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Organellar DNA diversity in some ornamental plants related to reproduction system and life strategy

PhD. thesis

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Declaration

I hereby declare that the work presented in this thesis is my own and was carried out entirely with help of literature and aid cited in the manuscript.

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1. List of abbreviations

Blast	Basic Local Alignment Search Tool
Bp	Base pair
cpDNA	Chloroplast DNA
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid triphosphate
ds	Double-stranded
EDTA	Ethylene diamide tetra acetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
Hour	hr
LB	Luria Bertani Broth
mtDNA	Mitochondrial DNA
M.W.	Molecular Weight
PCR	Polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
RT	Room Temperature
SD	Standard deviation
SDS	sodium dodecyl sulphate
SDW	Sterile distilled water
Seconds	sec
SSS	Substoichiometric shift
Tris	(hydroxymethyl) aminomethane;(HO-CH2)3C-NH2:2-amino-2-(hydroxymethyl)1,3-propanedioil.

Aims of the work

2. Aims of the work

1) To analyze the inheritance of mitochondrial and chloroplast genomes in a natural population of *Silene vulgaris* by means of PCR-RFLP in coding and Southern-RFLP in non-coding regions as well as sequencing of *atp1* and *cox1* genes. Paternal inheritance, vegetative sorting and de novo origin of mtDNA haplotype by recombination (substoichiometric shifting) are considered to explain non-maternal inheritance in the natural population.

2) To analyze the paternal inheritance of mitochondrial genome of *S. vulgaris* in the offspring of controlled crosses with a father bearing easily detectable mitochondrial marker.

3) *S. latifolia* ecological and life history characters are similar to the closely related species of *S. vulgaris* except for that *S. vulgaris* is gynodioecious plant whereas *S. latifolia* is a dioecious plant species. Here, a comparative study is carried out to examine if the breeding system is predictive of organellar DNA polymorphism and to investigate the association between mt and cpDNA haplotypes.

4) *A. vesiculosa* is a plant species with extremely low variation in DNA markers. It represents the opposition to *S. vulgaris*. Variations in cp and mtDNA in a world-wide collection of accessions of this species are studied.

Introduction

3. Introduction

3.1. Mitochondrial and Chloroplast Genome

Higher plants contain three genomes; nuclear located in the nucleus, mitochondrial and chloroplast genomes found in the cytoplasm. The two organelle genomes exhibit remarkably different tempos and patterns of evolutionary change. With a few notable exceptions, chloroplast genomes are highly conserved in size and gene arrangement, whereas mitochondrial genomes vary enormously in size and organization. It was believed that the sequence evolution of plant mtDNA is quite slow compared to the nucleus and chloroplast. The synonymous substitution rate of plant mitochondrial genes is a few folds lower than chloroplast genes, 10- to 20-fold lower than nuclear genes in both plants and mammals and 50- to 100-fold lower than mammalian mitochondrial genes (Wolfe *et al*., 1987; Palmer and Herbon, 1988; Muse, 2000). However, in recent studies exceptions of this pattern had been found in *Plantago* (Cho *et al*., 2004), *Pelargonium* (Parkinson, 2005), *Silene* (Stadler and Delph, 2002; Barr *et al*., 2007; Sloan *et al*., 2008; Touzet and Delph, 2009) and other plants (Mower *et al*., 2007). Angiosperms have by far the largest mtDNAs, at least 200 kb to over 2,400 kb in size, its larger in size and more complex than in animals, fungi and chloroplast. mtDNAs have different shapes linear, circular and complex molecules (Oldenburg and Bendich, 1996; Backert *et al.*, 1997). Chloroplast genomic size ranges from 120-217 Kb and maintains a highly conserved organization composed of double-stranded circular DNA molecule (Figure 1) with a quadripartite structure. In Spinach, as an example of Caryophyllales, chloroplast genome is composed of two inverted-repeat regions (IRA and IRB) of 25 kb separating a large (LSC) and a small single-copy region (SSC) of 82 kb and 17 kb, respectively and its typical of the majority of plastid chromosomes known (Palmer, 1990, 1992; Raubeson *et al*., 2005; Schmitz-Linneweber *et al*., 2001; Saski *et al*., 2005; Kubo and Mikami, 2007; Kubo and Newton, 2008).

The first complete sequence of an angiosperm mitochondrial genome was reported for *Arabidopsis thaliana* (Unseld *et al*., 1997) then for Sugar beet as shown in Figure (2) (Kubo *et al*., 2000), Rapeseed (Handa, 2003),Tobacco (Sugiyama *et al*., 2005), Rice (Notsu *et al*., 2002), Maize (Clifton *et al*., 2004) ,Wheat (Ogihara *et al*., 2005) and Grape (Goremykin *et al*., 2009). The angiosperm mitochondrial genome contains a number of small repeated sequence families with various sizes and sequences. The role of small repeated sequences appears to be particularly important for the evolution of the angiosperm mitochondrial

genome, including organizational alterations and the expansion of the genome (Lilly and Harvey, 2001).

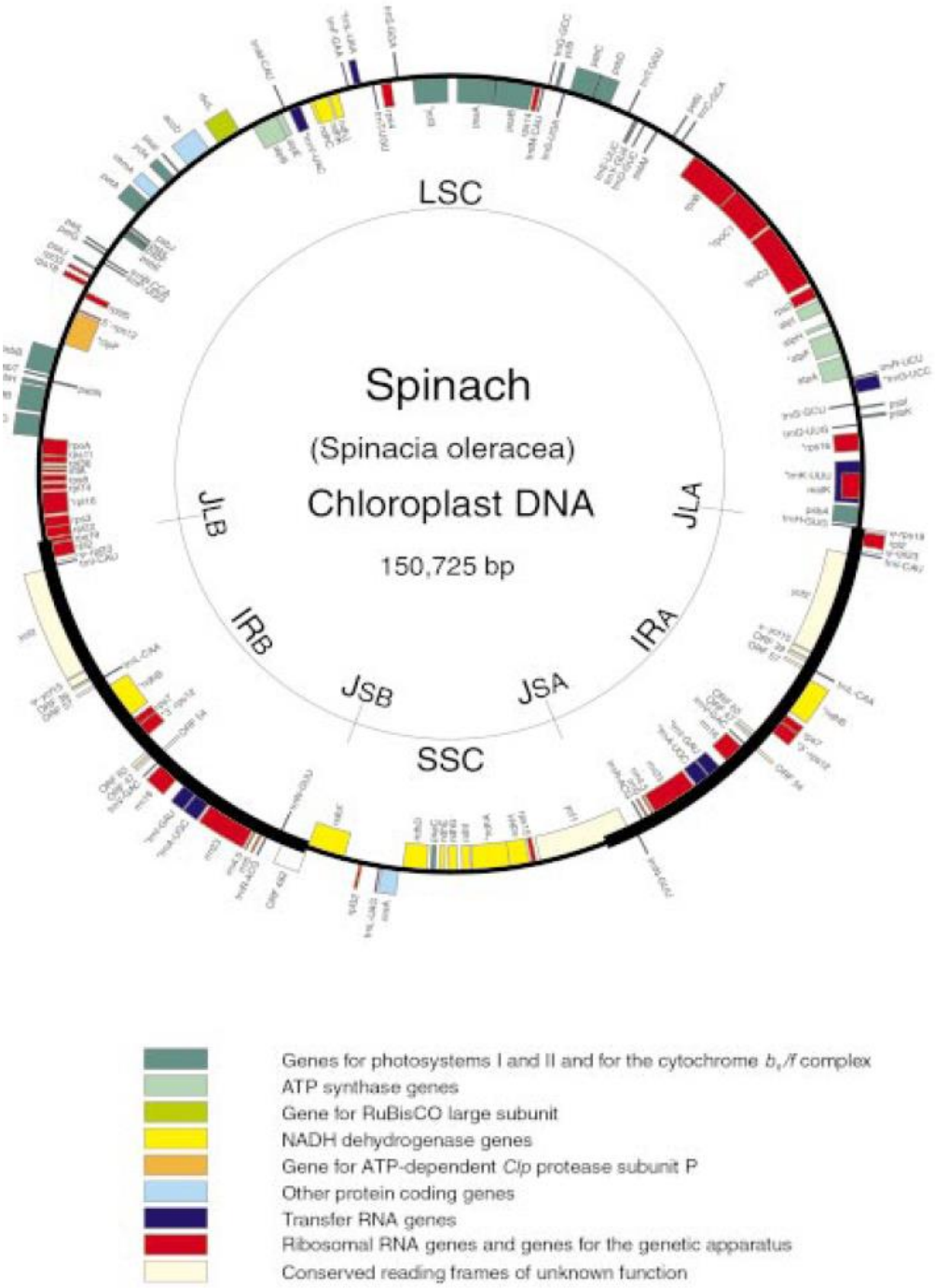


Figure (1): **Gene map of the plastid chromosome of *Spinacia oleracea* (accession number AJ400848)**. Genes drawn inside the circle are transcribed clockwise, those outside the circle counterclockwise. Asterisks indicate genes containing introns. Genes belonging to different functional groups are color-coded (Schmitz-Linneweber *et al*., 2001).

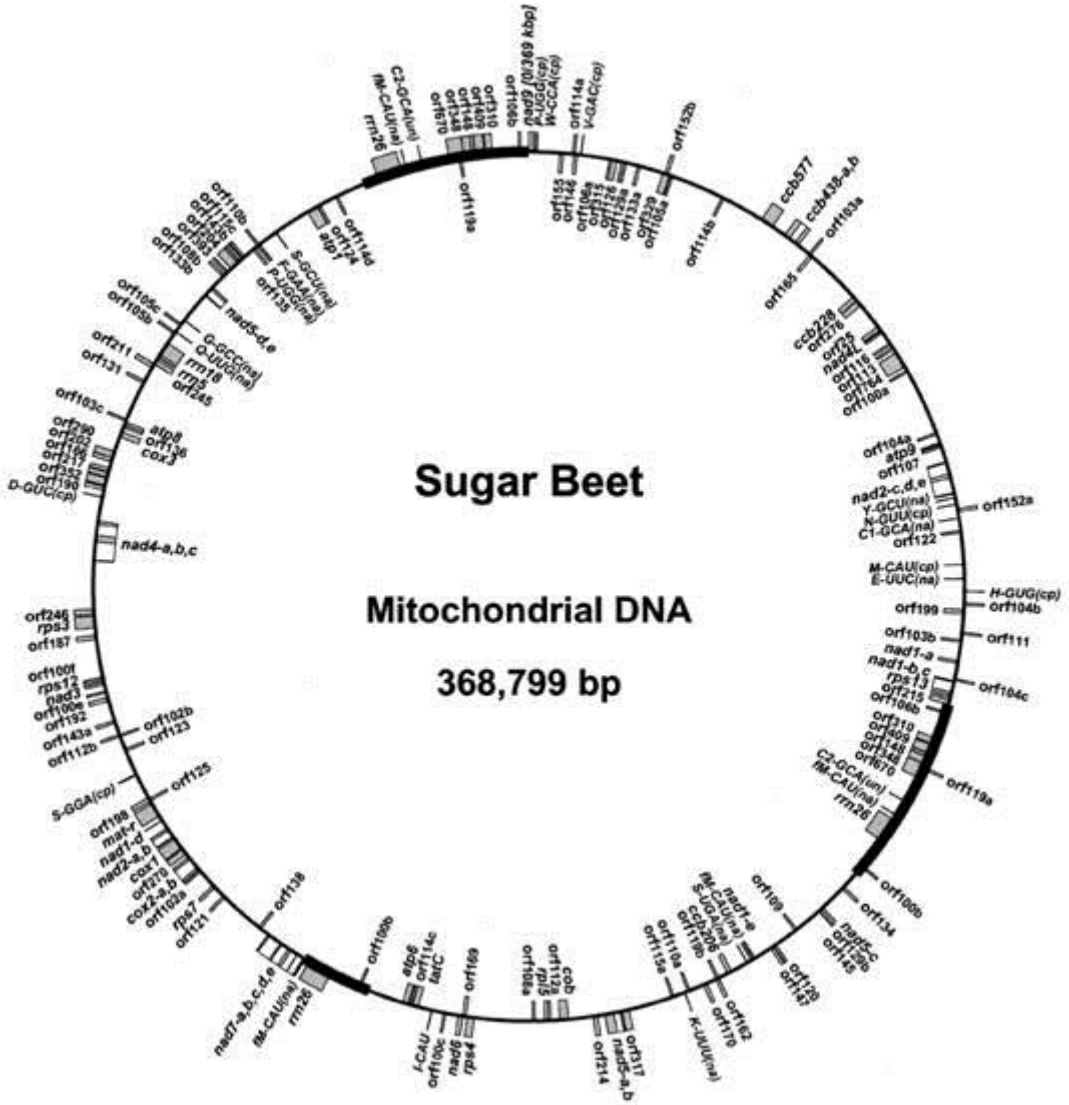


Figure (2): **Gene map of the sugar beet mitochondrial genome** (Kubo *et al*., 2000).

Genome sequences reveal that a flood of DNA from organelles has constantly been bombarding the nucleus since the origin of organelles (Figure 3). Recent experiments have shown that DNA is transferred from organelles to the nucleus at frequencies that were previously unimaginable. Endosymbiotic gene transfer is ubiquitous, continuing and natural process that pervades nuclear DNA dynamics. This relentless influx of organelle DNA has abolished organelle autonomy and increased nuclear complexity. The most extensively

studied example of mitochondrial gene transfer in flowering plants (or any group of Eukaryotes) is the cytochrome oxidase subunit 2 (*Cox2*) in legumes. *Cox2*, exist in the mitochondrion of virtually all plants, was transferred to the nucleus during recent legume evolution. On the other hand, mitochondrial genomes contain sequences homologous to chloroplast or nuclear DNA, the ratio of (chloroplast sequence: nuclear sequence) in mitochondria was variable among sequenced mitochondrial genomes, which may indicate less preferential migration of either of the DNAs (Timmis *et al*., 2004; Kubo and Mikami, 2007).

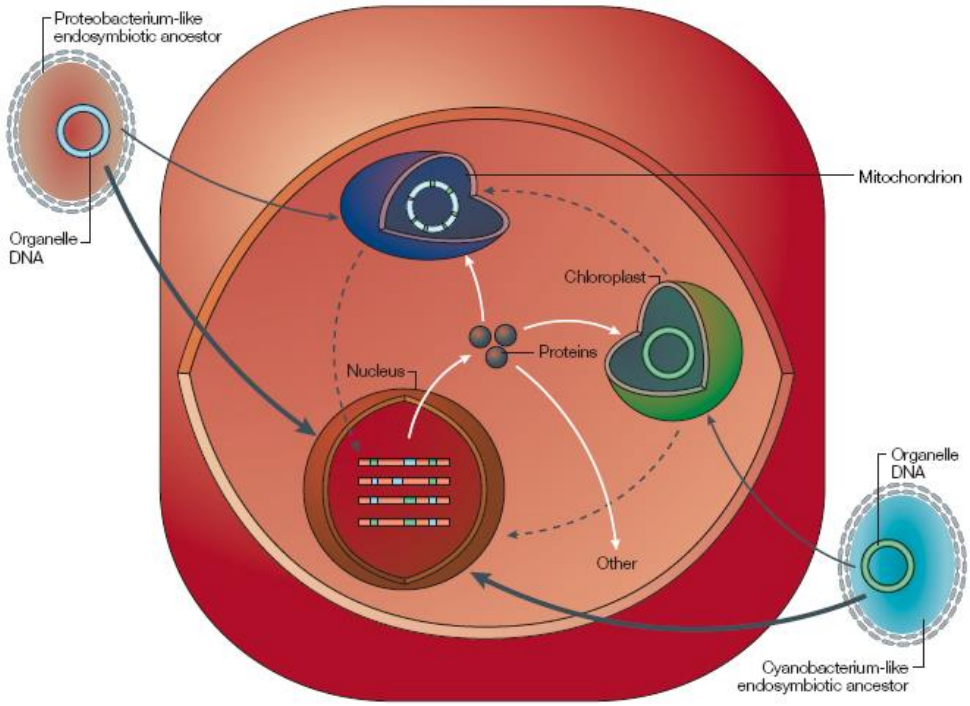


Figure (3): **The endosymbiotic theory and genetic mobility**. The eukaryotic mitochondrion is derived from a proteobacterial endosymbioticancestor but most of the genes that were originally present in this ancestor's genome have been transferred to the nucleus (thick black arrow), with only a small number being retained in the organelle (blue circle). Similarly, most of the genes from the cyanobacterial endosymbiont ancestor of the chloroplast were also transferred to the nucleus (thick black arrow). So, as a result, cytoplasmic organelles import more than 90% of their proteins from the cytoplasm (white arrows). The dotted arrows indicate that the process still going on (Timmis *et al*., 2004).

Angiosperm mitochondrial genomes have the potential to take up sequences of origins other than chloroplast and nuclear DNA, one such source is mitochondrial episomes (A segment of DNA that can exist and replicate either autonomously in the cytoplasm or as part of a chromosome) which have been found in some plant mitochondria. In Sugar beet mitochondria, sequence homology to mitochondrial episomes constitutes 3.6% of the genome.

The homology may be a remnant of the ancient integration of mitochondrial episomes into the main genome (Handa *et al*. 2002; Satoh *et al*., 2006; Kubo and Mikami, 2007).

3.2. Rapid changes within the mitochondrial genomes

It's well known that all sequenced mitochondrial genomes are presumed to undergo rapid changes in genome structure and gene copy number (Kubo and Newton, 2008). These rapid changes could lead to the coexistence of two types of mtDNA of different quantitative representation in one organism which is termed heteroplasmy (Kmiec *et al*., 2006). This Heteroplasmy could be generated first by nucleotide substitutions (Garcia *et al*., 2003; Allen *et al*., 2007) or by interamolecular recombination via repeated sequences in the mitochondrial genome that results in the generation of subgenomic molecules (loop out) or isomeric forms (flip flop) (Kmiec *et al*., 2006; Kubo *et.al*., 2007) which are termed sublimons (Small *et al*., 1987). The sublimons remain at substoichiometric level, where they could make a shift which is termed Substoichiometric shifting (SSS) (Small *et al*., 1987). The second way for the generation of heteroplasmy is the paternal leakage, were two mtDNA coexist in the zygote, one maternally and the second paternally inherited. The inheritance of mtDNA is mainly maternal but rare cases had reported occasional biparental inheritance (Kitagawa *et al*., 2002; Hattori *et al*., 2002; McCauley *et al*., 2005). The generated heteroplasmy has a rapid dynamic nature which could shift its proportions among plant tissues in wild populations (Arrieta-Montiel *et al*., 2001) or during the course of vegetative growth (Yamamoto and Newton, 1999). Recently it has been suggested that SSS, vegetative sorting and paternal transmission of mtDNA are mainly responsible for mtDNA polymorphism in natural populations (McCauley *et al*., 2005; McCauley and Olson, 2008; Pearl *et al*., 2009). Cytoplasmic male-sterility (CMS) is the best-known trait influenced by stoichiometric shifting (Janska *et al*., 1998; Motegi *et al*., 2003), though direct sterility-inducing factors have been shown to be chimeric genes present in mtDNA (Hanson, 1991). These novel chimeric genes are also thought to be created through dynamic, short-repeat sequence-mediated mtDNA rearrangement (Hanson and Bentolila, 2004).Under these mechanisms, the newly formed mtDNA are subject of the increase in frequency in different parts of the plant, this increase may result from SSS or from intraplant genetic drift termed mitochondrial (vegetative) sorting (McCauley and Olson, 2008). This sorting had been hypothesized to underlie the spatial sectoring of female and hermaphrodite flowers on the same individual (Andersson, 1999; Janska *et al.*, 1998)

In the three following subtitles, I will focus on paternal transmission of organelles, SSS&vegetative sorting of mtDNA and finally, on CMS in relationship of intramolecular recombination in the mtDNA.

