Key words: Molecular marker, RAPD, Sex determination, *Zamia fischeri*.

INTRODUCTION

Zamia fischeri Miq. (Zamiaceae, Cycadales), one of the oldest seed plants surviving in tropical and sub tropical areas since the Mesozoic era is uncommon in the wild. Given the level of habitat destruction it is considered highly threatened (The Cycad Pages *Zamia fischeri*, http://plantnet.rbgsyd.nsw.gov.au/cgi-bin/cycadpg?taxname = Zamia + fischeri). A relatively recent assessment of this plant by Donaldson (2003) and subsequent evaluation by Vovides and Chemnick (Cycad Red List Authority) has placed this plant in the endangered red list category (http://www.iucnredlist.org/details/42134: The IUCN Red List of Threatened Species). The more alarming scenario is the status of its dwindling population and the population growth is towards the negative direction. Hence, a coordinated effort for its conservation either *in situ* or *ex situ* is urgently required.

An effort towards this direction has been taken by the present group and it was noticed that the extreme slow growth of this plant and propagation by natural asexual means are major hindrances for increasing its population. The inherent slow growth rate of the Cycads in general is the bottleneck for any successful micro propagation programme (Dehgan, 1983). Attempt towards artificial pollination leading to recovery of large number of viable seeds is, hence, the only way to date, for scaling up the already diminishing population of this threatened plant. To fulfill the aforesaid objective substantial number of donor plants of both sexes is a prerequisite since, like most of the Cycads, *Zamia* is a dioecious plant and identification of sex at pre flowering stage by any of the phenotypic means is not possible.

Sex is the queen of problems in evolutionary biology (Bell, 1982) and sexual reproduction in plants is an interactive process, rather a communication between the plant and a dynamic environment (Willemse, 2003). Consequently, determination of sex in plants at pre flowering stage is an active area of research in recent times. This is gaining momentum both by the classical method of karyotyping targeted towards sex chromosome identification followed by *in situ* hybridization and also by more straight forward PCR-based approaches of molecular

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Figure 1. Male (A) and female (B) cone-bearing dioecious *Zamia fischeri* Miq.: Insets showing microsporophyll containing microsporangia and mature ovule in respective cases (Bar represents 1 mm).

sex marker development. To date, sex-linked RAPD markers have been developed in *Pistacia vera* (Hormaza et al., 1994), *Salix viminalis* (Alstrom et al., 1998), *Carica papaya* (Lemos et al., 2002), *Encephalartos natalensis* (Prakash and Staden, 2006), *Simmondsia chinensis* (Agrawal et al., 2007; Hosseini et al., 2011) and *Cycas circinalis* (Gangopadhyay et al., 2007); and reliable SCAR markers have been successfully derived from RAPD fragments in *Pistacia vera* (Hormaza et al., 1994), *Asparagus* (Jiang and Sink, 1997), *Actinidia* (Gill et al., 1998), *Cannabis sativa* (Mandolino et al., 1999), *Eucommia ulmoides* (Xu et al., 2004), *Rumex nivalis* (Stehlik and Blantner, 2004) and *Ginkgo biloba* L., a dioecious gymnosperm species, which has survived for more than 200 million years (Ling et al., 2003, Liao et al., 2009).

The genus *Zamia* has been worked out cytologically using fluorescence *in situ* hybridization technique (Tagashira and Kondo, 2001) and with particular reference towards sex chromosome determination by karyotyping. But sex determination was possible in only one species (*Z. pumila*), while it could not be resolved in two other species (Sangduen et al., 2007). *Z. fischeri*, the present material under study, was investigated using isozymic, two-dimensional gel electrophoresis of proteins and RAPD-based approaches to find out easy and reliable markers for sex determination of this endangered dioecious gymnosperm. The questions addressed for attaining this objective being: Whether (1) tissue specificity (vegetative/reproductive) can be resolved through isozymic analysis; (2) sex organ (microspore / ovule) specific unique protein spot(s) can be looked though proteome analysis; and (3) expression (sex organ) independent DNA based polymorphic marker can be identified from vegetative tissue itself, which will provide the cue for identification of sex in pre flowering condition.

MATERIALS AND METHODS

Plant material

Male and female cone-bearing plants (six each) of *Zamia fischeri* Miq. procured from a local nursery and subsequently maintained at Falta Experimental Farm (South 24 Parganas, West Bengal, India) of Bose Institute were used as study material.

Isozymic analysis

Peroxidase (PRX, E.C.1.11.1.7) isozymic analysis was done using both vegetative (youngest leaf) and reproductive (isolated microspores and ovules from plants of respective sex) tissue samples (Figure 1). Extraction was done using 1 g of fresh samples in each case following the protocol of Wetter and Dyck (1983). Samples were homogenized at 4°C with 1.5 cm^3 of 0.2 M Tris-HCl

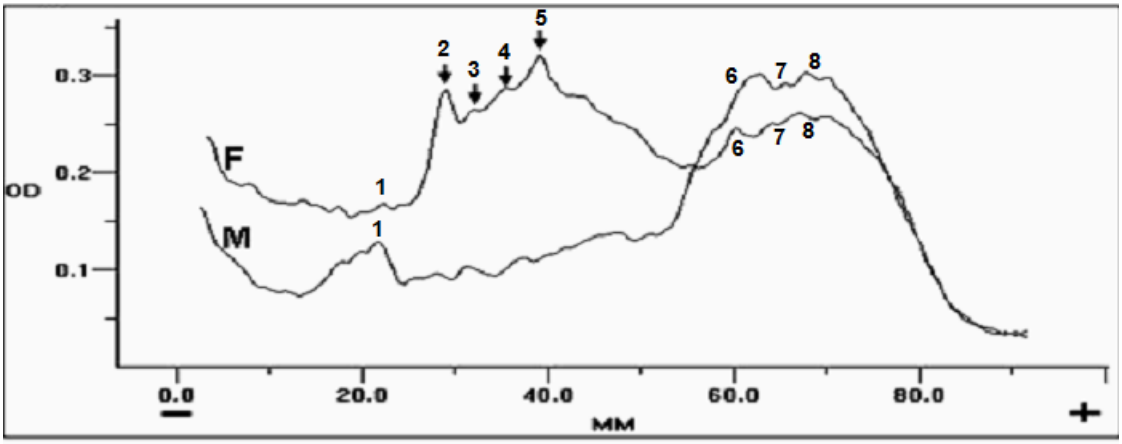

Figure 2. Densitometric scan of peroxidase isoenzyme of male (M) and female (F) reproductive structures of *Zamia fischeri* Miq. Appearance of unique bands has been indicated by arrow marks.

buffer (pH 8.5) containing 1 M sucrose and 0.056 M 2-mercaptoethanol. The macerate was centrifuged at 16000 g for 20 min at 4°C; the supernatant was used as source of crude enzyme. The protein content was estimated by the Folin-phenol method (Lowry et al., 1951). Equimolar amounts of protein (50 μg) samples of both the sexes were loaded in a native polyacrylamide gel. Electrophoretic runs were made for 3 to 4 h at 2 mA per lane at 4°C. The gel was developed by activity staining of peroxidase enzyme (Das and Mukherjee, 1997). Densitometric scan of the gels was done using BIO-RAD Gel Documentation System (Gel Doc 1000, version 1.5).

Two-dimensional gel electrophoresis of protein

Total protein was extracted from isolated microspores and ovules from plants of both the sexes using ReadyPrep™ Protein Extraction Kit of *BIO-RAD*. Isolated protein was subsequently quantified by *RC DC* protein Assay kit and purified by ReadyPrep™ 2-D Cleanup kit of *BIO-RAD*. The protein solutions (containing 70 μg protein) were loaded on dry IPG strips with re swelling buffer of pH gradient 3 to 10 NL (Immobiline dry strip, 13 cm, GE Healthcare). The strips were then re hydrated overnight, and isoelectric focusing was performed according to the following voltage gradient: 50 v for 4 h, 50 to 500 v for 30 min, 500 v for 2 h, 500 to 2000 for 1 h 30 min, 2000 v for 2 h, 2000 to 3500 for 1 h and finally 3500 v for 11 h for a total 38000 volt hours. Equilibration of gel strips was performed in an SDS equilibration buffer with DTT for 15 min and then with iodoacetamide for 15 min. Second dimension gel electrophoresis was performed on 10% PAGE followed by standard protocol of silver staining method for detection of protein spots. Gel images were digitized with a Bio-Rad Versa Doc.