3.2.1. Mode of inheritance of organellar DNA &mechanisms of paternal transmission

It is widely known that vegetative segregation efficiently removes mtDNA heteroplasmy at mitosis, with few reported cases of heteroplasmy, led to the assumption that individuals generally carry only one maternal mtDNA haplotype which is termed homoplasmy (Birky 1995, 2001; Mogensen, 1996). Researches on plants showed that maternal inheritance was not laws of organelle heredity, several exceptions to this rule were described (Nagata *et al*., 1999; McCauley *et al*., 2005; Guo *et al*., 2005; McCauley *et al*., 2007). Some plants produce progeny carrying a mixture of organelles inherited maternally, paternally, and biparentally (Figure 4). About 80% of angiosperms plastids are maternally inherited and the remainders have biparental inheritance (Matsushima *et al*., 2008). Recent findings suggest that paternal inheritance of mtDNA is more common than once believed (McCauley and Olson, 2008). It was suggested that organellar paternal transmission blockage could happen at any stages of Gametogenesis, Fertilization and Postfertilization by either segregation from the gamete during reproduction and before fertilization or degradation after fertilization (Birky, 2001; Barr *et al*., 2005; White *et al*., 2009). The majority of the previous studies of paternal transmission of mtDNA in plants focused on crosses between species, but the paternal transmission of mtDNA in natural plant populations has been little studied (McCauley and Olson, 2008)

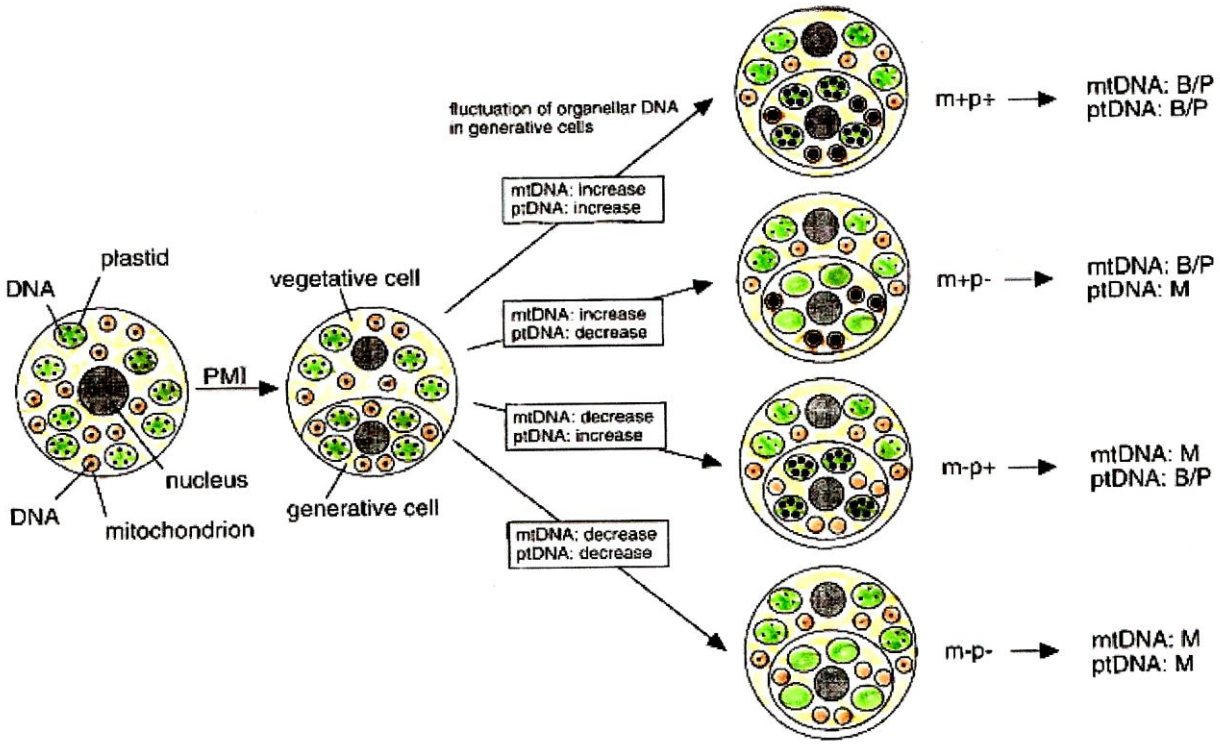


Figure (4)**: Organellar sorting in the course of pollen development.** In pollen development, the first step includes a tetrad formed by meiosis of the pollen mother cell then four microspores are formed. Each microspore undergoes pollen mitosis I (PMI) to divide into a vegetative cell (large) and generative cell (small).The generative cell matures and we can see four different cases : m+p+)biparental inheritance of both orgnelles DNA, m+p-)biparental inheritance of mtDNA accompanied with maternal inheritance of cpDNA, m-p+biparental inheritance of cpDNA accompanied with maternal inheritance of mtDNA, m-p-) the maternal inheritance of both organelles DNA (Nagata *et al*., 1999; McCormick, 2004; Matsushima *et al*., 2008). mtDNA) mitochondrial DNA, ptDNA) cpDNA, M) maternal inheritance and B/P) biparental/paternal inheritance. The photo is taken from Nagata *et al*. (1999) and colored.

3.2.2. Substoichiometric shifting (SSS) & vegetative sorting of mtDNA

SSS and vegetative sorting of mtDNA are now the widely accepted explanations in the studies of *S. vulgaris* mtDNA inheritance (McCauley and Olson, 2008; McCauley *et al*., 2005; Pearl *et al*., 2009).

SSS is a phenomenon rendering the plant mitochondrial genome unusually variable in structure is termed substoichiometric shifting. First reported in Maize (Small *et al*., 1987) as the stable presence of subgenomic mitochondrial DNA molecules within the mitochondrion at nearly undetectable levels, the process seems to be highly dynamic (Figure 5). Mitochondrial genomic shifting involves rapid and dramatic changes in relative copy number of portions of the mitochondrial genome over the time of one generation (Janska *et al*., 1998) These

substoichiometric forms have been estimated at levels as low as one copy per every 100–200 cells (Arrieta-Montiel *et al*., 2001). Generally, the rapid shifting process involves only a single subgenomic DNA molecule, often containing recombination-derived chimeric sequences, and the process is apparently reversible (Janska *et al*., 1998; Kanzawa *et al*., 1994). Genomic shifting can alter plant phenotype because the process activates or silences mitochondrial sequences located on the shifted molecule. Observed phenotypic changes have included plant tissue culture properties (Kanzawa *et al*., 1994), leaf variegation and distortion (Sakamoto *et al*., 1996), and spontaneous reversion to fertility in cytoplasmic male sterile crop plants (Janska *et al*. 1998; Smith and Chowdhury, 1991). It has been postulated that substoichiometric shifting may have evolved to permit the species to create and retain mitochondrial genetic variation in a silenced but retrievable form (Small *et al*. 1989).

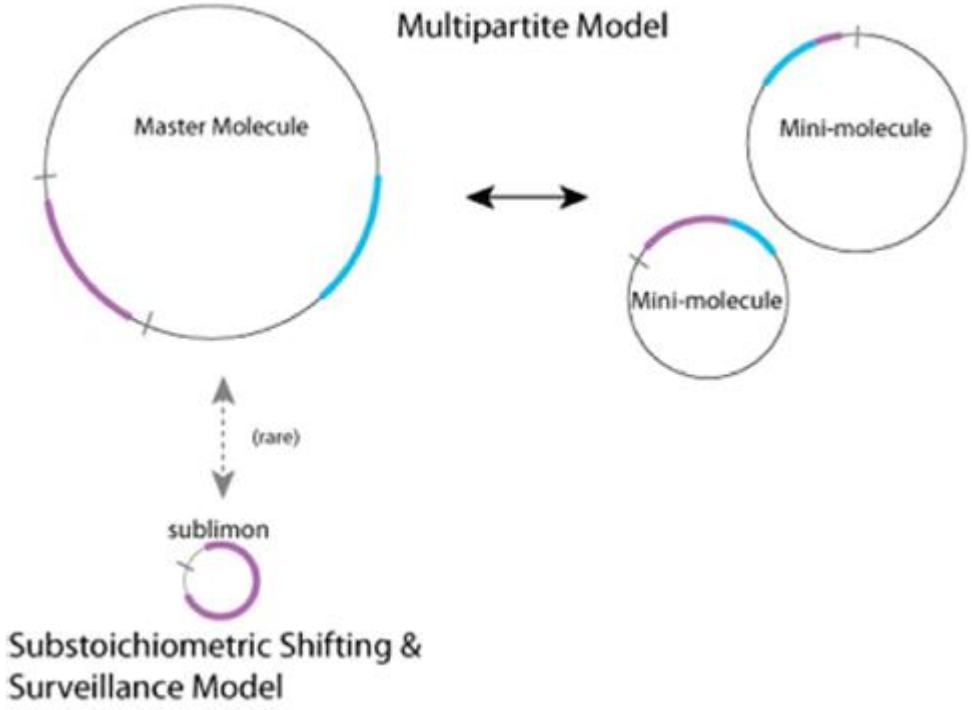


Figure (5): **Substoichiometric shifting**. The multipartite structure of plant mtDNA with a master molecule undergoes intragenomic recombination at large (>4 kb thick regions) direct repeats to create two or more subgenomic forms. Gray hash represent small repeats (<500 bp).
According to the Surveillance Model in *Arabidopsis* (Shedge *et al*., 2007), recombination-initiated replication is controlled by nuclear genes that allow replication only at large repeats and sometimes infrequent mistakes by surveillance genes allow the replication at the smaller repeats creating sublimons. On the other hand, the replication initiated in the large repeats results in the formation of both Master molecule and minimolecules which is termed the Multipartite model (Palmer and Herborn, 1989). The photo and description are taken from McCauley and Olson (2008).

This stoichiometry may change rapidly through the process of genomic shifting (Small *et al*. 1989; Arrieta-Montiel *et al*. 2001), in which low-copy-number substoichiometric mtDNAs become amplified into high-copy-number dominant ones, or vice versa. This process can be activated by stresses such as tissue culture cultivation (Fauron *et al*. 1990) or somatic hybrid regeneration (Sakai and Imamura, 1992; Motegi *et al*.,2003) and is controlled by nuclear genes including the Fr gene in *Phaseolus vulgaris* (Mackenzie and Chase, 1990; Janska *et al*. 1998) or *Msh1* and *RecA* in *Arabidopsis thaliana* (Abdelnoor *et al*., 2003; Sandhu *et al*.,2007). In *S. vulgaris* natural populations, the SSS had been suggested as an explanation for the heteroplasmic status of some individuals (McCauley *et al*., 2005),

Mitochondrial (vegetative) sorting exist in heteroplasmic individual, and was described as the process whereby random transfer of different numbers of copies of each allele from one mitochondria and cell to the next. This process results in a change in the frequency of mitochondrial alleles within different plant tissues (McCauley and Olson, 2008). They suggested that this process is analogous to random genetic drift in natural populations. They hypthesized that unlike sorting, shifting is a directed process that is more likely to occur when nuclear genes controlling mitochondrial replication are nonfunctional (they presented here another point of view). What is importatnt here that if we have a cryptically heteroplasmic mother, this mother could occasionally produce an offspring in which her low copy mtDNA haplotype becomes numerically predominant in an offspring owing to chance events associated with mitochondrial population bottlenecks (vegetative sorting) during the cell divisions leading to gametogenesis.

Mitochondrial vegetative sorting was suggested as an explanation for the changes in the degree of heteroplasmic state of *S. vulgaris* individuals (Pearl *et al*., 2009). In fact the heteroplasmy was found in several studies in *Silene* and also quantified (Andersson, 1999; Stadler and Delph, 2002; Welch *et al*., 2006; Pearl *et al*., 2009). Andersson (1999) found that female and hermaphroditic flowers co-occurring on the same plant when crossed with common pollen donor produced offspring that differ significantly from each other. This could be explained by that different parts of the same maternal plant carry significantly different mitochondrial populations that include different forms of CMS (Welch *et al*., 2006)

3.2.3. CMS as the consequence of intramolecular recombination within mtDNA

Cytoplasmic male sterility (CMS) is a common trait in higher plants, mainly inherited maternally and associated with the failure to produce functional pollen. Open reading frames (ORFs) associated with CMS often represent chimeric genes that origin from recombination of either important mitochondrial genes (like *atp1* for example) or their 5′- and 3′-flanking region, or from sequences of unknown origin which could be named mitochondrial CMS associated genes/protein. Mitochondrial CMS associated genes are transcribed because they either fuse to promoter sequence or cotranscribed with mitochondrial genes. Dominant nuclear genes which are named restorers of fertility (*Rf*) is known to act as a suppressor of male sterility phenotype, then the fertile phenotype could be observed in plants have CMS mitochondrial genomes. While CMS associated genes have been identified for a number of CMS systems, we have only now started to learn about the molecular features of restorer genes and their proteins. One of the important protein families which are encoded by *Rf* genes are pentatricopeptide repeat proteins (PPR proteins).They function on preventing the expression of CMS associated genes, also they were found in several plants and nowadays, they are considered to be the key regulators of plant mitochondrial gene expression. CMS and fertility restoration represent important agronomic traits in crops such as Petunia, Common bean, Maize, Sorghum, Sunflower, Rice and Rapeseed, which are essential for the production of hybrid seeds on a commercial scale. Hybrid breeding allows exploitation of heterosis and leads to higher yields and more yield stability. In addition to facilitating the commercial exploitation of CMS-Rf systems, detailed studies of CMS and *Rf* genes provide us with information that can increase our understanding of nuclear-cytoplasmic interactions (Schnable and Wise, 1998; Hanson and Bentolila, 2004; Eckardt, 2006; Chase, 2007; Fujii and Toriyama, 2008; Schmitz-Linneweber and Small, 2008).

There are many examples of CMS as the consequence of intramolecular recombination within mtDNA. In Petunia, Nivison and Hanson (1989) identified the gene product of the CMS-associated *pcf* gene by using antibodies produced against a synthetic oligopeptide. The 43 kDa protein encoded by *pcf* is post-translationally processed at the N-terminus to give a 25-kDa protein which disrupts pollen development (Nivison *et al*., 1994). Petunia *Rf* locus was composed of duplicated genes containing a pentatricopeptide repeat (PPR) motif. One of them

encodes a 592 amino acid protein and is able to restore fertility when transferred to CMS plants (Bentiola *et al*., 2002).

In Maize, Dewey *et al*. (1987) identified an additional protein of 13 kDa to be expressed in the T-cytoplasm, which is encoded by the unique coding region *T-urf13*. The 13 kDa protein represents a prominent mitochondrially encoded protein, which confers sensitivity to the T-toxin. The T-toxin produced by the T race of *Cochliobolus heterostrophus* (fungal plant pathogen), causing the southern corn leaf blight, interacts with the protein, which is located in the inner mitochondrial membrane in an oligomeric arrangement. This induces the formation of a pore that makes the membrane leaky (Dewey *et al*., 1987; Korth *et al*., 1991). The binding of the pathotoxin renders the mitochondria incapable of performing oxidative phosphorylation (Kaspi and Siedow 1993; Rhoads *et al*., 1995). For the disruption of pollen development, it is assumed that the 13-kDa protein might interact with an anther-specific substance in a way similar to that observed with the T-toxin (Flavell, 1974). The *Rf2a* in Maize was cloned and encodes a protein with aldehyde dehydrogenase activity (Touzet, 2002; Liu *et al*., 2001). So far this is the only *Rf* gene which doesn't encode PPR proteins **(**Fujii and Toriyama, 2008)

In *Sorghum bicolor*, Bailey-Serres *et al*. (1986) investigated the in organello translation products of a number of CMS systems. For CMS Milo, they identified an additional 65 kDa protein, for CMS 9E an additional 42 kDa protein and the absence of a 38 kDa protein (Dixon and Leaver, 1981). For CMS IS II2 and MS M35-1(B), additional proteins of 12 kDa and 82 kDa, respectively, were detected (Bailey-Serres *et al*., 1986). The locus *Rf1* in sorghum was determined and it was found to encode a pentatricopeptide repeat protein (Klein *et al*., 2005).

In Sunflower, a new open reading frame orfH522 in the 3′-flanking region of the *atp1* gene could be associated with the CMS phenotype PET1 (Köhler *et al*., 1991; Laver *et al*., 1991).

In *Phaseolus vulgaris*, the product of *pvs-orf239* CMS associated postulated gene was identified using antibodies. A part from the predicted protein of 27.5 kDa, a second protein of 21 kDa could be identified, which might be a degradation product. The important here is that the 27.5 kDa protein in *Phaseolus vulgaris* can only be detected in the pollen mother cells and the developing microspores or in other words the reproductive tissues (Abad *et al.*, 1995).

The *Msh1* (formerly *CHM*) in *Arabidopsis thaliana* appears to be involved in the suppression of illegitimate recombination in plant mitochondria and encodes a homolog of the *E. coli MutS* mismatch repair. The mutation of the *Msh1* locus results in reproducible mitochondrial rearrangements in *A. thaliana* (Abdelnoor *et al*., 2003). Disruption of *Msh1*in transgenic

Tobacco and Tomato leads to a type of mitochondrial DNA rearrangements associated with CMS in plants. In both species, these experiments resulted in reproducible mitochondrial DNA rearrangements and a condition of male (pollen) sterility (Sandhu *et al*., 2007). *RecA3* (*E. coli RecA* homolog) and *Msh1* genes were suggested to control plant mitochondrial genome maintenance and that the loss of both genes functions simultaneously has large effect on growth rate and reproduction (Shedge *et al*., 2007).

In gynodioecious plants (in which hermaphroditic and functionally female individuals co-occurr in the same population), it's believed that CMS associated genes/protein disrupt proper pollen development, thus converting otherwise hermaphroditic plants to functional females. Under the cytonuclear system of sex determination believed to be prevalent in gynodioecious populations, individuals bearing a CMS gene are females "male-sterile" unless they carry appropriate nucleus-encoded "restorer" alleles (Schnable and Wise, 1998; Charlesworth and Laporte, 1998).

3.3. Silene *as a model system in Ecology and Evolution*

The genus *Silene* (Caryophyllaceae) has many characters that support the current trend of making it as a model system in ecology and evolution. First, it contains many species through the world and has large variety of ecological and morphological characters. Second, *Silene* species show variety of breeding systems, including ones that are hermaphroditic (*gallica, apetala* etc…), gynodioecious (*vulgaris, acaulis, coeli-rosa, nutans* etc…), and dioecious (*latifolia, dioica* etc…) (Desfeux *et al*., 1996). Third, several species are easily bred, cultivated, have short life cycle and produce a lot of seeds (Cros *et al*., 2003, Bernasconi *et al*., 2009). Fourth, researchers can depend on many ecological studies carried out on different members of the genus.