RAPD analysis

Genomic DNA was isolated from vegetative (youngest leaf) tissue (100 mg each) of both male and female *Z. fischeri* plants of known sex (three each) using the QIAquick DNeasy plant minikit (Qiagen). DNA concentration in the samples was adjusted to 25 mg dm^{-3} for PCR reaction in each sample. RAPD analysis was performed according to the method of Williams et al., (1990) using ten oligonucleotide (decamer) primers, OPA 01–OPA 05 and OPB 01–OPB 05 (Operon Tech., Alameda, USA). Amplifications were carried out in a thermal cycler (MJ Research) with an initial denaturation of 120 s at 94°C and the temperature profile of each cycle was as follows: 60 s denaturation at 94°C, 60 s annealing at 35°C and 120 s for extension at 72°C. The reaction continued for 45 cycles followed by 300 s hold at 72°C to ensure that the primer extension was completed. The PCR reaction mixture of 0.025 cm^3 consisted of 1× PCR buffer (including $MgCl_2$), 200 μM of each dNTP, 0.4 μM of primer, 25 ng of genomic DNA and 1 unit of Taq DNA polymerase (Sibenzyme). The PCR products were resolved in 1.6% agarose gel (TAE, 7 V/cm) and detected by ethidium bromide staining. RAPD-derived unique male and female-specific bands of *Z. fischeri* were eluted using QIAquick Gel Extraction kit (Qiagen). The eluted DNA fragment was later re amplified using the respective primer followed by purification using QIAquick PCR Purification kit (Qiagen) and subsequently cloned by conventional TA cloning method in pGEM-T Easy vector (Promega). Cloned DNA fragments were sequenced from DNA Sequencing Facility at University of Delhi South Campus, New Delhi, India. The sequences were subsequently submitted to NCBI, GenBank database for onward processing to obtain accession numbers (GQ141708, GQ141709). Sequence homology was studied by BLAST searching (Altschul et al., 1990).

RESULTS

Peroxidase isozymic profiling

The sexual demarcation of *Z. fischeri* could not be obtained by peroxidase isozymic profiling when the vegetative (leaf) tissue was used as the enzyme source since both male and female plants showed identical profile in comparable developmental stage (result not shown). The use of sex organ specific tissue for enzyme source (microspores and ovules from male and female plants respectively), however, resulted in marked differentiation in peroxidase profiles. Of the total eight iso forms of peroxidase resolved in native gel, four (R_{mf} 0.28, 0.32, 0.35 and 0.63) were absolutely unique for ovules since those were totally absent in case of male plants (Figure 2).

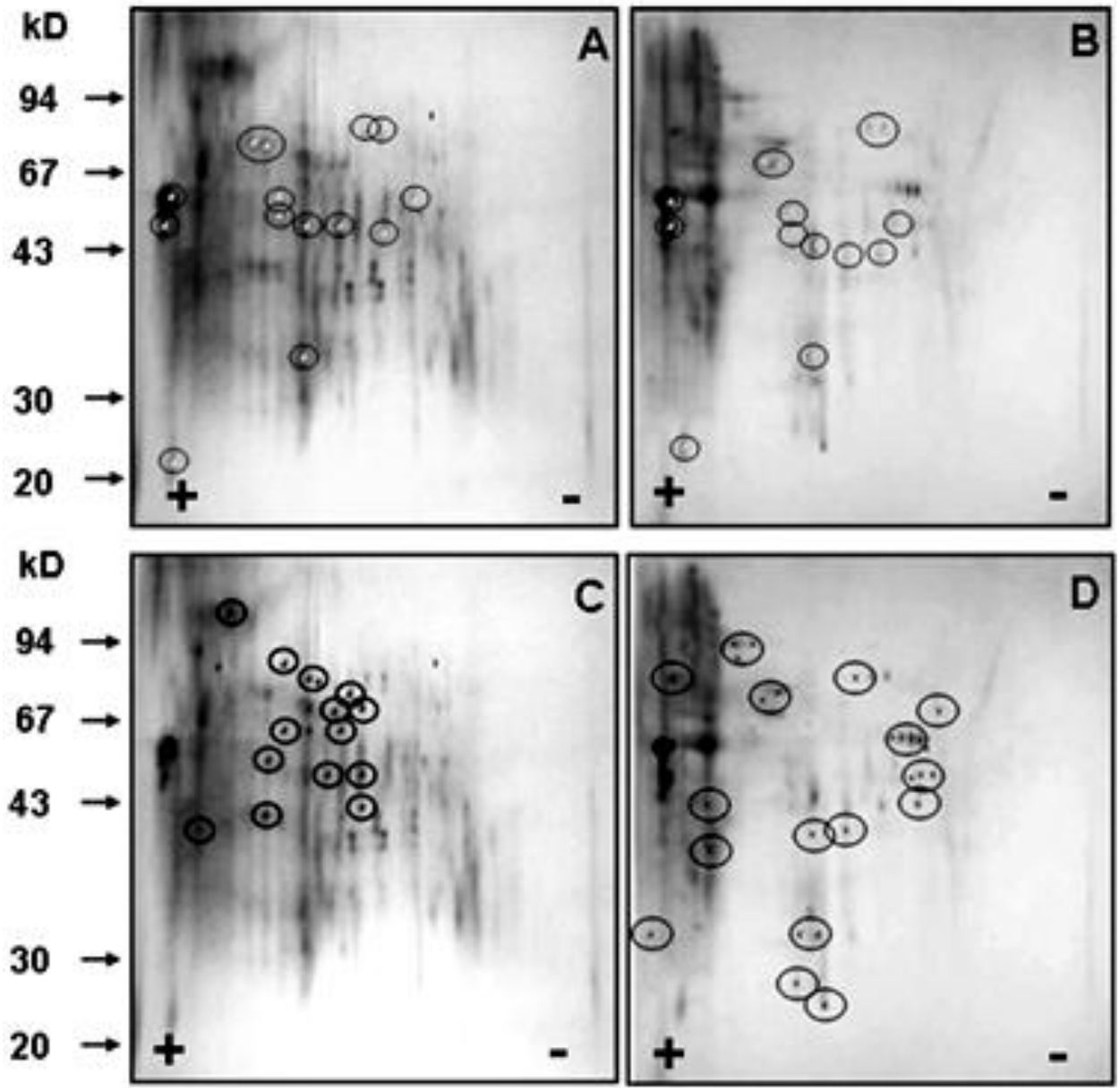

Figure 3. Two dimensional gel electrophoresis of total protein extracted from reproductive structures of male and female *Zamia fischeri* Miq. Common protein spots of male (A) and female (B) samples; unique protein spots of male (C) and female (D) samples have been differentially marked with software.

Two-dimensional gel electrophoresis of protein

Of the large number of protein spots resolved after two-dimensional gel electrophoresis of male and female *Z. fischeri* using microspore and ovules as the protein source, fourteen were identical in both the cases, which can conveniently be designated as the common protein spots (marked in Figures 3A, B). Fifteen unique spots of relatively high molecular mass was noted in microspore derived protein (male) sample (marked in Figure 3C) while twenty five unique spots ranging between 20 to 94 kD were identified in ovule derived protein (female) sample (marked in Figure 3D).

RAPD analysis

Of the ten RAPD primers tested, significant polymorphism between male and female *Z. fischeri* plants was recorded in case of OPB03 and OPB04. The RAPD profiles of both OPB03 and OPB04 primers showed one male and one female-specific DNA fragments in each of