S. vulgaris (Caryophyllales) is a perennial herb which has multiple uses. It has edible, medicinal, landscaping, xerogardening uses beside its recent application for phytoremediation of soils contaminated with heavy metals like Zinc. It's successful as pot plant (Figure 6) and it can tolerate semi-arid conditions which raise the possibility to be used as a ground cover on road sides in deserted areas (Harmens *et al*., 1993; Chaney *et al*., 1997; Ernst and Nelissen, 2000; Olson *et al*., 2005; Arreola *et al*., 2004, 2006; Franco *et al*., 2006). *S. vulgaris* became a model plant during the last two decades for the study of DNA polymorphism in mitochondrial genes in natural populations around the globe because, its widely spread across the world,

easy to grow and to be crossed as the remaining of the species of its genus also it produces a lot of seeds (Cross *et al*., 2003). Gynodioecy is associated with CMS and female individuals could contain CMS factors. From this point of view *S. vulgaris* is used for the studies of CMS in natural populations. Previous studies revealed mitochondrial genes polymorphism in *cox1* flanking regions of *S. vulgaris* in the USA (Olson and McCauley, 2002) and Central Europe (Štorchová and Olson, 2004).There were high substitution rates in coding regions of several mitochondrial genes and moderate substitutions in chloroplast genes (Houliston and Olson, 2006; Barr *et al*., 2007;Touzet and Delph, 2009) also mitochondrial heteroplasmy were found in many individuals in *S. vulgaris* (McCauley *et al*., 2005;Welch *et al*., 2006; Pearl *et al*., 2009).

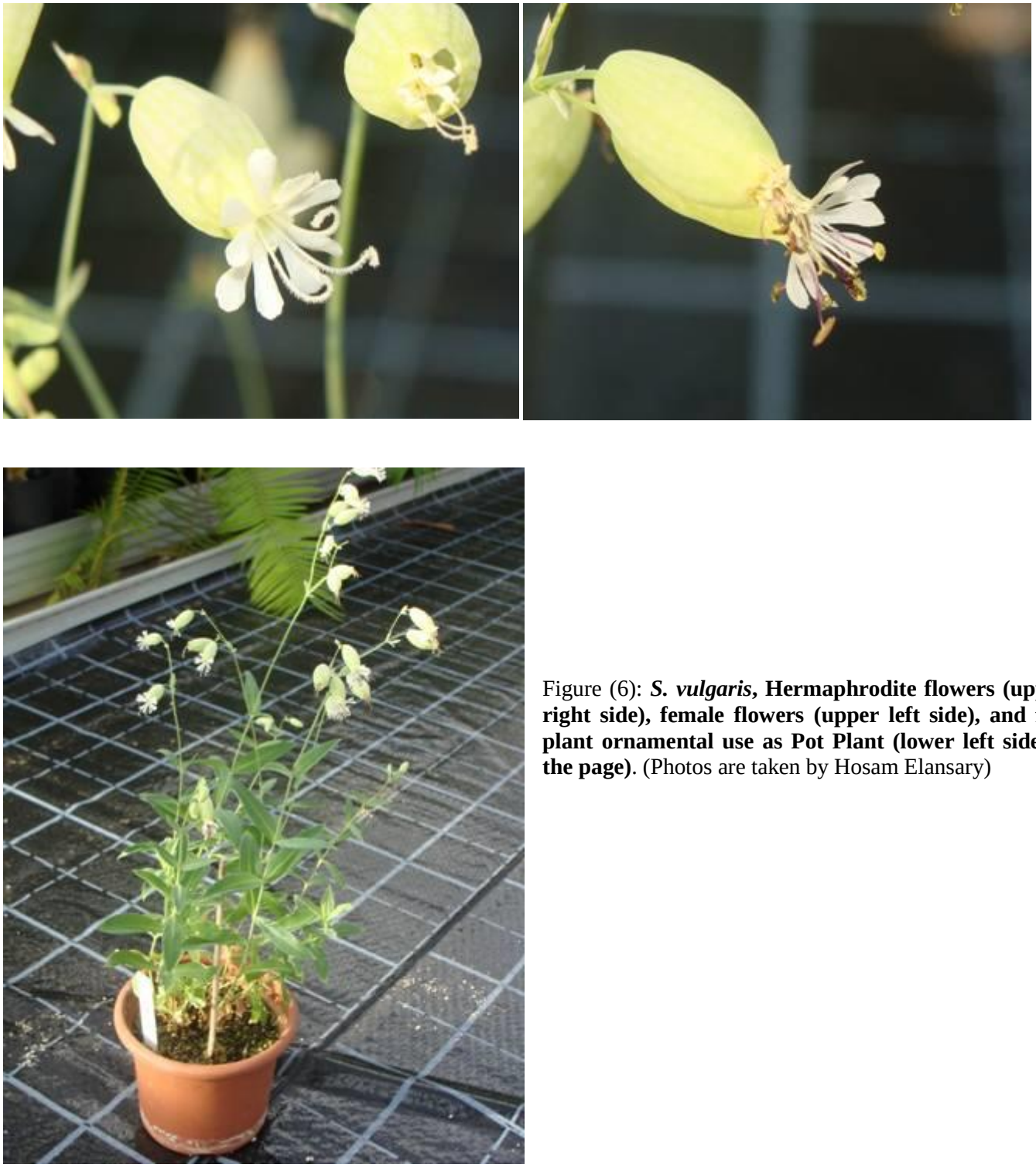


Figure (6): ***S. vulgaris*, Hermaphrodite flowers (upper right side), female flowers (upper left side), and full plant ornamental use as Pot Plant (lower left side of the page)**. (Photos are taken by Hosam Elansary)

3.4. What is known about inheritance of mt and cpDNA in S. vulgaris*?*

Rare paternal transmission of mtDNA was documented in *S. vulgaris* (McCauley *et al*., 2005; Pearl *et al*., 2009) and nonmaternal inheritance of cpDNA (McCauley *et al*., 2007). The association between mtDNA and cpDNA was confirmed (Olson and McCauley, 2000; Štorchová and Olson, 2004). The paternal transmission of mtDNA documentation is becoming much more important subject nowadays (McCauley and Olson, 2008). The most up to date work was done by Pearl *et al*. (2009). They utilized Q-PCR of *atp1* and *cox1* to evaluate the origin, extent and transmission of mtDNA. They quantified the heteroplasmy in 408 *S. vulgaris* individuals sampled from 22 natural populations located in USA also they compared the mother and offspring from 71 families to determine the rate of heteroplasmy gained and lost via paternal leakage and vegetative sorting across generations. They found evidence on paternal transmission of the mtDNA especially from the study of homoplasmic mothers and their respective offspring. When the mother is heteroplasmic, the mother-offspring differences in mtDNA haplotype could mainly due to vegetative sorting of mtDNA (McCauley and Olson, 2008) during the cell division rather than to paternal inheritance. They found that about 4% of the offspring of the homoplasmic mothers (complete or partial replacement of the maternal genome counted together) was heteroplasmic and they assumed that the mother-offspring differences most likely arise due to paternal inheritance at the time of offspring zygote forming during fertilization. They concluded that the paternal inheritance of mtDNA was relatively rare event in *S. vulgaris* and if it happened, the contribution of the pollen donor to biparental inheritance was often limited. They found 61 heteroplasmic individuls, an increase in heteroplasmy in the offspring of homoplasmic mothers and a decrease in heteroplasmy in the offspring of heteroplasmic mothers. They explained the decrease by the vegetative sorting of mtDNA and partially by paternal transmission but they could not distinguish for what extent is the contribution of each process, becuase they didnt have important inforrmations like the number of mitochondria transmitted from mother to daughter cell and the number of cell divisions separating the leaf sampled from the mother and the leaf sampled from the offspring for DNA extraction. The 4% ratio of the paternal transmission in the case of homoplasmic mothers' offspring (Pearl *et al*., 2009) is the same as the 4% paternal transmission found before by McCauley *et al*. (2005). McCauley *et al*. (2005) analyzed 318 individuals distributed among 23 controlled crosses in the USA using PCR-RFLP of *atp1*and *cox1* mitochondrial genes. The maternal inheritance was indicated in 96%

of the individuals studied, one or more individuals from each of four sib groups displayed a genotype that was identical to the father, or that did not match either parent. Given evidence that inheritance is not strictly maternal, it was hypothesized that some individuals could carry a mixture of maternally and paternally derived copies of the mitochondrial genome. *S. vulgaris* heteroplasmy was quantified before by Q-PCR (Welch *et al*., 2006) and they found that the mitochondrial variation within individuals constituted 26% of the total in one population which is consisted with the biparental model of mtDNA inheritance which is in agreement with Pearl *et al*. (2009). These studies demonstrated that heteroplasmy is a widespread phenomenon in *S. vulgaris* natural populations and could be used as an effective tool to track paternal transmission of mtDNA (McCauley and Olson, 2008).

The nonmaternal inheritance of cpDNA has been documented before in *S. vulgaris* by McCauely *et al*. (2007). They used the PCR-RFLP method to compare mother and offspring of cpDNA haplotypes. Two classes of individuals were used, the first consisted of a set of parents collected from 12 natural populations in Virginia and New York and their offspring obtained from greenhouse controlled crosses, the second set consisted of individuals obtained by collecting seed capsules and corresponding maternal leaf tissues from two natural populations one in Virginia and one in New York The nonmaternal inheritance was inferred in the first class when the offspring matched the pollen donor rather than the pollen recipient, while in the second set, the nonmaternal inheritance was inferred when the haplotype of an offspring didn't match its mother but rather that of another cpDNA haplotype known to occur in the population under study, though the father was not known. From 215 individuals analyzed in the first class of individuals, they found 10 individuals (4.7%) had cpDNA haplotype that matched their father. From 156 individuals analyzed in the second class (48 from Virginia and 108 from New York), they found three individuals (1.9%) that differed than their mother cpDNA haplotype but they carried haplotypes correspond to their respective populations.

The genetic association between chloroplasts and mitochondria has been documented in many plant species like in populations of *B. vulgaris* (Cuguen *et al*., 1994; Desplanque *et al*., 2000), *Thymus vulgaris* (Belhassen *et al*., 1993) and in *S. vulgaris* (Olson and McCauley, 2000; Štorchová and Olson, 2004). Olson and McCauley (2000) utilized PCR-RFLP and sequencing of either introns or intergenic spacers to detect cpDNA variation, also they used Southern-RFLP of *cox1* mtDNA to estimate the linkage disequilibrium between the two organellar DNAs. They found complete linkage disequilibrium between cpDNA and mtDNA haplotypes

in 86 individuals from 17 populations of *S. vulgaris* in Virginia, USA. The mtDNA haplotypes were always nested within cpDNA haplotypes in a pattern consistent with the co-transmission of cp and mtDNA, also they hypothesized that the evolutionary histories of the mitochondria and chloroplasts are congruent within *S. vulgaris*. This study was followed by a study of European populations, carried out by Štorchová and Olson (2004). They studied 86 individuals from eight populations from central Europe (Czech Republic, Germany and Austria). They utilized Southern-RFLP of *cox1* of mtDNA and sequencing of *psbA-trnH* intergenic spacer of cpDNA. Their mt and cpDNA association data didn't always follow the previous pattern of nesting the mtDNA within cpDNA haplotypes (Olson and McCauley, 2000). Interestingly, they found three cases where one mtDNA haplotype was associated with more than one cpDNA haplotype and three cases where one cpDNA haplotypes was associated with more than one mtDNA haplotype. They supported the early conclusion that the mitochondrial and chloroplast genomes appear to be primarily co-inherited in *S. vulgaris*. Houliston and Olson (2006) sequenced three mtDNA genes (*atp1*, *atp9*, *Cob*) and two cpDNA genes (*matK* and *ndhF*) in *S. vulgaris* populations from USA (Virginia and New York from the same populations analyzed before by Olson and McCauley, 2000), central Europe (Czech Republic/Germany) and Asia (Russia). They found incongruence in the phylogenetic patterns of organelle genes in the mitochondria and chloroplasts which indicated the potential for independent evolutionary trajectories. They explained the contrast of the data with those achieved before by Olson and McCauley (2000) that their data include DNA sequences from coding regions only, whereas Olson and McCauley (2000) used noncoding regions in the cpDNA and flanking regions in *cox1* mtDNA and its well known that the RFLP flanking regions and intergenic spacers evolve faster than coding regions. Interestingly, no study in plants so far analyzed the frequency of paternal co-transmission of both organelles.

3.5. The gender and mitochondrial haplotype

Approximately 7% of Angiosperm species exhibit gynodioecious breeding systems in which individuals are either female or hermaphroditic (Olson and McCauley, 2002). Population sex ratios in gynodioecious species can be highly variable. The relative number of females within gynodioecious populations is thought to be under frequency-dependent selection (Frank, 1989; McCauley and Brock, 1998), female frequencies range from 0% to 75% female in *S. vulgaris* (McCauley *et al*., 2000b). In *S. vulgaris*, the gender is typically regulated by a cytonuclear interaction involving cytoplasmic male sterility (CMS) factors associated with the

mitochondrial genome. The entire mitochondrion is inherited as a single linkage unit. Thus, a polymorphic marker anywhere in the genome could be in linkage disequilibrium with a CMS factor, creating nonrandom associations between mtDNA haplotypes and sex expression in natural populations this associations have been documented in many species like *Thymus vulgaris* (Belhassen *et al*., 1993), *B. vulgaris* (Cuguen *et al*., 1994), *Plantago lanceolata* (De Haan *et al*., 1997), *S. acaulis* (Stadler and Delph, 2002), and *S. vulgaris* Virginia population in USA. They hypothesized that the haplotypes were in linkage disequilibrium with different CMS factors, consequently the determination of the frequency of females within populations could estimate if different CMS types are present or not (Olson and McCauley, 2002).

3.6. Silene latifolia *(White Campion), dioecious species, studied for comparison with* S. vulgaris

The study of *S. latifolia* gave us the opportunity to analyze various characters from the point of view of reproduction system. The polymorphism in flanking regions of mitochondrial genes and association between chloroplast and mitochondrial markers was the topic of interest in a comparative study between *S. vulgaris* and S. *latifolia*, as it has not been so far analyzed.

3.6.1. S. latifolia *is closely related to* S. vulgaris

Silene latifolia is a flowering diploid (2n = 24) dioecious species with an X/Y chromosomal sex determination system. It is short-lived perennial herb (Figure7) belonging to the family Caryophyllaceae. It is native to Eurasia and North Africa (Morocco to Egypt), could be originated in the Middle East or Mediterranean region then invaded Europe with the spread of agriculture then introduced to North America in the middle eighteenth century from Europe. *S. latifolia* became a model system in different fields of science, including host–pathogen interactions, sex chromosome evolution, sexual dimorphism, sex-ratio distortion, reproductive ecology, hybridization and biological invasions (Westergaard, 1958; Richards *et al.*, 2003; Taylor and Keller, 2007; Teixeira and Bernasconi, 2008). Ecological and life history characteristics are similar in the closely related species of *S. vulgaris* and *S. latifolia* except for that *S. vulgaris* is gynodiousy plant whereas *S. latifolia* is dioecious plant with no recent

history of CMS. Crossing studies in *S. latifolia* have shown no cytoplasmic genetic effects on sex ratio or sex allocation (Taylor, 1994; Ingvarsson and Taylor, 2002).

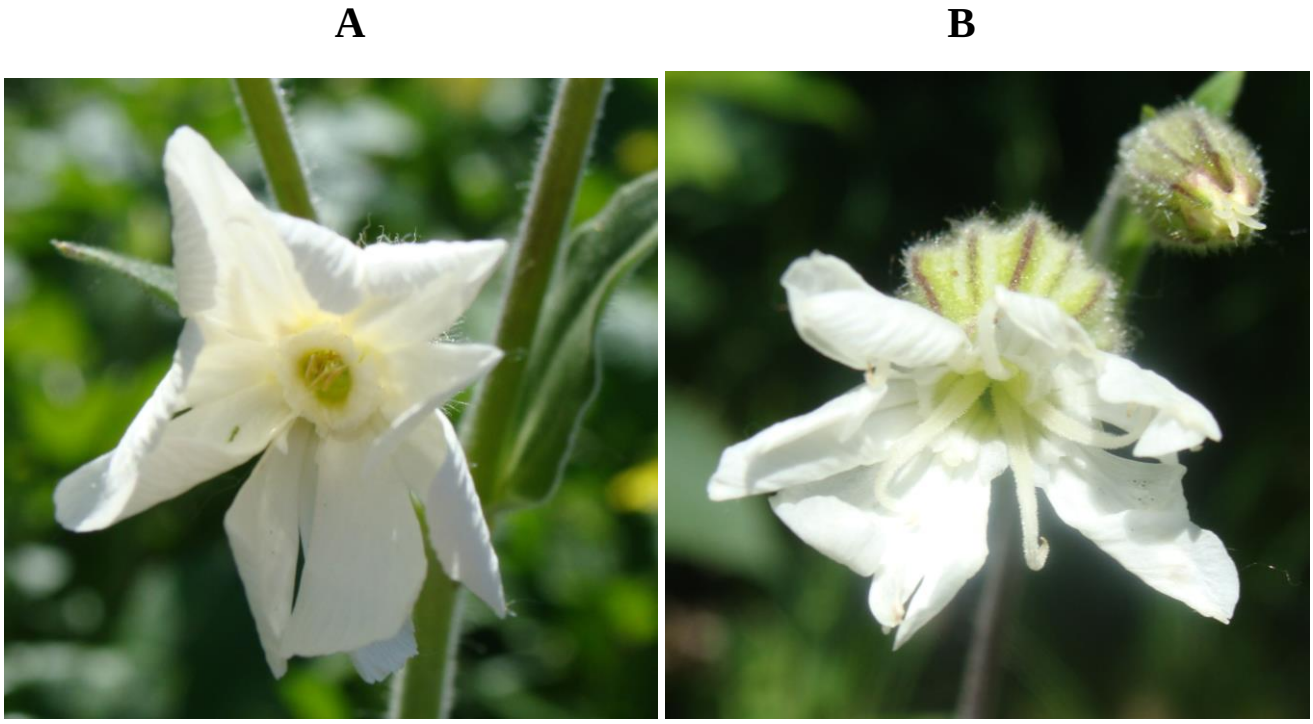


Figure (7): ***Silene latifolia* male flower (A) and female flower (B).** Photos are taken by H.Elansary.

3.6.2. Mitochondrial and chloroplast DNA polymorphism

Several studies were carried out to study mitochondrial and chloroplast genomes polymorphism within the *Silene* which have led to contradictory conclusions. Sequence analysis at nuclear and cytoplasmic loci in *S. vulgaris* and *S. latifolia* showed higher diversity in the later (Ingvarsson and Taylor, 2002; Taylor and Keller, 2007) suggesting recurrent selective sweeps caused by epidemic dynamics, but a comparison of cytoplasmic and nuclear loci found substantially higher polymorphism in *S. vulgaris* than *S. latifolia* (Sloan *et al*., 2008). In seven mitochondrial genes, they found 57 segregation site in *S. vulgaris* (in total) compared to only 16 in *S. latifolia* , also on the nuclear level, the same trend was found, and they explained it by the difference in mutation rates between lineages. Finally, Touzet and Delph (2009), conducted comparative analysis within *Silene* using sequences of *Cox1* and *Cob* mitochondrial genes and found striking differences in nucleotide polymorphism, with gynodioecious species (*S. vulgaris*) exhibiting more segregating sites and more haplotypes than non-gynodioecious species (*S. latifolia*) in *Cob* ,but they found limited number of

haplotypes of *cox1* in *S. vulgaris* (2) which was lower than *S. latifolia* (3), and they concluded that balancing selection acts on genetic factors underlying gynodioecy alter the pattern of nucleotide polymorphism in mitochondrial genes in gynodioecious rather than non-gynodioecious and they suggested that the breeding system has an effect on nucleotide diversity.

The genetic variation among *S. latifolia* European /American populations has been studied by allozymes (McCauley, 1994; Richards *et al*., 2003), sequencing of cpDNA (Taylor and Keller, 2007; Sloan *et al*., 2008) and microsatelites (Jolivet and Bernasconi, 2007). None of the previous studies utilized Southern hybridization, capable to reveal polymorphism in gene flanking region as a technique for the detection of variation among or within populations of *S. latifolia*. This is the first study to use Southern hybridization in the analysis of genetic variation among and within *S. latifolia* European populations. Association between chloroplast and mitochondrial markers has been analyzed in *S. vulgaris* (Olson and McCauley, 2000; Štorchová and Olson, 2004), but so far not in *S. latifolia*. The uniformity of the coding regions of mitochondrial genes in *S. latifolia* prevents the application of readily available PCR-generated markers derived from these genes. Thus, Southern RFLP markers could be utilized to examine not only the diversity of mitochondrial markers, but also their association with chloroplast markers in *S. latifolia*.

3.7. Aldrovanda vesiculosa (Droseraceae), *an example plant of low genetic variation within organelles*

A. vesiculosa is very distantly related to *Silene*, belonging to different family of the same phylogenetic branch Caryophyllales. It respresents the apposite to *S. vulgaris* in regards with genetic variation being extremely uniform (Maldonado San Martín *et al*., 2003; Hoshi *et al*., 2006). However, organellar markers have not been yet studied in *A. vesiculosa*. A unique world wide collection of *A. vesiculosa* in the Institute of Botany of the Czech Academy of Sciences (CAS), in Třebon, Czech Republic (Adamec, 1997 a, b) was used to analyze within species variation in mt and cpDNA. In this chapter, I will describe some physiological and genetic data about this collection which will guide us in understanding the importance of studying this low genetic variation plant.

3.7.1. Botanical description

A. vesiculosa belongs to carnivorous plants (Plants which catch and digest animals for their nutrition). These plants have specific morphological adaptations enabling them to catch small animals. Carnivorous plants have been used extensively for medication and other purposes because plants in the past were virtually the only source of medicinal preparations (Pietropaolo and Pietropaolo, 2005) beside its ornamental uses. An important family of f carnivorous plants is the family Droseraceae which includes four genera *Drosera*, *Drosophyllum*, *Aldrovanda*, and *Dionaea*. The last three of them are monotypic. The genus *Aldrovanda* consists of one species *A. vesiculosa* L., *Dionaea* consists of one species *D. muscipula* Ellis, *Drosophyllum* consists of one species *D. lusitanicum* L. and *Drosera* includes nearly 150 species (Rivadavia *et al*., 2003). Droseraceae are herbs with roots or rootless like *A. vesiculosa*, the traps are either composed of sticky leaf glands with slow enclosure of the prey by movement of the blade (*Drosera, Drosophyllum*), or steel-trap-like leaf blades with spring shut (*Aldrovanda, Dionaea*). Leaves usually show basal aggregation; they could be whorled and submerged (*Aldrovanda*). They are hermaphrodites and the flowers are solitary, or form inflorescences. Fruits are non-fleshy; dehiscent (nearly always), or indehiscent (*Aldrovanda*) and the seeds are copiously endospermic (Watson and Dallwitz, 1992 onwards)

3.7.2. Distribution ,habitat ,morphological and physiological variation among Populations

A. vesiculosa is endangered aquatic plant used in Aquarium (ornamental uses); it was first cited as *Lenticula palustris Indica* ín 1696 by Plukenet. In 1747 Monti described and named it *Aldrovandia* in honor of the Italian naturalist Ulisse Aldrovandi (1522-1605). Finally in 1753 Linnaeus used the name *Aldrovanda vesiculosa* which is now commonly known as the Waterwheel Plant (Breckpot, 1997). It is distributed across the World in patches, nowadays it became endangered, and some of its local races are extinct. It is extinct in Bangladesh, Denmark, France, Germany, Italy ,Japan and now it is endangered in Poland, Switzerland, and vulnerable in Bulgaria, China, Croatia, the Czech Republic, Hungary, and Russia (Kondo *et al.*,1997 ; Maldonado San Martín *et al.*, 2003) . The rootless plant floats just below the water surface,(free-floating) ,it occurs irregularly in shallow standing dystrophic waters like lakes, bog and fen, pools, backwater pools, and peaty fishponds with a low mineral nutrient

(nitrogen and phosphorus) concentration but a high $C0_2$ concentration beside sufficient preys, and enough light for optimum growth. Flowering of the European ecotype is very rare consequently seeds and reproduce vegetatively by apical branching of the shoots (Berta, 1961; Kaminski, 1987).

There are four main differences between the European temperate and the Australian (sub) tropical strains of *Aldrovanda*; they are the color, overwintering characteristics, axillary buds characters, and Boron sensitivities. Temperate Europeans are light green ,contain large amount plumbagin (a sulphur-yellow pigment) and no anthocyanin, more adapted to cold overwintering, they form axillary branches poorly, and mainly when their apices are damaged while the Australians are green, or rose to deep purple because they contain anthocyanins as well as plumbagin. The European and Japanese plants are light green and contain a great amount of plumbagin (a sulphur-yellow pigment) but no anthocyanins. The color of Australian plants may be green, or slightly rose to deep purple because they contain anthocyanins as well as plumbagin (Figure 8), more weakly dormant turions and during hot weather, they frequently form branches from axillary buds (Adamec, 1999). An exception is a red Hungarian population recently discovered (Borhidi and Járai-Komlódy, 1959; Adamec, 2005).

3.7.3. Low genetic variation among populations

The low genetic diversity among the accessions of *A. vesiculosa* is evident from three previous studies, the first used allozymes and it revealed no variation among plants from NE Poland, east Poland, and south Germany at 15 loci of seven enzymatic systems tested (Adamec and Tichý, 1997).The second study used 151 RAPD primers for amplifying DNA of plants from Europe, Japan and Australia. They found 21 primers of 151 tested primer with 79 polymorphic markers which confirmed the previous results (Maldonado San Martín *et al.*, 2003). A third comparative study was carried out to infer intraspecific diversity of six population from Australia, Italy, Poland, Ukraine and two from Japan (all plants were green except Australia was red) using sequences of *45S* ribosomal DNA and they revealed homologue sequences of the six populations, they concluded that *A. vesiculosa* was very conservative in its sequences (Hoshi *et al*., 2006).

A

B **C**

Figure (8)**: *A. vesiculosa* entire plant and the trap.** Entire plant from north Australia (B) grown in indoor aquarium (Adamec, 1999) , European (C) flowering at an artificial site at Karlstejn, S. Bohemia, Czech Republic and the trap (A). Photos are taken from http://www.butbn.cas.cz/adamec/ &http://www.carnivorousplants.org.

3.7.4. Phylogenetic relation among Aldrovanda vesiculosa, Dionaea muscipula *and* Drosera regia

Despite *A. vesiculosa* Plant similarity in leaf morphology to *D. muscipula* most taxonomists were classifying the *A. vesiculosa* within *Droseraceae* because of its floral similarities with *Drosera* more than *D. muscipula.* Now, *A. vesiculosa* is considered to be the closest relative of *D. muscipula*. Boesewinkel (1989) considered the ovules and seeds of *A. vesiculosa* to be more similar to those of *D. muscipula* than to any other taxon in *Droseraceae* also *A. vesiculosa* has similar trapping mechanism as *D. muscipula* (compare Figures 8 and 9) that they work by rapid transmission (6–17 cm/s) of action potentials between excitable cells but the traps are smaller and function under water in *A. vesiculosa* while the genus *Drosera* has an active flypaper traps and capture their prey with mobile glandular hairs that are present on the adaxial leaf surface and the velocity in some *Drosera* species are 10% slower. Cameron *et al*. (2000) sequenced the nuclear *18S* and plastid *rbcL*, *atpB,* and *matK* genes and concluded from morphological characters and the structure as well as from of the molecular cladogram that *Aldrovanda* was sister to *Dionaea*, and this pair was sister to *D. regia*. They also recommended that *Aldrovanda*, *Dionaea*, and *Drosera*, but not *Drosophyllum*, as members of Droseraceae, they also hypothesize that the snap-traps of *Aldrovanda* and *Dionaea* were derived from a common terrestrial ancestor. Rivadavia *et al.* (2003) confirmed the previous results by studying the combined *rbcL* and *18S* rDNA sequences and they concluded that, the snap traps of *Aldrovanda* and *Dionaea* are homologous despite their morphological differences.

Both of. *D. muscipula* and *D. regia* were used as outgroups in within species study of *A. vesiculosa* populations. The sequence information was further used to calculate genetic distances among the three species. Interesting hypothesis about partially independent evolution of chloroplast and nuclear DNA was suggested based on the results. In general, this work provided an insight into variation in organellar, particularly mitochondrial, genomes in higher plants and contributed to understanding the forces which influenced this variation.

A **B**

C **D**

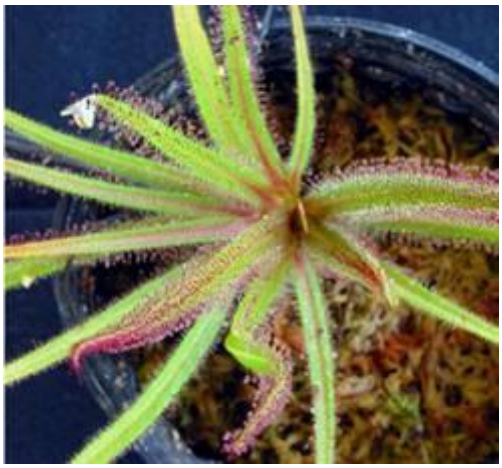


Figure (9): ***D. regia*** **(C),** ***D. muscipula*** **(D) entire plants and their traps (A&B, respectively).** Photos are taken from:http://www.carnivorousplants.org, http://www.jardin-botanique-lyon.com

Material &Methods

4. Material and Methods

4.1. Chemicals, equipments and software

4.1.1. Chemicals

Agarose	Serva,Germany
Metaphor	Cambrex Bio Science,USA
Chlorophorm	Fluka, Japan
D-Sorbitol	Sigma, USA
EDTA	Sigma, USA
Hydrochloric acid	LACHEMA, CZ
Isopropanol	Lach-Ner , CZ
Phenol	Lach-Ner , CZ
CTAB	USB, CZ
Sodium chloride	Lach-Ner, CZ
Sodium hydroxide	Lachema, CZ
Maleic acid	Acros Organics, CZ
Sucrose	Lach-Ner, CZ
Tris	Serva, CZ
Triton X-100	Serva, CZ
Agar	Serva, Germany
Anti digoxigenin-AP conjugate, Fab fragments	Roche, Germany
CDP-Star	Roche, Germany
DIG Easy Hyb	Roche, Germany
DNA Molecular-Weight Marker VII, DIG-labeled	Roche, Germany
dNTP	Fermentas, Lithuania
Glycerol	Sigma, USA
IPTG	ICN biomedicals, Germany
SDS	Fluka, Japan
Tween20	MP Biomedicals, France
X-Gal	MP Biomedicals, France
Na3Citrate.2H2O	Lachema, CZ
Lysozyme TypeVI	MP Biomedicals, France
Kristalon	(Hydro Agri Rotterdam, Holland; AGRO CS, ČR)
Lambda DNA/PstI Marker, 24	Fermentas, Lithuania
Baycor 25WP	Bayer, CZ
Omite	Opava, CZ

4.1. 2. Antibiotics

Ampicilin	Sigma, Germany
Tetracycline	Duchefa, Holland
Kanamycin	Sigma, Germany

4.1.3. Enzymes

Lysozyme Type VI	MP Biomedicals, France
Ribonuclease A From Bovine Pancreas	Sigma Aldrich, USA

4.1.3.1. Restriction enzymes

EcoRI	Fermentas, Lithuania
HindIII	Fermentas, Lithuania
SmaI	Fermentas, Lithuania
AluI	Fermentas, Lithuania
MspI	Fermentas, Lithuania
DdeI	Fermentas, Lithuania

4.1.3.2. Polymerases

Taq DNA polymerase	Promega, USA
Phusion high fidelity polymerase	Finnzymes, Finnland

4.1.4. Kits

BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems, USA
QIAquick® Gel Extraction Kit	Qiagen, Germany
QIAquick® PCR Purification Kit	Qiagen, Germany
pGEM-T® Easy kit	Promega, USA
PCR DIG Probe Synthesis Kit	Roche, USA
REDExtract-N-Amp Plant PCR Kits	SIGMA, USA

4.1.5. Media and buffers

4.1.5.1. Media for growing Bacteria

LB (Luria Bertani Broth) preparation was as following:

pH 7-7.5 (adjusted with 5 N NaOH)

Peptone (Trypton) 10g/L

Yeast extracts 5g/L

NaCl 10 g/L

According for Maniatis *et al*. (1986).

4.1.5.2. Media for growing plants

1:1:1 Coccopress: Vermiculite: Perlite

Coccopress	Serva-Seed, Slovakia
Vermiculite	Agro, CZ
Perlite	Agro, CZ

4.1.5.3. Buffers

1x TAE (1 L): 100 ml 0,5 M EDTA pH 8; 242 g TRIS; 57,1 ml concentrated CH3COOH

5x TBE (1 l): 0,45 M TRIS-borate (54g TRIS; 27,5 g Boric acid)

0,01 M EDTA (20 ml 0,5 M EDTA)

1x TBE: 5x dilution of 5x TBE

4.1.6. Equipments

Horizontal Electrophoresis	Owl separation systems, USA
ABI PRISM 3100 Genetic Analyzer	Applied Biosystems, USA
Centrifuge Hettich	Hettich, Germany
Crosslinker ULTRA-LŪM CEX-800	ULTRA-LŪM, USA
Deep Freezer -80	SANYO, Japan
Hybridization oven/shaker	Amersham, UK
ProBlot Hybridization oven	Labnet , CZ
Cultivation box Sanyo MLR-350HT	Sanyo Electronic ,Japan
T Gradient cycler	Biometra, Germany
XP Cycler	KRND,CZ
Hybridization tubes	Amersham, UK
Hybridization membrane Hybond N+	Amersham, UK
Hyperfilm	Amersham, UK
Whatman	Maidstone, UK

4.1.7. Software

Photo-communication system and software

PAUP* 4,0 b10 (http://paup.csit.fsu.edu/), (Swofford, 2000)

Primer3 (http://frodo.wi.mit.edu/)

VectorNTI software Suite 9 (InforMax, Inc.; Invitrogen, UK)

BioEdit (Hall, 1999), (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html)

MEGA4 (http://www.megasoftware.net/), (Kumar *et al*., 2004).

Kodak Digital Science 1D v.2.0.3 (Eastman Kodak Company, USA)

MODWEB (http://modbase.compbio.ucsf.edu/ModWeb20-html/modweb.html) (Pieper et al., 2006)

Cn3D 4.1 (www.ncbi.nlm.nih.gov)

PYMOL program (http://www.pymol.org/) (DeLano Scientific LLC)

DnaSp 4.9 program (http://www.ub.edu/dnasp/)

GDA 1 program (http://lewis.eeb.uconn.edu/lewishome/software.html)

4.1.8. Vector

pGEM-T Easy (Promega, USA)

4.1.9. Bacteria

E. coli XL-1 Blue: endA1, gyrA96, hsdR17, *lac* –, recA1, relA1, supE44, thi-1, [F' lacI qZ ΔM15, proAB, Tn10], tetracyclin resistant (Bullock *et al*., 1987)

4.2. Plant material

4.2.1. Silene vulgaris *natural population*

Ten *S. vulgaris* (Moench) Garcke (Caryophyllaceae) maternal plants from the population Kováry Meadows (Czech Republic), located on the hillside 10 km west of Prague, at the altitude 300 m (Štorchová and Olson, 2004) were used in the study(Figure 10). One branch carrying at least 15 mature capsules from each individual was sampled by H.Štorchová. The leaves of each individual were also sampled. The DNA of the maternal plants was extracted by H. Štorchová before starting my research. Unfortunately, the DNA did not reach the quality allowing reliable Southerns. so it was used for PCR analysis only to provide comparison with progeny. The seeds obtained from these ten mother plants were grown by H. Elansary (April 2006) in the greenhouse of the Institute of Experimental Botany. These seeds produced a population (Kovary population) composed of 331 individual resulted from natural pollination.

4.2.2. Controlled crossed between S. vulgaris *Kovary and geographically distant populations*

The first group of controlled crosses was done between the population of S. *vulgaris* from Krnasnjarsk (Krn) in south central Russia near Lake Baikal and *S. vulgaris* Kovary (Kov) natural population previously studied here. The second group of crosses was done between Moutain view (MV) in Virginia (USA) and Kovary (Kov) population (Czech Republic) were done in the greenhouse of the Institute of Experimental Botany AS CR, Prague by H.Elansary (Figure 10). Controlled crosses within Kovary population was conducted also but the detection of paternal transmission of mtDNA was not possible in it because of the lack of specific mtDNA PCR markers the can detect the paternal inheritance within this population. The focus here will be on the controlled crosses either between Kov X Krn or KovX MV. Both of Krn or MV populations bear easily detectable mtDNA marker which could be easily detected by PCR.

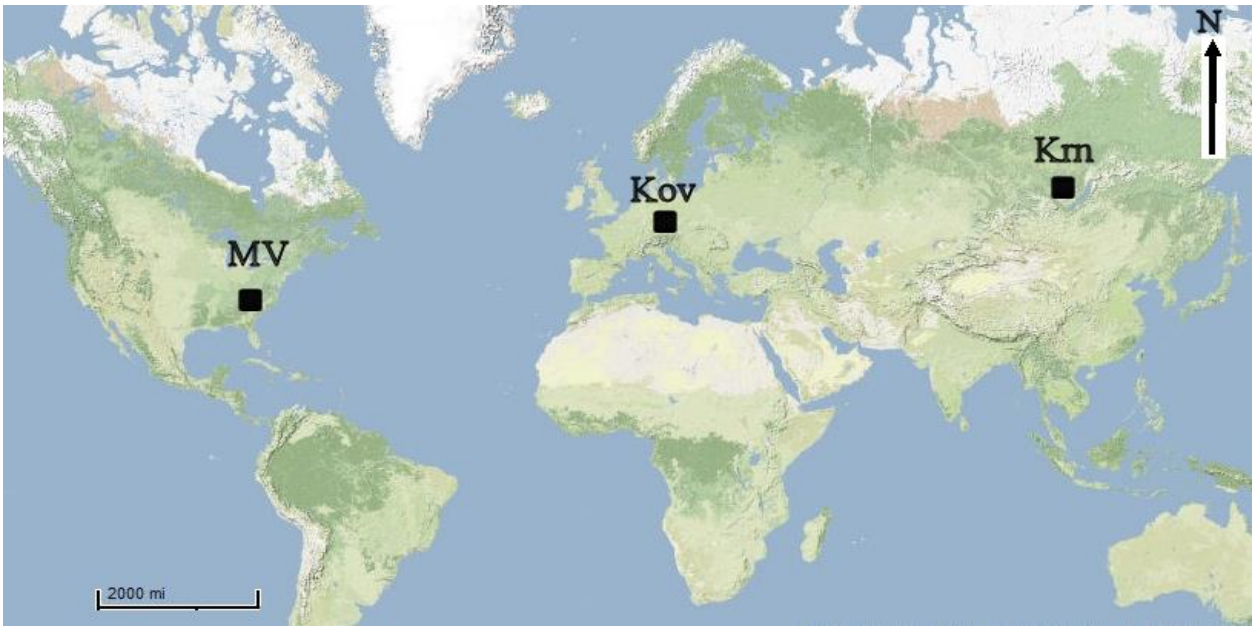


Figure (10): The location of the plants used for controlled crosses.