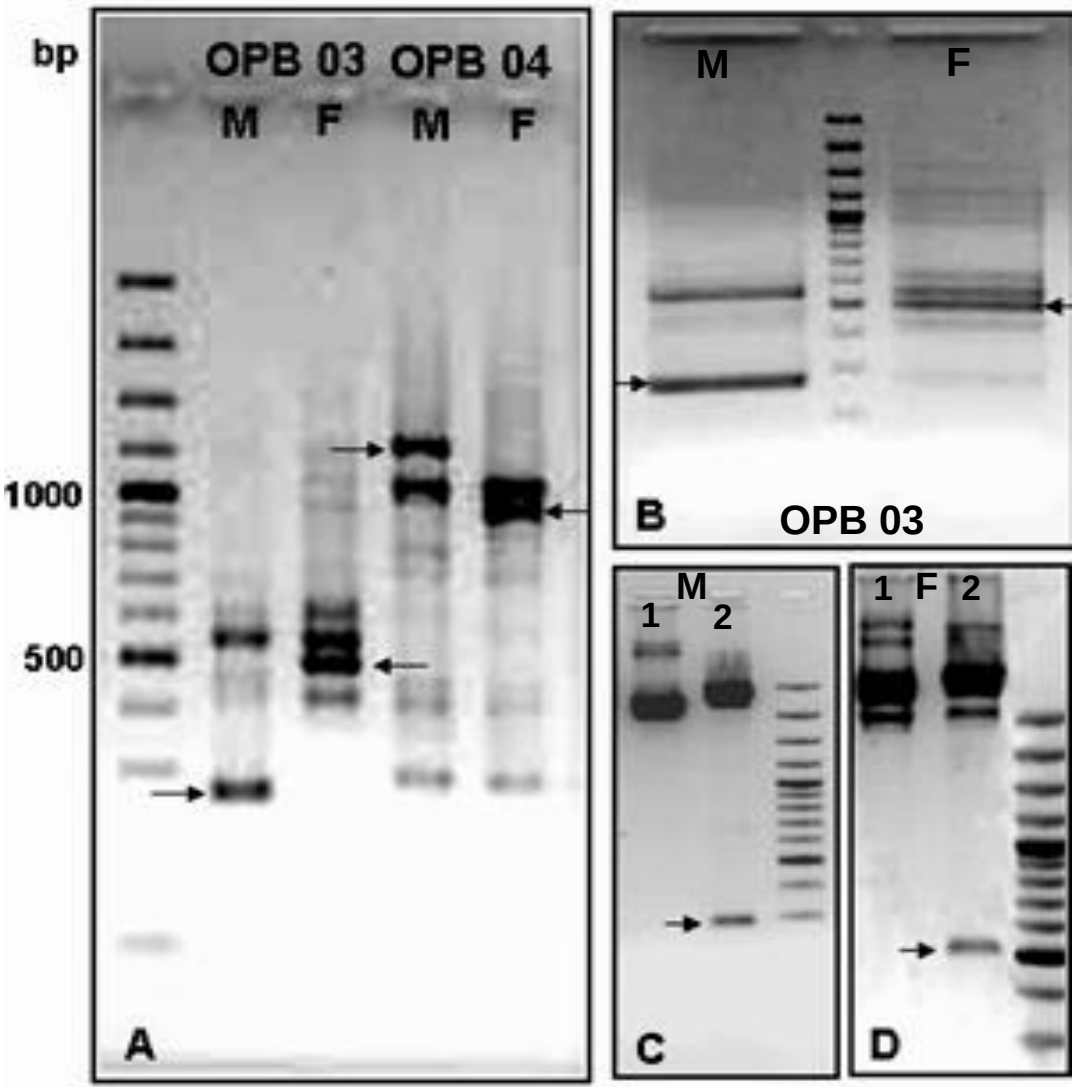

Figure 4. RAPD derived male (M) and female (F) specific bands of *Zamia fischeri* Miq. (A) Polymorphism in RAPD profiles using primers OPB 03 and OPB 04; (B) purified eluted bands of OPB 03 primer (M and F); (C,D) purified plasmids (Lane 1) from the clones (primer OPB 03) and their respective restriction enzyme (*Eco*R1) digested fragments (Lane 2) showing the inserts (all bands of interest are arrow marked)

the cases. The molecular mass of male and female-specific fragments in case of OPB03 were 294 and 534 kb (Figure 4A), while those were 1320 and 1015 kb respectively in case of OPB04 (Figure 4A). The specific DNA fragments of both male and female samples of OPB03 only were eluted out from gel (Figure 4B), cloned (Figures 4C AND 4D) and subsequently sequenced. Sequencing of these two cloned DNA fragments, followed by BLASTN searching, revealed homology with *Araucaria angustifolia* clone AAng27 microsatellite sequence (maximum identity 83%; GenBank accession no. AY865591) in case of male specific DNA fragment (GQ141708), while the female specific DNA fragment (GQ141709) did not result in any relevant homology with the available database.

DISCUSSION

Gymnosperms include the earliest living lineages with innovations of a greatly reduced male gametophyte (pollen), pollination as well as seeds with extremely large genome. Recent molecular phylogenetic studies indicate that the five major lineages of extant gymnosperms (cycads, *Ginkgo*, Gnetales, Pinaceae, and all other conifers) form a monophyletic group that is sister to angiosperms, although an alternate placement of Gnetales as sister to the angiosperms cannot be unambiguously rejected. Cycads represent the likely sister lineage to all other extant gymnosperms and are consistently included in comparative developmental and molecular systematic studies. As a basal lineage, cycads

provide exemplars to help ascertain the generalized gymnosperm reproductive features from which flowering plant morphology and genetic controls were likely modified. Of the cycads, *Zamia*, in particular, has been targeted for extensive sequencing and expression profiling (http://www.greenbac.org/tree.html: The green plant BAC library project).