In total, 1007 individuals distributed among 29 crosses were analyzed by PCR (Figure11) and some of them by Southern Hybridization for detecting paternal transmission, vegetative sorting and determining sex ratio.

In KrnXKov crosses the offspring (631 individuals) was screened using several primer pairs to detect paternal transmission including (BobKr_188For- BobKr_393Rev; Cob443R-putORF atpA 56F2; InvBobAtp_For1- InvBobAtp_Rev2) (Table 2).

In MVXKov crosses the offspring (376 individuals) was screened using one primer pair (putORF atpA 56F2-putORFatpA 279R2) (Table 2). The primers which were used were specific for a chimeric gene. Most of the offspring were screened with two or three primers to be confident of the result of the screen.

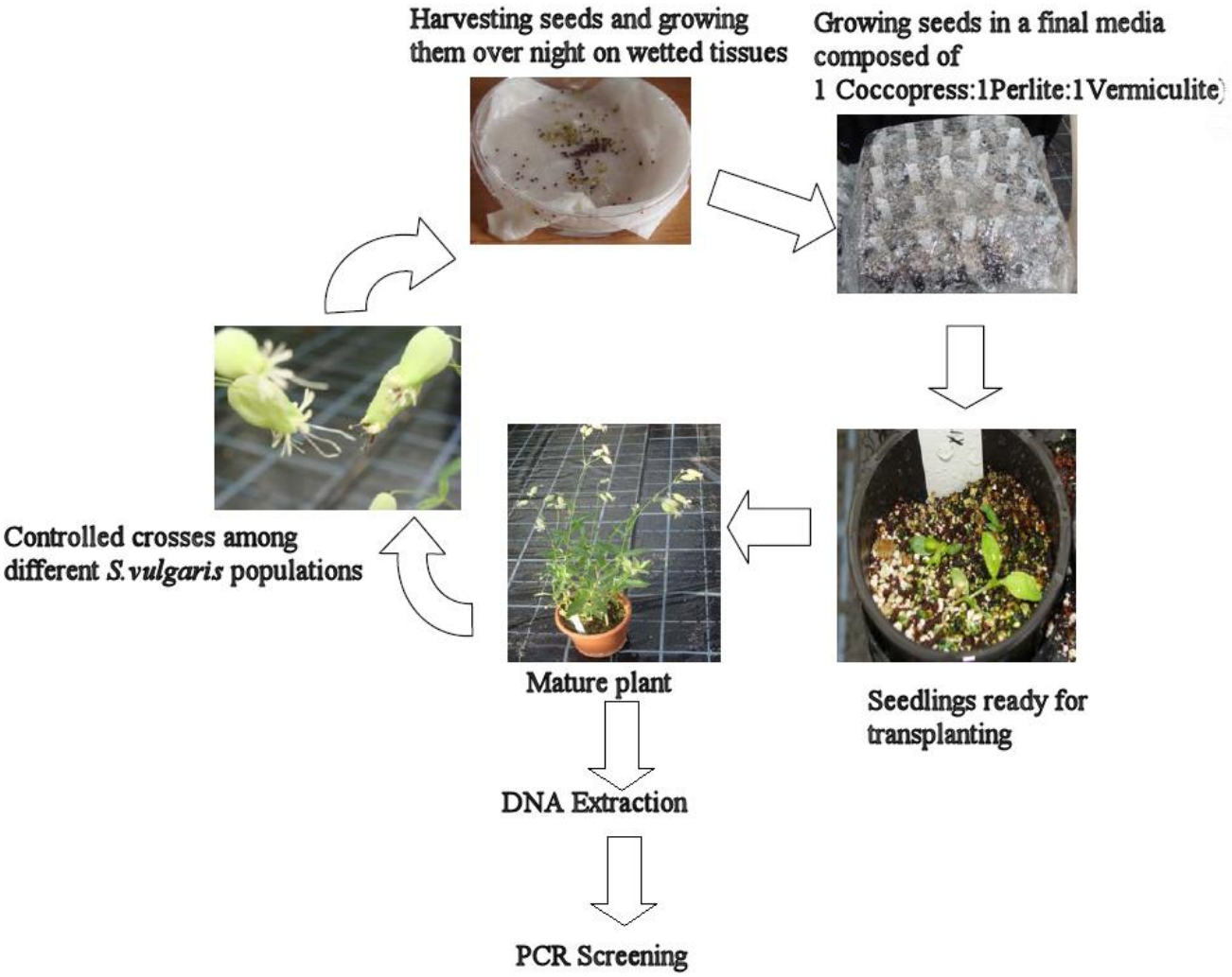


Figure (11): **Schematic theme for controlled crosses among different *S. vulgaris* populations**. DNA extraction and PCR screening for the purpose of the detection of paternal transmission.

4.2.3. S. latifolia *from Europe*

Leaves were sampled (by G. Bernasconi) from 48 plants from eight populations in Northern Europe (Figure 12), four populations from Sweden [Norra Åsum (N), Jönköping (J), Kåseberga 1 (K) and Gärds Köpinge (G)], two from Netherlands [Tolkamer (T) and Renkum (R)] , one from Denmark [Århus (A)] and one from Germany [Stechendorf 2 (S)]. The leaves were dried and stored in silica gel. DNA was extracted (in Prague by H. Elansary) from dried leaves. The DNA was analyzed by Southern-RFLP and PCR-RFLP using the same markers previously used with *S. vulgaris*. The aim was to compare mt and cpDNA polymorphism and mt and cpDNA haplotypes association of dioecious plant (*S. latifolia*) and the gynodioecious (*S. vulgaris*).

Figure (12): **Geographic location of *Silene latifolia* populations studied.** GPS co-ordinates were: N, 55°59'117" N/14°09'506" E; J, 57°46'356" N/14°09'.464" E; K, 55°22'919" N/14°03'613" E; G, 55°56'709" N/14°08'853" E; T, 51°51'.491" N/06°05'157" E; R, 51°59'512"N/05°46'353" E; S, 49°54'535" N/11°19'217" E; A, 56°14'745" N/10°08'522" E. Black dots represent the location of the population. (The Photo is created by H. Elansary).

4.2.4. Aldrovanda vesiculosa *L. accessions.*

A unique world-wide collection of 15 accession of *A. vesiculosa* is kept at the Institute of Botany, in Třebon, Czech Republic as shown in Table (1) (Adamec, 1997a, b). It comprises plants originating from Europe, Japan, Australia and Africa in addition for *Drosera regia* and *Dionaea muscipula. D. regia* and *D. muscipula* were intended to be used as an out-group for making phylogenetic tree. The availability of cultivated plants from four continents allowed the estimation of the genetic variation within cp and mtDNA of this endangered species using molecular tools. *Aldrovanda v.* accessions, *D. regia and D. muscipula* plants used are listed in Table (1).

Table (1): *Aldrovanda v.* accessions, *D. regia and D. muscipula* locations and references.

Abbreviation or species	Country of origin	Site of collection	Latitude	Longitude (◦E)	Year of collection	No. of collected plants	Start of cultivation at Třebon	No. of stocked plants	Reference or vaucher
EP	East Poland	Lake Długie, Łeczna-Włodawa Lake District	51º26N	23º06E	1993	31	1993	31	Kamiński (1987)
UK	North UKrnaine	Western bank of the Kiev reservoir (on the Dn'epr river) near Stracholes' village, at the T'et'erev river estuary	51º03N	30º25E	1997	6	1997	6	Adamec (1995)
HU	SW Hungary	Lake Baláta-tó, Somody County	46º19N	17º12E	2003	12	2003	12	Borhidi and Járai-Komlódy (1959)
LI	Lithuania	Lake Ruzhas in the Ignalina district, NE Lithuania	55º30N	25º28E	2003	10	2003	10	Vilkonis (2003)
RO	Romania	Shallow lake on Obretin island near the Sulina branch in the Danube delta	45º11N	29º19E	1998	20	1998	20	Maldonado San Martín et al. (2003)
SW	Germany (Switzerland)	Artificial site, Lake Metmenhaslisee near Kloten in the Zurich Canton, Switzerland; plant origin from pool Bühlweiher at Lake Constance near Lindau, SW Bavaria, Germany	47º34N	9º41E	1994	2	1994	2	Koch (1950)
SEA	SE Australia	Longvale Swamp near Broulee at Batemans Bay at the East Coast, NSW	35º35S	150º09E	1997	12	1997	12	Adamec (1999a)
NA	North Australia	Girraween Lagoon, ca. 30 km SE of Darwin, NT	12º31S	131º05E	1994	2	1998	3	Wilson (1995); Adamec (1999a)
SWA	SW Australia	Coastal lake near Esperance, W Australia	33º48S	121º49E	2002	5	2002	5	Gibson (2004)
KA	North Australia	Leach Lagoon near Katherine, NT	14º38S	132º37E	2003	5	2003	5	--
AR	Australia Armidale	Billybung Lagoon near Gyura, Armidale County, NSW	30o06S	151o47 E	2006	3	2006	3	--
NR	North Russia	Shallow lake near the estuary of the Sviri river on the southeastern bank of Lake Ladoga	60º29N	32º57E	1997	8	1997	8	Afanas'ev (1953)
JPT	Japan, Tokyo	Hozoji pond near Hanyu City in Saitama Prefecture, Honshu island	36º12N	139º42E	1980s	?	1993	12	Komiya (1966)
JPK	Japan, Kyoto	Lake Ogura-Ike, Uji near Kyoto (plants from Osaka Botanical Garden, Japan)	35º01N	135º47E	1955	?	2001	8	--
AF	Botswana	Shallow swamp in Okavango delta	19º33S	23º13E	2005	12	2005	12	Obermeyer (1963-1988)
DM *Dionaea muscipula*	SE USA	Liberec Botanical Garden, Czech Rep.	?	?	?	?	--	--	CPV1d
DR *Drosera regia*	South Africa	Liberec Botanical Garden, Czech Rep.	?	?	?	?	--	--	CPD74

4. 3. Growing plants

All *S. vulgaris* plants in the study were grown by H.Elansary in a glass greenhouse at the Institute of Experimental Botany AS CR, Prague. Seeds were grown first on Petry dishes covered by wetted tissues overnight for the stimulation of the embryo and weakening of the seed coat. Then they were grown in plastic pots on the surface of a media (1 Coccopress: 1 Perlite: 1 Vermiculite) treated with Fungicides then covering the pots with transparent plastic covers. Seeds start to germinate after 48-96 hrs from growing under high humidity conditions.

After germination, the seedlings are fertilized with compound fertilizer (Kristalon 15-5-30+MgO) (1000 ppm). After 2 weeks, Seedlings are transplanted into separated plastic pots. Plants were grown under long day conditions until flowering and determining gender. Flowers with at least two anthers were considered as hermaphrodite. If the individual changed its gender during the course of cultivation, so the shifting plants (from female to Hermaphrodites or vise versa) were determined. In *S. vulgaris* natural population the gender was determined during two years observation period.

In *S. vulgaris* controlled crossed the gender was determined twice to extinguish the shifting plants (if any). Crosses were done using defined parents in a special area in the greenhouse with special care toward using suitable insecticides to eliminate insect driven pollination. Seed capsules were collected from mother plants in the greenhouse after 3-4 weeks from pollination. The seeds were dried then grown as mentioned before.

4. 4. DNA extraction from leaves

A sorbitol extraction method (Štorchová *et al*., 2000) was used to purify DNA from 100 mg of fresh leaf tissue from a single leaf, or two paired leaves from the same branch in *S. vulgaris*. To determine if there is within-individual variation in the same plant using the southern-RFLP, two samples of total genomic DNA were extracted with the same method from two different branches from the same plant in the same time. This experiment was done on 11 individuals of family Kov52 and five individuals from the family Kov45. *S. latifolia, A. vesiculosa* accessions, *D. regia* and *D. muscipula* were extracted with the same method.

The details of the protocol are:

Extraction buffer:

0.34 M D-Sorbitol,

0.1 M Tris-HCl pH 7.6,

5mM EDTA,

0.2% v/v β-mercaptoethanol

Lysis buffer:

0.2 M Tris-HCl pH 7.56,

2M NaCl,

0.05 M EDTA,

2% CTAB

Chloroform: isoamylalcohol

Chloroform is mixed with isoamylalcohol in a proportion of 24:1

Isopropanol (-20º C)

80% EtOH

1. Frozen leaves were ground to a fine powder in liquid nitrogen in a mortar using a pestle.
2. Immediately, the powder was suspended in an extraction buffer, adding 1ml for 0.1 g of tissue; after which the homogenate is incubated for 10 minutes at RT in 2ml Eppendorf and centrifuged at 8000 rpm/5min at RT.
3. The supernatant was then eliminated and the pellet is suspended in 0.3ml extraction buffer. Then 0.3ml lysis buffer were added and mixed. Samples were incubated at 65ºC/30min in a thermo-block.
4. The tubes were then left to cool down to RT, about 10 min. Protein were extracted by mixing the tube contents with 0.6ml of chloroform: isoamylalcohol, which was carefully added in the hood, due to the volatility of the chloroform. The mixture was then centrifuged at 7000rpm/5min at RT.
5. The upper phase was carefully transferred into a new micro tube, trying not to mix the two phases that formed.
6. Two thirds volume of ice cold isopropanol was added and mixed well by hand. The extract was stored at -20º C for at least 30 min.
7. The tubes were centrifuged at 13000rpm /15min at -4º C.
8. The pellet was then washed with 80% EtOH to remove residuals of salts and centrifuged again under the same conditions; supernatant is eliminated and samples are dried briefly at RT.
9. The DNA was dissolved in approximately 40ul SDW and left overnight at 4º C to complete dissolving.

4.5. Extraction of PCR fragments from Gel

DNA is run on agarose gel and checked on the U.V. transilluminator, in order to observe when the band with the size of interest is well separated, and then the fragment is excised from the gel with a clean sharp scalpel. The intensity of U.V. light was decreased in order to prevent DNA damage of the material to be cloned. Then the DNA was extracted and purified from the agarose gel following the procedure of QIAquick Gel Extraction Kit (QIA Gene,

Cat. No.28704). Briefly, after weighing the gel slice in a micro tube, three volumes of buffer QG are added to one volume of gel. The mixture is incubated at 50ºC for 10 min. or until the gel is melted. Then the sample is placed in a spin column and centrifuged at 13000 rpm. 0.75 ml of Buffer PE are then added to the spin column and centrifuged again. The spin column is then placed into a clean micro tube, adding 30ul of water and storing the sample at -20.

4.6. Gel Electrophoresis

Gel electrophoresis was done in OWL apparatus using ethidium bromide staining in 1XTAE buffer. It was used to examine the quality of DNA extracted samples, PCR products and for Southern-RFLP.

4.7. Determining DNA concentration

This step was done for samples intended to be analyzed by Southern-RFLP and for direct sequencing of PCR products. Using a U.V. spectrophotometer:
1. First dilute your DNA (I usually use 2.5 uL in 497.5 uL).
2. Then adjust the spectrophotometer at 260 nm with your blank.
3. Measure your DNA sample.
4. You can then use the following formula to estimate the DNA concentration:
concentration = 260 reading * Absorbance factor * dilution factor (in my it is 200)

The Absorbance Factor of
ssDNA: 37 µg/ml
dsDNA: 50 µg/ml
ssRNA: 40 µg/ml

4.8. Cloning of PCR products

Cloning of PCR products was adopted for the purpose of sequencing long stretches of nucleotides otherwise direct sequencing was used. Cloning was used for the purpose of

sequencing fragments of *atp1* and *cox1* genes in *S. vulgaris*, *atp1* gene in *A. vesiculosa*, *D .regia*, *D. muscipula*.

The following steps were done:

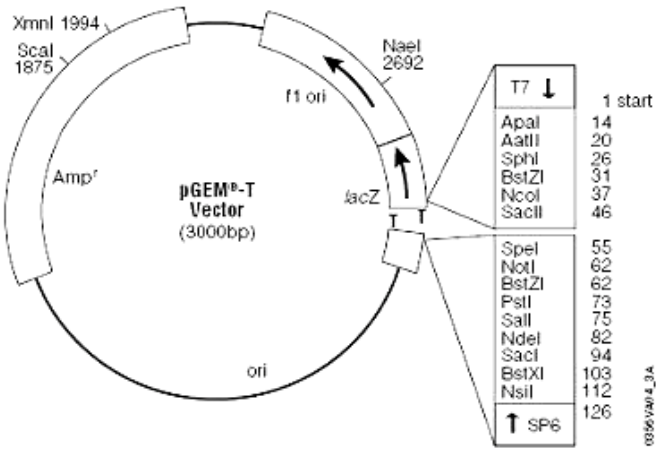
4.8.1. Preparing PCR fragment

PCR fragments which don't have visible primer dimmers (on gel) were first cleaned using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). In case that the PCR product contains primer dimmers or other products, the fragment of interest is sliced from gel using QIAquick Gel Extraction Kit. Final cleaned PCR product in both cases was eluted in a final volume of 30uL to increase the concentration of the PCR product and increase the effectiveness of the ligation. Finally the 30ul are once again concentrated by heating on 55ºC/20min.

4.8.2. Ligation

After purification, PCR product was cloned into pGEM-T Easy Vector (Promega), following the protocol provided in the technical manual. The vector contains a 3̀ terminal thymidine at ends, enabling the insertion of the PCR product with oligo A overhangs, and preventing recircularization as well as multiple restriction sites that allow for the release of the insert (Figure 13). The reaction was set up as follows:

Components	Standard reaction	Positive control
SDW	------	1ul
2x rapid ligation buffer	5ul	5ul
pGEM-T Easy Vector 50ng	1ul	1ul
PCR product	3ul	-----
Control insert DNA	----------	2ul
T4 DNA ligase (3Weiss unites/ul)	1ul	1ul
Final volume	10ul	10ul



pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
*lac*Z start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

Figure (13): **pGEM-T Easy Vector circle map and sequence reference points.**

The reaction was incubated 15 hrs/4ºC then 15 min/65ºC in TRIO Thermoblock. Finally we get the ligation mix.

4.8.2. Preparation of competent cells

The following *E.coli* strain was used:

E. coli XL-1 Blue: endA1, gyrA96, hsdR17, *lac* –, recA1, relA1, supE44, thi-1, [F' lacI qZ ΔM15, proAB, Tn10], tetracyclin resistant (Bullock *et al*., 1987)

5ml culture of *E.coli* in LB medium is incubated overnight at 37ºC in a rotary shaker.

1- The culture is then added to 100 ml of LB medium and cultivated for 3 hrs at 37ºC, until OD at 650 wave length =0.3-0.4.
2- Cultures are then chilled on ice for 10 min. and centrifuged at 4000rpm/10min. at 4ºC.
3- The pellet is resuspended in 400ul of 0.1 M $CaCl_2$, kept on ice for 25 min. and then centrifuged at 4000rpm for 10min at 4ºC.
4- Pellet is again resuspended in 2 ml of 0.1M $CaCl_2$, and then 0.4ml of sterile glycerol to make 20% final solution.
5- The cells are immediately frozen in liquid nitrogen and stored at -70ºC. Cells shouldn't thaw.

4.8.3. Transformation of Competent cells

Competent cells were heat-shock transformed with PCR products ligated to the vector, following the protocol described by Clark (1997):

1- Competent cells are thawed on ice for 10 min.
2- From ligation mix, 20ng/ul are used for transformation, in this case, 1 ul of ligation mixture for every 30ul of competent cells culture. It's left on ice for 10-20min.
3- Cells are then heat-shocked at 42ºC / 2 min.
4- Cells are left 2 min. on ice.
5- Culture is then transferred to 900ul of LB medium and left 1 hr in the shaking incubator at 37ºC for recovery.
6- Cells are centrifuged at 3500rpm/3 min, supernatant is removed, leaving about 100ul, which is pipetted to a plate containing LB medium with Ampicillin (0.05mg/ml).
7- They are left to grow overnight at 37ºC.
8- White colonies are counted dividing the plate in four parts to simplify the estimation, and some of them chosen to be further inoculated into a new plate containing LB medium with Ampicillin, and left to grow overnight at 37ºC.

4.9. Plasmid isolation

4.9.1. Using CTAB method

Plasmids were isolated following the protocol described by Del Sal *et al*. (1989).

1- Each colony is inoculated into liquid LB medium with Ampicillin (50ug/ml) and left to grow overnight in shaking incubator at 37ºC.
2- Samples are then centrifuged at 3000rpm/4 min at 4ºC.
3- The pellet is then resuspended in 200 ul of STET. Ten ul of lysozyme (50mg/ul) are added, mixed by hand and left at room temperature (RT) for 5 min.
4- Microtubes are put in boiling water for 45s.
5- Samples are centrifuged at 12000rpm for 10min at RT.
6- The pellet is eliminated using toothpick and the rest is incubated with 10ul of RNAse (10ng/ul) for 10min at 55ºC.
7- Then 10ul of 5% CTAB are added, the mixture is now shortly vortexed, left at RT for 10min.
8- Samples are centrifuged at 12000rpm for 5 min at RT.
9- The pellet is then resuspended in 300ul of 1.2 M NaCl and 750 ul of EtOH 96%, vortexed and incubated for 10min at RT.
10- Samples are centrifuged 10min at 13000rpm at 8ºC.
11- Supernatant is eliminated and 500ul of 80% EtOH are added.
12- Samples are centrifuged again under the same conditions for 15 min.
13- After supernatant is eliminated, samples are left to dry at RT.
14- They are then resuspended in 20ul of TE buffer and left at 7ºC overnight.
15- Finally 3ul are loaded in 1% Agarose gel to check the concentration and quality of plasmid DNA obtained.

STET 8% sucrose
50 mM Tris-HCl, pH 8,0
0,1% Triton X-100
50 mM EDTA

5% CTAB 5g CTAB
In 100 ml 0,5 M NaCl

Digestion with restriction nucleases was then followed to check if the plasmid contains the fragment of interest, restriction digestion with endonucleases was performed using EcoRI and according to the following protocol:

SDW 14ul

EcoRI buffer 2ul

Plasmid DNA 3ul

EcoRI enzyme 1ul

Final volume 20ul

Finally, run the samples on 1.8% Agarose gel to check the molecular weight of the insert beside Molecular Marker.

4.9.2. Using rapid toothpick isolation for examining insertions in plasmid

1- Preparing lytic solution by mixing of 275 ul sterile water, 25 ul 1M Tris pH 8.0, 50 ul 0.5 M EDTA, 50 ul RNAse A (10 mg/ml), 100 ul lysozyme in water (5 mg/ml))(Keep frozen -20).

2-Pipette 15 ul per sample, using a sterile toothpick touch 1 colony and resuspend it in the lytic solution, incubate 5 min. at room temperature, then add 2 ul of equilibrated phenol (phenol is warmed on 50ºC), briefly vortex, centrifuge 2 min. at 12000 rpm. Load 8 ul taken from surface. Run with the loading dye on 0.7% agarose, 1xTAE.

4.10. Southern- RFLP

RFLP variation was assessed for the HindIII and EcoRI restriction sites flanking *atp1* and *cox1* mitochondrial genes in two separate assays in the case of *S. vulgaris* and in *S. latifolia* but in *A. vesiculosa* EcoRI restriction enzyme only was applied.

The protocol was developed originally by Southern (1975) with modifications. The technique adobted in this study utilize DIG hybridization and chemiluminescent detection system. The advantages of using the DIG system include avoidance of using radioactive labels, ease of probe preparation, extended storage and multiple use of the same probe.

One of the advantages of this protocol is the use of post-hybridization washes of very high stringency, then the bands achieved using this method cannot represent non specific targets (in case of using target specific primers). These bands identify *cox1* or *atp1* homologs, either full length or truncated, that are present either in the nucleus or in the mitochondrion, although the location in chloroplast cannot be also excluded. If there's no restriction sites of the enzymes

used (EcoRI or HindIII) within the gene of interest (*atp1* or *cox1*), therefore one Southern band corresponds to one gene copy (full length or truncated).

The procedure of Southern here, is designed for blotting an agarose gel onto positively charged nylon membrane. The protocol is divided into 3 stages. **First**, preparing the DNA samples and running them into agarose gel, and before blotting, the agarose gel is pretreated by soaking in a series of solutions that depurinate, denature, and neutralize the DNA and gel matrix. Then the DNA transfer itself, which occurs by upward capillary action. Then DNA is immobilized on the nylon membrane by U.V. irradiation. **Second**, Chemiluminescence detection of DIG-labeled probes with alkaline phosphatase labeled antibodies. **Third**, stripping step to facilitate further detection using different probes with the same membrane. The following is the description of each step.

4.10.1. Preparing DNA fragments, blotting and immobilizing DNA by U.V.

1- Determine DNA concentration.

2- Digest the DNA samples (as shown in the following protocol) with appropriate restriction enzyme, run in an agarose gel (0.7- 0.9% 1X TBE) with appropriate DNA size markers, stain with ethidium bromide, and photograph with a ruler laid alongside the gel (so that band positions can later be identified on the membrane).

Component	Volume in ul
Water	9
Buffer	3.5
Enzyme (EcoRI or HindIII)	2.5
Total genomic DNA	20
Total reaction volume	35

3- Rinse the gel in distilled water and place it in a clean plastic or glass tray containing in Denaturation buffer twice for 30 min. Wash it with distilled water for 10 min. Shake the gel in neutralization buffer twice for 30 min. Shake in 20xssc for 10 min.

4- Put the gel on wetted 3MM Whatman bridge (Figure14). Squeeze out air bubbles by rolling a glass pipet over the surface.

5- Cut a piece of nylon membrane just large enough to cover the exposed surface of the gel and place it on the gel. Cut three sheets of Whatman 3MM paper to the same size as the membrane and place these on top of the membrane. Squeeze out air bubbles.
6- Cut paper towels to the same size as the membrane and stack these on top of the Whatman 3MM papers to a height of 10 cm.
7- Lay a glass plate on top of the structure and place a weight on top to hold everything in place. Leave overnight.

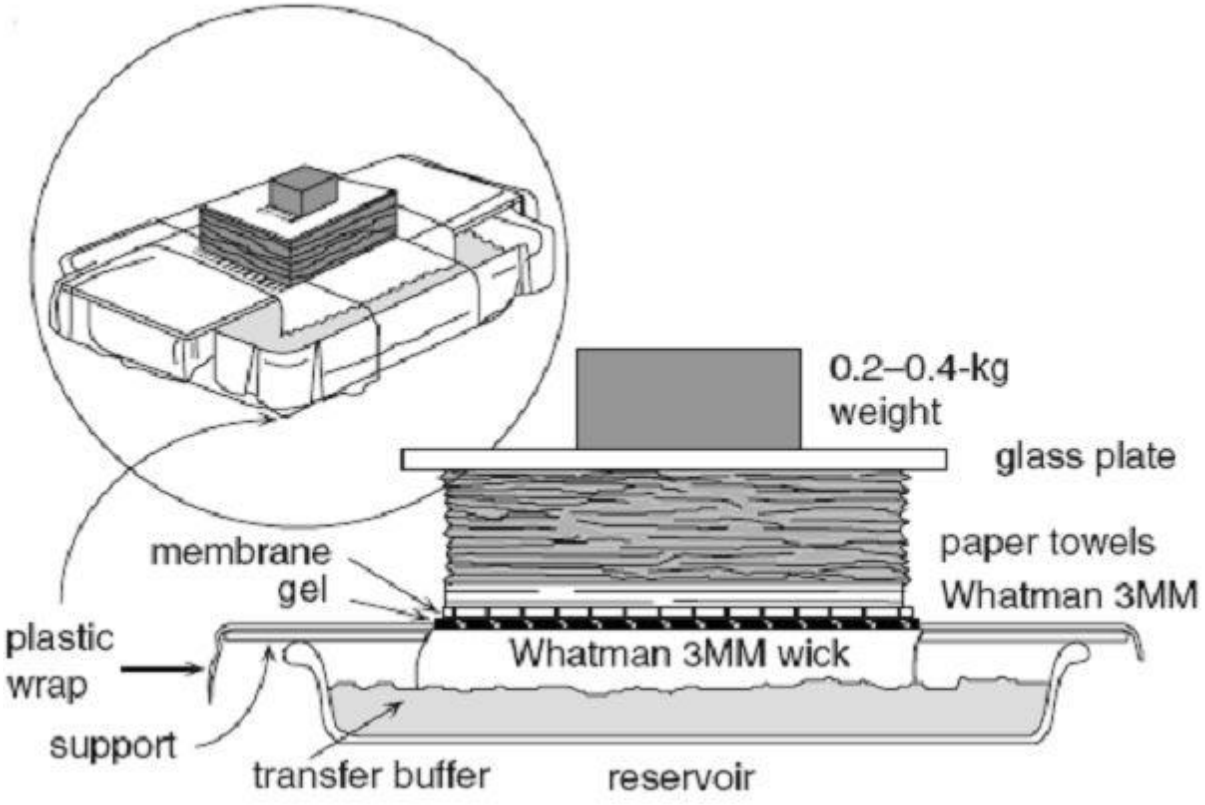


Figure (14): **Southern Hybridization technique used in the study.** The photo taken from Brown (2004).

8- In the second day, disassemble the transfer pyramid.

9- Remove the paper towels and filter papers and recover the membrane. Mark in pencil the position of the wells on the membrane and ensure that the up-down and back-front orientations are recognizable.

11- To immobilize the DNA, on a wetted Whatman piece, crosslink the membrane in the U.V. illuminator (the energy and time depend on the machine).

12- Rinse the membrane directly in water for one min and then let it dry for 1 hr inside a pocket of Whatman.

4.10. 2. Probe Digoxigenin labeling using PCR and detection

4.10.2.1. Probe preparation

Three probes were prepared in this study *atp1*, *cox1* and *cob*. The first two probes were applied for *S. vulgaris* and *A. vesiculosa* and the three of them were applied for *S. latifolia*. Probe labeling was done using the PCR DIG probe Synthesis Kit (Roche, USA).

The PCR mix in *atp1* probe is as follows:

Component	Dig labeled probe (ul)	Control
Water	16.4	16.4
dNTP 2mM	1.25	2.5
Dig NTP	1.25	------
10x Buffer with MgCl	2.5	2.5
atpUp	0.6	0.6
atplo	0.6	0.6
High fidelity polymerase (Roche)	0.4	0.4
DNA	2	2
Total volume	25	25

The PCR mix in *cox1* probe is as follows:

Component	Dig labeled probe (ul)	Control (ul)
Water	16.2	16.4
dNTP 2mM	1.25	2.5
Dig NTP	1.25	------
10x Buffer with MgCl	2.5	2.5
cox1 F28	0.7	0.6
cox1 R1.6K	0.7	0.6
High fidelity polymerase (Roche)	0.37	0.4
DNA	2	2
Total volume	25	25

The PCR mix in *Cob* probe is as follows:

Component	Dig labeled probe (ul)	Control (ul)
Water	16.8	16.4
dNTP 2mM	1.25	2.5
Dig NTP	1.25	------
10x Buffer with MgCl	2.5	2.5
cobIP59	0.4	0.6
cobIP64	0.4	0.6
High fidelity polymerase (Roche)	0.4	0.4
DNA	2	2
Total volume	25	25

PCR conditions and primers used are in Tables 2 and 4. After finishing the PCR, the quality of the probe was examined by running (5 ul probe+5ul SDW water+1.2 ul loading die) on 0.9 % agarose and examining that the probe used has higher M.W. than the control which is not labeled with Digoxigenin. Figure (15) illustrate the DIG incorporation during PCR technique which was used in this study.

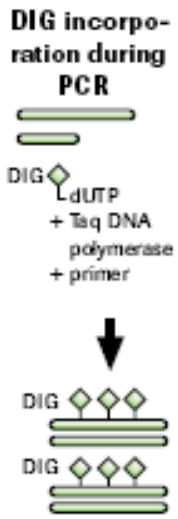


Figure (15): Probe preparation during PCR. Photo is taken from Roche Applied Science Manual.

After preparing the probe using PCR, the following was done:

1. 18 micro liter PCR product +100 ul double distilled water (the amount depend on many factors)
2. keep the probe tube in boiling water for 10 min
3. Put the tube directly on ice for 10 min.
4. Add to 8 ml of Dig Easy Hyb granules solution preheated on 42ºC.

The probe is then used directly for hybridization or stored in -20ºC. In case that the probe was used before, heat it for 10 min on 68ºC in water path, so it's suitable for using again.

4.10.2.2. Detection

For chemiluminescence detection of DIG-labeled CDP-Star (alkaline phosphatase labeled antibodies) was used (Figure16). This substrate belongs to the group of the dioxetane phenyl phosphates and after dephosphorylation by alkaline phosphatase, an intermediate is formed whose decomposition results in light emission which can be recorded on X-ray film. The

preparation of antibody, blocking buffer, detection buffer, denaturation buffer, neutralization buffer, due and fixation solutions are in Appendix E.

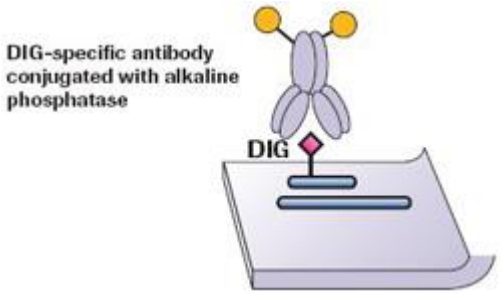


Figure (16): Detection principle. The photo is taken from Roche Applied Science DIG-labeling manual.

1. The membrane is prehybridized with 20-40 ml of preheated (42ºC) Dig Easy Hyb (the volume depend on the size of the membrane) in the oven on 42ºC for 3 hrs.
2. Pour off the Dig Easy Hyb and hybridize with the probe. Let it overnight.
3. Pour off the probe and start the stringency washes.
4. Wash twice 5 min in 2x SSC containing 0.1% SDS at 42ºC to remove nonspecifically bound probe.
5. Wash twice 15 min in 0.1x SSC containing 0.1% SDS at 65ºC to remove undesired hybrids of low homology.
6. Pour off the previous washing solution and then add blocking buffer 1x and shake for 30min on RT.
7. Pour off blocking buffer then add the antibody and shake for 30 min.
8. Pour off and add detection buffer for 5 min.
9. Pour off and pipet the CDP* solution on the membrane, incubate for 5 min then discard the excess CDP* and cover it with plastic and expose the membrane for X-ray film <1hr.

4.10.3. Stripping and reprobing the membrane

1. Rinse the membrane in distilled water for 1min.
2. Wash with 0.2 M NaOH/0.1% SDS, 2 x 15 min on 37° C.
3. Rinse in 2x SSC, 5 min. Cover the membrane with plastic foil and store it in 4° C until the next use.

4.11. PCR-RFLP

The technique was used to distinguish variation within coding regions of *atp1* and *cox1*. Based on the sequencing of *atp1* and *cox1* genes, enzymes were chosen to cut PCR fragments of these genes and visualized on gel electrophoresis. PCR-RFLP was applied for *atp1* and *cox1* PCR products in *S. vulgaris*, once using AluI and once MspI for *atp1*, but using MspI& DdeI (double digest) for *cox1* gene PCR product. It was also applied using EcoRI for *cox1* PCR product in *S. latifolia* in the same method as *S. vulgaris*.

The following steps were done using total genomic DNA prepared as described before:

1. I amplified the two mitochondrial genes, *atp1* and *cox1*, mtDNA markers portions of two mitochondrial genes, *atp1* and *cox1* (using amplification conditions as described in Table 3) from all the 331 offspring of *Silene vulgaris*.
2. A 10 ml aliquot of the *atp1* PCR product was digested once with AluI and once with MspI. The resulting fragments visualized by electrophoresis on a 3 % Metaphor agarose gel stained with ethidium bromide.
3. A 10 µl aliquot of the *cox1* PCR product was double digested with MspI& DdeI, the digestion products were visualized by electrophoresis and ethidium bromide staining.
4. Observed differences between individual *atp1* sequences included the gain or loss of several AluI restriction sites and MspI restriction sites were documented, and the same method were applied for *cox1* PCR digested products.

4.12. Heteroplasmy detection by PCR-RFLP"knock back"

The PCR-RFLP approach developed by McCauley *et al*. 2005 was used to reveal additional rare copies of the *atp1* (heteroplasmy) in two families in *S. vulgaris* population Kov45 and Kov52. Sequencing of *atp1* molecule revealed that there's three haplotypes A, B and C. Haplotype A differed than others that it has SmaI restriction site, so it was possible to detect heteroplasmy in all the individuals carrying haplotype A (families Kov45 and Kov52). Suitable restriction sites in *atp1* genes were not available in B and C haplotypes in the remaining families, preventing application of "knock back" approach.

The following protocol was used:

1. Digesting (200ng) 1ul genomic DNA of each individual with SmaI enzyme then using the digest as a template for *atp1* PCR amplification

2. In case that there's no PCR product it was scored as homoplasmic individual for haplotypes B and C but in case that there were PCR product, the PCR product was digested with SmaI again
3. To confirm that this PCR Product is the *atp1* molecule of haplotype B or C.
4. In case that the PCR product is knocked down it was scored as homoplasmic individual and that the first product was formed because of partial digestion otherwise it was scored as heteroplasmic individual. Control sample carrying haplotype A was used as a control after replacing the enzyme with water then using it for PCR amplification.