While looking for easy-to-score peroxidase isozymic marker in *Zamia fischeri*, demarcation between male and female genotypes could not be attained in the present study using vegetative tissue as the starting material thus eliminating the credential of peroxidase isozyme as a candidate to determine sex at pre flowering stage. However, significant alterations in iso peroxidase profile were observed when reproductive tissue samples (isolated microspores and ovules from plants of respective sex) were used as the isozyme sources, thus indicating the involvement of this developmental stage regulated isozyme with sex expression, which warrants further basic investigation in this area. Peroxidase isozymes, due to its developmental stage specificity, have long been used in demarcation of sex in dioecious plants (Parthasarathi and Angadi, 1984; Sharma et al., 2010). Gymnosperms usually have higher levels of isozymic variability than angiosperms and this variation has been utilized in population study of the giant Sequoia (*Sequoiadendron giganteum*) (Fins and Libby, 1982). As a prelude to the reporting of the remarkable effort of conservation of endangered *Cupressus dupreziana* using a surrogate mother from related species *C. sempervirens* (Pichot et al., 2001), the same group noticed the lack of mother tree alleles in zymograms of two isozymes in *C. dupreziana* embryos (Pichot et al., 2000).

The advent of proteomic research has revolutionized the study of plant developmental biology in recent times. With an aim to characterize the unique proteins responsible for gymnosperm pollen biology, efforts have been made to evaluate the pollen drops (Wagner et al., 2007) and pollen tube development (Wu et al., 2008). Our endeavor to distinguish the microspore and ovule specific proteins by two-dimensional gel electrophoresis resulted in both identical as well as sex specific proteins. Further characterization of the specific proteins would probably give insight into the evolution of sexual dimorphism in *Zamia*.

Determination of sex at pre flowering stage though has been becoming an active area of research but the effort has been targeted mainly towards the angiosperms, with obvious ready to be implemented reason for planting and propagation of crop plants of commercial interest. The research on gymnosperm in this area is relatively few due to paucity of materials for study as a consequence of dwindling population status of most of the rare and endangered gymnosperms, which otherwise have great aesthetic presence in the wild. Reports in this direction are available in *Encephalartos natalensis* (Prakash and Staden, 2006), *Cycas circinalis* (Gangopadhyay et al., 2007), *Cycas tanqingii* (Jing et al., 2007), and *Ginkgo biloba* (Liao et al., 2009), the living fossil. The basic experimental approach of all these three was to look for sexual dimorphism using arbitrary primer (RAPD) exploiting the dioecious nature of the plants and to develop specific molecular marker, which probably has culminated in the work with *Ginkgo biloba*, where anonymous marker has been transformed successfully into SCAR marker (Liao et al., 2009).

Plants are essentially hermaphrodite but the gymnosperms, in particular, show complete dioecy in most of the cases, plausibly due to their early origin in the evolutionary time scale than angiosperms, which have appeared relatively recently. This is reflected beautifully in the RAPD profiles of contrasting sex within same genotype of gymnosperms and *Zamia fischeri*, the present study material, is no exception to that since distinct differences between male and female was observed in at least two primers out of the ten tested. Though four specific DNA fragments were obtained in the present study, two each of two contrasting sex but only two (one each from male and female) were successfully cloned for further analysis while the other two could not presently be worked out. Of the two DNA fragments, the homology of the male specific one (GQ141708) with micro satellite sequence of *Araucaria angustifolia* (Schmidt et al., 2007), another gymnosperm, evokes interest since micro satellites are the repeat regions of genome, which have become time-tested during the course of evolution. Repetitive elements, the satellite repeats and the dispersed elements showing differential representation pattern within the genome of *Zamia* species has been recently examined by Southern blots analysis (Cafasso et al., 2009).

Understanding of sex expression in plants, particularly in the absence of well defined sex chromosomes is a challenging area and in the quest towards this phenomenon the present group ventured with hermaphrodite *Carica* (Gangopadhyay et al., 2007), dioecious *Trichosanthes* (Roy et al., 2008) and *Cycas circinalis* (Gangopadhyay et al., 2007). The reason behind choosing a wide range of plant species ranging from gymnosperm to angiosperm is to understand the biology and the evolution of sexuality in higher plants. In almost all the cases the work ended up in isolating a small fragment of DNA, which in comparison of the total genome is although insignificant but until such time we have the full genome sequence perhaps our knowledge will far from complete and till such time we have to be satisfied with such small fragments.

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