Reaction conditions for DNA digestion were:

DNA	1ul
Water	16ul
10X Buffer Tango	2ul
SmaI	2ul
Total volume	20ul

Reaction conditions for PCR digestion were:

PCR	10ul
Water	18ul
10X Buffer Tango	2ul
SmaI	2ul
Total volume	30ul

Total volume was mixed gently, span down for few second and incubated for 16 hrs on 30° C.

4.13. Direct Sequencing

Direct sequencing reaction using Cycle Sequencing (Figure 17) was applied in *Silene vulgaris* population. PCR products of *atp1* from 10 representatives of the ten families, *psbA-trnH* of 36 plants of *S. vulgaris* (2-3 per family) and for confirming the sequence of the internal part of *atp1* gene.

The technique was also used in *A. vesiculosa atp1* gene sequencing (to confirm the sequence of cloning) and in 6 chloroplast loci including *trnS-trnG*, *trnP-trnW*, *Cpa-Cpb*, *rpl20-5'-rps12*, *rbcL60-atpB1* and *psbA-trnH* of *A. vesiculosa* (14 accession), *D. regia*, and *D. muscipula*. (Only *psbA-trnH* was done before By H.Štorchová).

In *S. latifolia cox1* (of 4 plants), *atp1* (of 4 plants), *psbA-trnH* (of 48 plants) were sequenced in the same manner.

PCR products were cleaned using QIAquick PCR purification kit (Qiagen, Valencia, CA). *The* direct sequencing was carried out by BigDye terminator reaction mix (Applied Biosystems) as shown below.

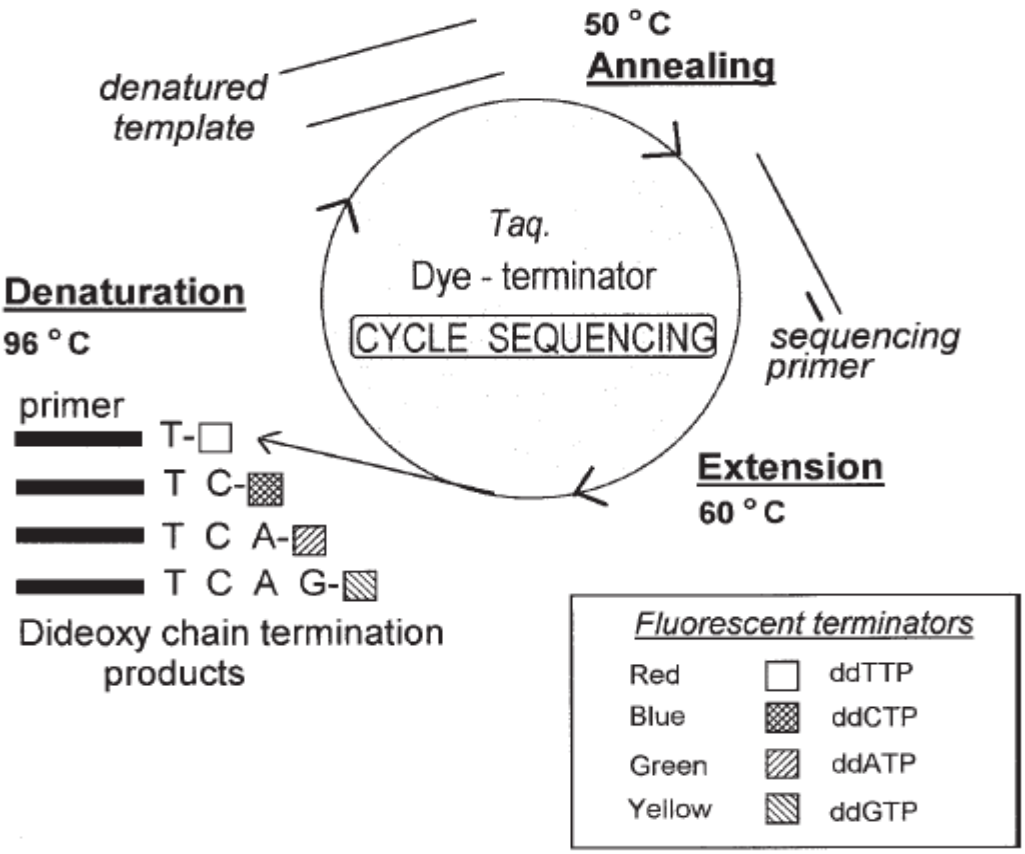


Figure (17)**: Cycle sequencing reaction.** Taken from Graham and Hill (2002).

Direct sequencing procedures:

1. Prepare the Cycle Sequencing reaction as follows:

PCR product	2-4 ul (PCR product)/ 5ul (in case of plasmid)
Sequencing buffer	1.5ul / zero (in case of using 4ul BigDye)
BigDye	1ul
Primer	1.6ul
SDW	complete to 10ul final volume

2. After PCR, spin down PCR tubes
3. Transfer the volume (10ul) into new 0.2ml microtubes and add 1.5ul 3M Sodium Acetate then 40ul 96% EtOH and vortex well.

4. Leave the microtubes on the bench for 30-60min.
5. Centrifuge the tubes at 12500rpm at 6ºC for 20min.
6. Very carefully discard supernatant and add 250ul 80% EtOH and vortex briefly.
7. Centrifuge the tubes at 12500rpm at 6ºC for 20min.
8. Discard supernatant and dry pellets for 30min at room temperature upside down on a paper towel.
9. Keep the samples in 4ºC.

Then running the cycle sequencing purified product on an ABI3130 sequencer (Applied Biosystems).

4.14. Sequences and Statistical Analysis

4.14.1. Contig assembly and alignments

Editing and contig assembly were accomplished using Vector NTI Suite 9 (InforMax, Invitrogen, Paisley, UK). Alignment of sequences was done using CLUSTALW embedded within Vector NTI Suite 9, BioEdit (Hall, 1999) and MEGA 4 (Kumar *et al*., 2004). Manual editing was done using BioEdit (Hall, 1999).

S. vulgaris sequences of mitochondrial and chloroplast genes were deposited in GenBank (EU805575-EU805579) and (EU805570 to EU805574), respectively. Sequences of mitochondrial and chloroplast regions of *A. vesiculosa, D. regia and D. muscipula* were also deposited in GenBank (FJ764808 - FJ764834). Sequences and alignments *of S. latifolia* are illustrated in Appendix C. Alignments of *S. vulgaris* sequences are in Appendix B, *A. vesiculosa*, *D. regia* and *D. muscipula* in Appendix D.

Atp1 Proteins of *A. vesiculosa*, *D. regia and D. muscipula* were estimated using Vector NTI Suite 9. Three dimentional model was created for the Atp1 protein of *A. vesiculosa* using MODWEB (Pieper *et al*., 2006), and aligned using PYMOL (DeLano Scientific LLC).

4.14.2. Genetic Distances

Genetic distances (GD) among *A. vesiculosa*, *D. regia* and *D. muscipula* were calculated with PAUP 4.0 b10 software (Swofford, 2000) using the HKY85 evolutionary model for nucleotide substitution. Data was calculated once for each intergenic spacer and once for the combined data set. Alignment of the six chloroplast region including, *trnS-trnG*, *trnP-trnW*, *Cpa-Cpb*, *rpl20-5'-rps12*, *rbcL60-atpB1* and *psbA-trnH* was adjusted to minimize the number of insertion/deletion events (indels) and nucleotide polymorphisms in the vicinity of the indels then the alignment was used for measuring genetic distance (Alignments are in appendix D).

4.14.3. Statistical analysis

The frequencies of sexes (Females, Hermaphrodites, and Shifting females) among progeny families from individuals with different *atp1* and *cox1* haplotypes were compared using a logistic regression implemented in SAS 9.1 using PROC GLIMMIX (SAS Institute 2004). PROC GLIMMIX allowed replicates within each mitochondrial haplotype to be treated as random effects for hypothesis testing (done by M.S.Olson).

The number of segregation sites and nucleotide diversity value which is the average number of nucleotide differences per site between a pair of randomly chosen sequences (Nei, 1987) were calculated for *atp1*, *cox1* and *psbA-trnH* in *S. latifolia* and *S. vulgaris* using DnaSP v4.9 (Rozas *et al*., 2003). The population genetic structure of *S. latifolia* mt and cpDNA were assessed by calculating Θ_{ST}, a statistic analogous to Wright's F_{ST} (Weir, 1996) using the GDA computer program (Lewis and Zaykin, 2001). The linkage disequilibrium which is the non-random association between specific cp and mtDNA haplotypes (Olson and McCauley, 2000). To assess whether the association between mt and cpDNA haplotypes was consistent across populations in *S. latifolia*, Fisher's exact was used with 10000 run in the GDA (Lewis and Zaykin, 2001).

Phylogenetic analysis of *psbA-trnH* haplotypes of *S. latifolia* was performed using PAUP 4.0 b10 software (Swofford, 2000). Maximum Parsimony criterion was used to construct the phylogenetic tree and based on 10000 bootstrap replicates.

4.15. PCR primers and conditions

The following Tables (2 and 3) illustrate primers used in the study and PCR conditions (Table 4) used for each primer pair.

Table (2): ***Silene*** **primers used in the study.**

Silene **primers**				
Region	**Primer name**	**Primer sequence (5´-3´)**	**Purpose**	**Citation**
atp1**coding region**	**atp1 lo**	**TCT AGT GGC ATT CGA TCA CAG A**	***atp1*** **amplification, probe synthesis and sequencing**	**McCauley *et al*. (2005)**
	atp1 up	**TAC ACG AAT TTT CAA GTG GAT GA**		
atp1 **internal**	**atp1297F**	**TCG ACG TGT CGA AGT GAA AG**	***Internal atp1*** **primers designed for sequencing**	**H. Štorchová**
	atp11170R	**TCT GAG CCA AAT TGA GCA AA**		
Cob	**cobIP59**	**GAT TAT CCA ACC CCG AGC**	**Probe synthesis**	**Houliston and Olson, 2006**
	cobIP64	**GAA TGG GCG TTA TGG C**		
Cob **and Specific chimeric gene**	**cob443R**	**GGC CAG ATG AAG AAG ACT GG**	**Screening for chimeric gene cotranscription with *Cob* in KovXKrn crosses**	**H. Štorchová**
	putORF atpA 56F2	**GGC AGG AGG CTC TCT AGG AG**		
cox1 **coding region**	***cox1*** **F82**	**CCG CGG AGT GAT GGG CAC ATG CTT CT**	***cox1*** **amplification, probe synthesis and sequencing**	**Olson and McCauley (2002)**
	cox1 **R1.6K**	**AAG GCT GGA GGG CTT TGT AC**		
cox1 **coding region**	***cox1*****RevHos**	**GGA TGA CCG AAA AAC CAA AA**	**Used with *cox1* F82 for internal *cox1* amplification**	**H. Elansary**
Specific chimeric gene	**BobKr_188For**	**GTC CTG GTT CCC GTC ATA AA**	**Screening for chimeric gene in KovXKrn crosses**	**H. Štorchová**
	BobKr_393Rev	**GAC CGC ATT TGG CAT CTG**		**H. Štorchová**
Specific chimeric gene	**InvBobAtp_For1**	**TTA CGC AGC TGT CAA TGG AT**	**Screening for chimeric gene in KovXKrn crosses**	**H. Štorchová**
	InvBobAtp_Rev2	**CAT GAT ACG AAA ACC AAA ATC AGA**		
Specific chimeric gene	**putORF atpA 279R2**	**TGG ATT CAA TGT TGT CCG TGA**	**Used with 56F2(the above one) for chimeric gene screening in KovXMV crosses**	**H. Štorchová**
psbA trnH**(GUG)**	**trnH**	**ACT GCC TTG ATC CAC TTG GC**	**sequencing**	**Hamilton (1999)**

	psbA	**CGA AGC TCC ATC TAC AAA TGG**		
Sequencing primers				
pGEM-T Easy	**M13(JL)**	**GTAAAACGACGGCCAGTG**	**Sequencing of plasmid insertions**	**Jiří luboš**
	M13rev(JL)	**GCTATGACCATGATTACGC**		

Table (3): *A. vesiculosa* primers used in the study.

***A. vesiculosa specific* primers**				
atpB1-rbcL60	**atpB1**	**GAA GTA GTA GGA TTG ATT CTC AT**	**sequencing**	**Savolainen *et al*.(1994)**
	rbcL60R	**CAG GAG TAT AAT AAG TCA TTG**		**H. Štorchová**
Internal *atpB1-rbcL60*	**AtpRbc F1**	**TTA GCA CTC GAT TTC GTT GG**	**Internal primers of *atpB1-rbcL60* for sequencing**	**H. Štorchová**
	AtpRbc R2	**CGA CAT GAA TTA GGC GTT ACT G**		
Internal *Cpa-Cpb*	**aldro_cpaF1**	**CCC CTA TTT GGT GCA ATC AG**	**Internal *Cpa-Cpb* chloroplast fragment sequencing**	**H. Štorchová**
	aldro_cpbR1	**CAT AAT CTC GAA TAT GAG TTC AA**		
rps12* exon1-*rpl20	**5'-rps12**	**GTC GAG GAA CAT GTA CTA GG**	**sequencing**	**Hamilton (1999)**
	rpl20	**TTT GTT CTA CGT CTC CGA GC**		
***trnP*(UGG)-*trnW*(CCA)**	**(trnP)PW_f**	**GAT TTG AAC CTA CGA CAT CG**	***trnP-trnW* chloroplast fragment sequencing**	**GenBank AB298100**
	(trnW)PW_r	**GAT GTG GCG CAG CTT GGT AG**		
***trnS*(GCU)-*trnG*(UCC)**	**trnS**	**GCCGCTTTAGTCCACTCAGC**	**sequencing**	**Hamilton (1999)**
	trnG	**GAA CGA ATC ACA CTT TTA CCA C**		

Table (4): **PCR conditions used in the study after optimization.**

PCR primers	Pre heating	Denaturation	Annealing	Extension	No. Of cycles	Final extension
Fragments amplification						
aldro_cpaF1-aldro_cpbR1	**94º/2min**	**93º/1min**	**55º/1min**	**72º/2min**	**35**	**72º/5min.**
atpB1- rbcL60R	**94º/2min**	**93º/1min**	**50º/1min**	**72º/2min**	**35**	**72º/5min.**
AtpRbc F1- AtpRbc R2	**94º/2min**	**93º/1min**	**50º/1min**	**72º/2min**	**35**	**72º/5min.**
atpUp-atp lo	**94º/2min**	**93º/1min**	**55º/1min**	**72º/2min**	**35**	**72º/5min.**
atp1 1170- atp1 297	**94º/2min**	**93º/1min**	**60º/1min**	**72º/2min**	**35**	**72º/5min.**
***cox1* F28-*cox1* HosRev**	**94º/2min**	**93º/1min**	**60º/1min**	**72º/1min**	**35**	**72º/5min.**
***cox1* F28-R1.6K**	**94º/2min**	**93º/1min**	**58.7º/1min**	**72º/2min**	**35**	**72º/5min.**
Direct Sequencing reaction	**96º/1min**	**96º/10sec**	**50º/5sec**	**60º/4min**	**30**	**------**
InvBobAtp_For1-InvBobAtp_Rev2	**94º/2min**	**93º/30sec**	**60º/30sec**	**72º/1min**	**35**	**72º/5min.**
Put ORF atp1 56 F2-279 R2	**94º/2min**	**93º/30sec**	**60º/30sec**	**72º/1min**	**35**	**72º/5min.**
PW_f- PW_r	**94º/2min**	**93º/1min**	**60º/1min**	**72º/2min**	**35**	**72º/5min.**
rps12-rpl20	**94º/2min**	**93º/1min**	**60º/1min**	**72º/2min**	**35**	**72º/5min.**
psbA-trnH	**94º/2min**	**93º/40sec**	**55º/30sec**	**72º/1min**	**35**	**72º/5min.**
trnS-trnG	**94º/2min**	**93º/1min**	**60º/1min**	**72º/2min**	**35**	**72º/5min.**
Probe synthesis						
atpUp-atp lo	**94º/2min**	**93º/40sec.**	**55º/40sec.**	**72º/1.5mi**	**35**	**72º/5min.**
cobIP59- cobIP64	**94º/2min**	**93º/1min.**	**58º/1min.**	**72º/2min**	**35**	**72º/5min**
***cox1* F28-R1.6K**	**94º/2min**	**93º/30sec.**	**56º/30sec.**	**72º/1.5min**	**35**	**72º/5min**

Results

5. Results

The results of this thesis are composed of four parts. The first part will show the results of *S. vulgaris* natural population investigation, the second part focuses on the controlled crosses of *S. vulgaris*, the third part will deal with *S. latifolia* and finally the fourth part will cover *A. vesiculosa* results.

5.1. Inheritance of mitochondrial and chloroplast genomes in a natural population of S. vulgaris

In this chapter of results, I will describe the results concerning organellar DNA diversity of the natural population of *S. vulgaris*. This part of results is the fruit of more than two years of intensive work.

5.1.1. Haplotype comparison among families

The naturally pollinated population of *S. vulgaris* is composed of 331 individual plants. This population was under investigation by several methods (PCR-RFLP, DNA Sequencing and Southern-RFLP) to distinguish the different mtDNA haplotypes. They were also analyzed by sequencing of cpDNA *psbA-trnH* intergenic region as explained in the Material and methods. The father or the fathers of this population is unknown because, they were naturally pollinated. The mother plant DNA quality was low and not sufficient for Southern-RFLP analysis so they were used only in PCR-RFLP"Knock back".

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5.1.1.1. PCR-RFLP of mitochondrial genes cox1 and atp1

This analysis revealed 3 haplotypes in *atp1* coding region identified by AluI and MspI fragment profiles (A, B and C) and two *cox1* haplotypes (A or B). One representative of each family is shown in Figure (18). The summary of the results is shown in Table (5). Of 331 individuals (99.4%) carried the maternal haplotype and 2 individuals (0.6%) carried different haplotype than their siblings from families Kov53 and Kov52.

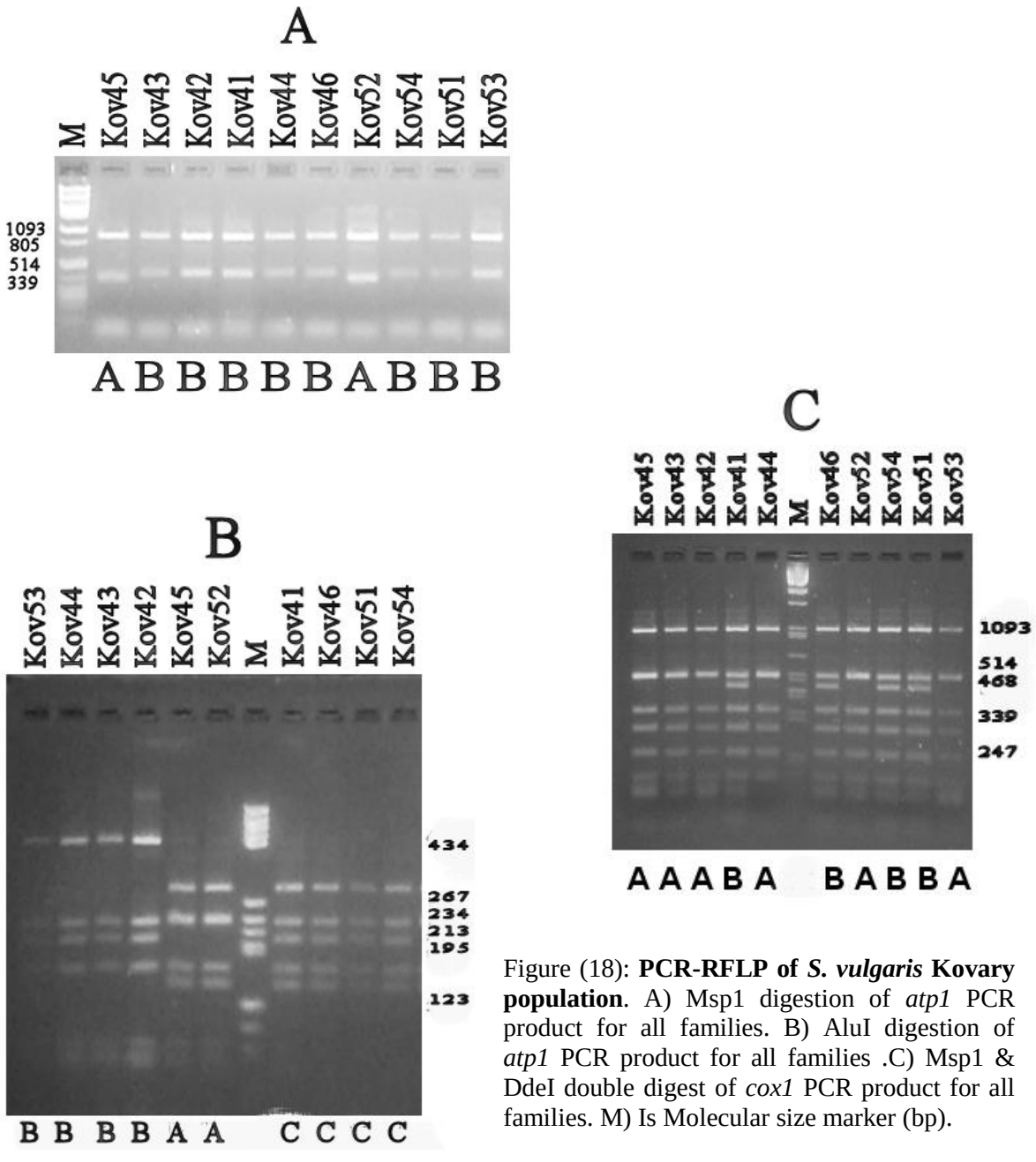

Figure (18): **PCR-RFLP of *S. vulgaris* Kovary population**. A) Msp1 digestion of *atp1* PCR product for all families. B) AluI digestion of *atp1* PCR product for all families .C) Msp1 & DdeI double digest of *cox1* PCR product for all families. M) Is Molecular size marker (bp).

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Table (5): **Representatives of the families and their haplotypes derived from PCR-RFLP of the *cox1* and *atp1* coding sequences**

	PCR- RFLP			
PCR fragment	*atp1*	*atp1*	*atp1*	*cox1*
Family \ enzymes	AluI	MspI	AluI &MspI	Msp1&Dde1
Kov45	B	A	A	A
Kov52	B	A	A	A
Kov53	A	B	B	A
Kov44	A	B	B	A
Kov43	A	B	B	A
Kov42	A	B	B	A
Kov41	C	B	C	B
Kov46	C	B	C	B
Kov54	C	B	C	B
Kov51	C	B	C	B
variations				
(Kov53) 195	C	B	C	B
(Kov52) 316	C	B	C	B

Sequencing of *atp1* and *cox1* genes in one representative of each family and some additional individuals revealed that there occurred 3 haplotypes in the coding region of *atp1* gene and 2 haplotypes in *cox1* gene as shown in Figure (19).The sequences of the *cox1* and *atp1* coding regions matched the previous *S. vulgaris* records in GenBank, originating in Europe or North America as shown in Table (6). This finding supports the view of *S. vulgaris* haplotypes being scattered across distribution range without clear phylogeographic signal (Taylor and Keller, 2007).

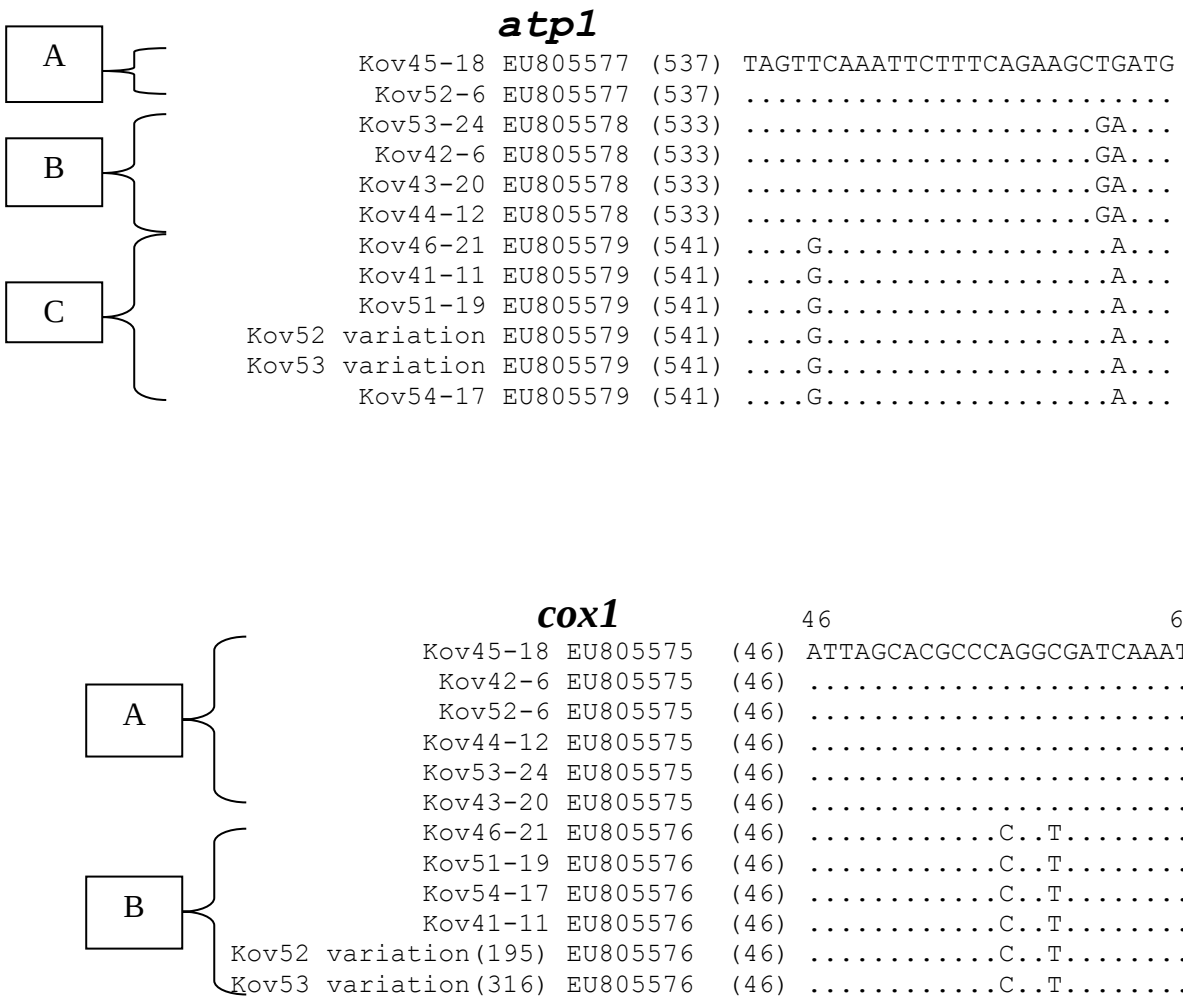

Figure (19): **Various haplotypes of *atp1* gene (upper alignment) and *cox1* gene (lower alignment)**. The letter at left indicates the corresponding *atp1* haplotype found in Kovary population.

Table (6): ***atp1* and *cox1* coding regions that matched the previous records in GenBank, originating in Europe or North America**.

mtDNA haplotype	Matching sequence from Europe (Location and accession No.)	Matching sequence from N. America (Location and accession No.)
atp1 EU805579	Estonia EF139470	USA EF139480
atp1 EU805577	Estonia EF673791	N.America DQ841788
atp1 EU805578	Italy EF139479	USA EF139481
cox1 EU805575	-	N.America EU440347
cox1 EU805576	-	N.America EU440348

5.1.1.2. Analysis of Paternal transmission and heteroplasmy using PCR-RFLP"Knock back"

Knock back was applied using SmaI enzyme digestion of *atp1* PCR products generated from all individuals, including mothers, of the two families Kov45 (heteroplasmic mother) and Kov52 (homoplasmic mother). The proportion of heteroplasmic individuals were 46% and 20% in these families, respectively (Table 7). Seven heteroplasmic individuals of family Kov52 were carrying 2 haplotypes of *atp1* gene, either A and B, or A and C. The amplification of maternal DNA, pre-digested with SmaI, generated no bands. It means that that the low amount of B or C mitochondrial genome could be transmitted from a mother, but most likely came from the father by paternal transmission. In the case of Kov45 there were 18 heteroplasmic individuals and 21 homoplasmic individuals originating from heteroplasmic mother, which may implement that the heteroplasmic character of 21 individuals was lost during fertilization or in the course of vegetative growth.

Table (7): **Heteroplasmy in two families of *S. vulgaris* Kovary using PCR-RFLP method**.

	Heteroplasmic offspring	Homoplasmic offspring	Total no.	% Heteroplasmy	Mother
Kov45	18	21	39	46%	heteroplasmic
Kov52	7	28	35	20%	homoplasmic

5.1.1.3. Among family variation revealed by Southern-RFLP

Southern hybridization revealed 5 different RFLP haplotypes in the *cox1* region and 6 different haplotypes in the *atp1* region (Table 8). Additional faint bands were observed in nearly all RFLP patterns (94%) in at least one combination probe/restriction enzyme. These faint bands may correspond to the mitochondria with different haplotype being present in the same heteroplasmic individual. Another explanation is genome of different architecture co-existing with master genome in the same mitochondria. Finally, faint bands may be derived from the region of lower homology to the probe (e.g. chimeric genes), residing in the same mitochondrial genome as major genes. The fact that the position of faint bands vary among individuals makes first two explanations more likely.

Two individuals which showed different pattern than their sisters were found (Figure 20). One of them belongs to family Kov52 (316) and carried haplotype corresponding to the family Kov54 and the second one was from Kov53 (195) and carried haplotype corresponding to the family Kov41 (Figure 20). Another plant, discovered in the Kov43 (273) family, was identical to its siblings except for unique *cox1* flanking RFLP pattern, not corresponding to any other haplotype in this study (Figure 21).

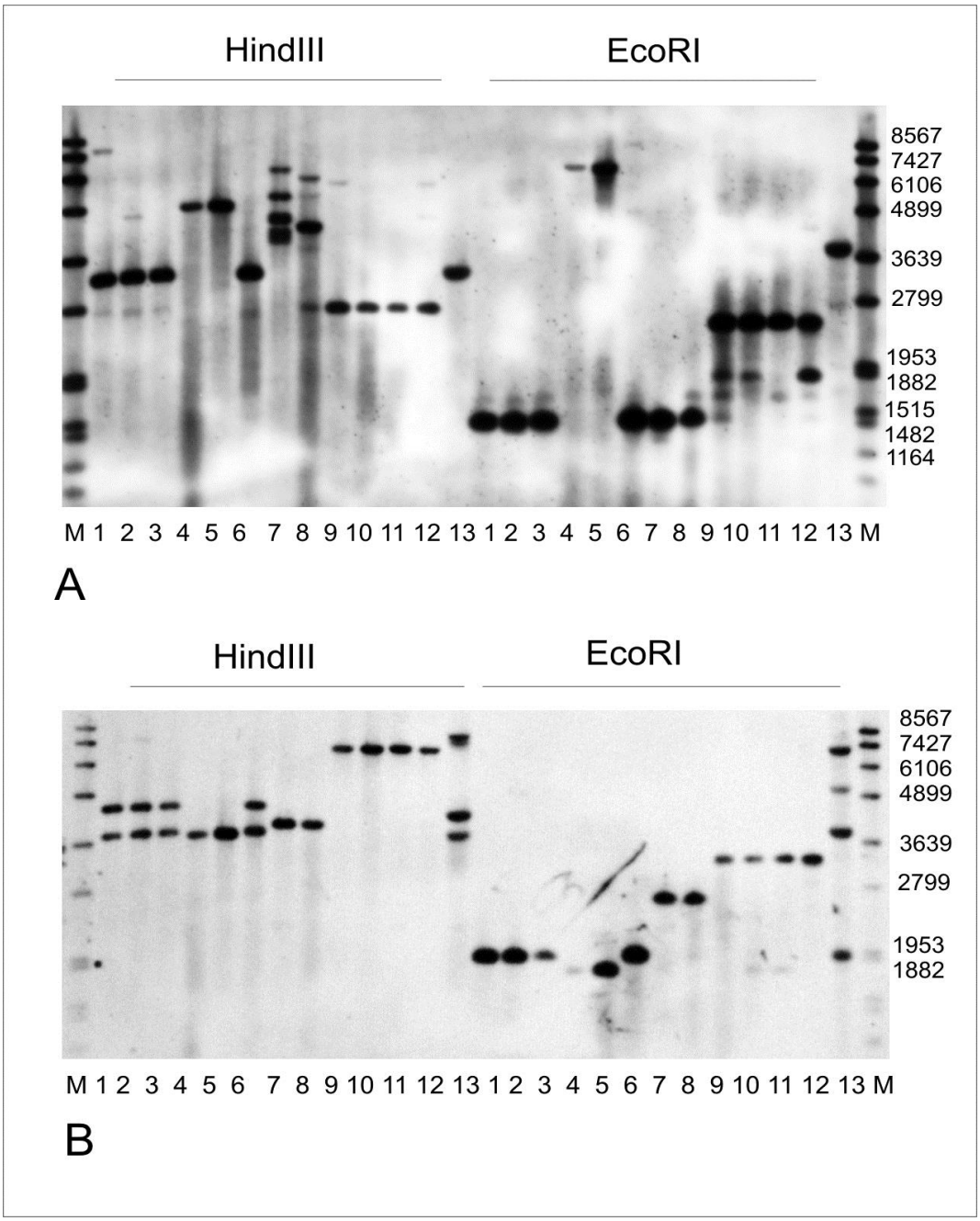

Figure (20): **High variation in mitochondrial Southern-RFLP patterns among representatives of ten families from the *S. vulgaris* population**. Total DNA was digested with HindIII and EcoRI and hybridized with DIG labeled *atp1* (A) and *cox1* (B) probes. The individual plants belong to the following families: 1)Kov42, 2) Kov53, 3) Kov43, 4) Kov53 (variation), 5) Kov54, 6) Kov53, 7) Kov45, 8) Kov52, 9) Kov52 (variation), 10) Kov41, 11)Kov46, 12)Kov051, 13)Kov44. M – DNA molecular size marker (bp)

Table (8): **Family codes, mtDNA and cpDNA haplotypes, offspring size** .The families originated from ten maternal plants growing in Kovary Meadows (Czech Republic). Molecular weights are shown below each mtDNA haplotype.

	PCR - RFLP		**Southern - RFLP** Numbers indicate fragment size (kb)				**DNA sequence**	**Number of individuals**
	cox1	***atp1***	*cox1* EcoRI	*cox1* HindIII	*atp1* EcoRI	*atp1* HindIII	**Chloroplast *psbA-trnH***	
Kov41	Kov**B**	Kov**C**	**A** 3.2	**A** 7.3	**A** 2.5;1.8	**A** 2.8	Kov 41	18
Kov46	Kov**B**	Kov**C**	**A**	**A**	**A**	**A**	Kov 41	32
Kov51	Kov**B**	Kov**C**	**A**	**A**	**A**	**A**	Kov 41	26
Kov54	Kov**B**	Kov**C**	**E** 1.7	**E** 3.8	**D** 7.2	**D** 4.9	Kov 54	38
Kov42	Kov**A**	Kov**B**	**C** 1.9	**C** 4.6**;**3.9	**B** 1.5	**C** 3.1	Kov 42	32
Kov43	Kov**A**	Kov**B**	**C**	**C**	**B**	**C**	Kov 42	38
Kov53	Kov**A**	Kov**B**	**C**	**C**	**B**	**C**	Kov 42	38
Kov44	Kov**A**	Kov**B**	**F** 7.3;5.0; 3.8;1.9	**F** 8.3;8.0; 4.1;3.8	**E** 3.8	**E** 3.2	Kov 44	36
Kov45	Kov**A**	Kov**A**	**B** 2.6	**B** 3.9	**B** 1.5	**F***	Kov 52	39
Kov52	Kov**A**	Kov**A**	**B**	**B**	**B**	**B**	Kov 52	34

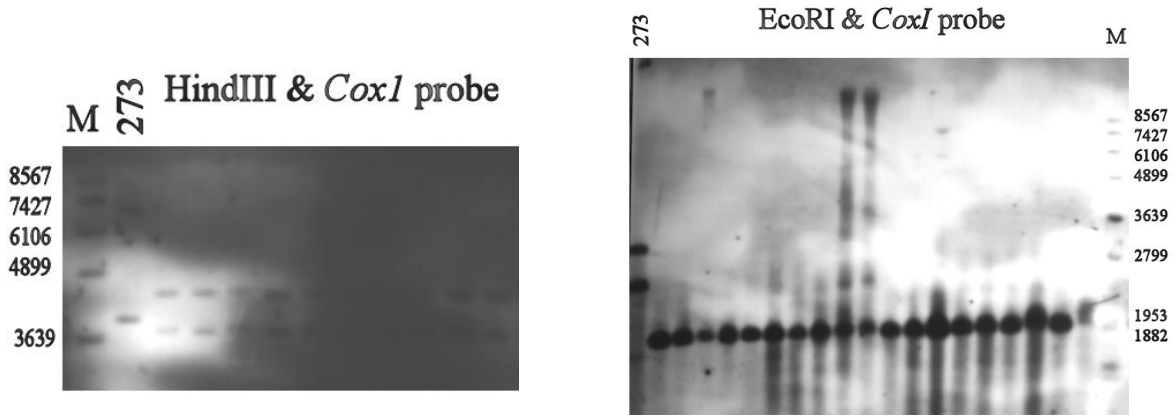

Figure (21): ***S. vulgaris* plant No.273 (family Kov43) carried a different *cox1* haplotype than present in other members of the same family.** This haplotype was unique among plants under study and was not observed it in any other individual. M. is Molecular size marker (bp).

5.1.1.4. Comparison of chloroplast haplotypes among families

Chloroplast haplotypes were determined in the representatives of each family by the sequencing of the chloroplast *psbA-trnH* intergenic region. This region is highly variable, frequently used in angiosperm genetic studies and suggested as plant barcode (Kress and Erickson, 2008; Kress *et al*., 2005). According to our expectation, high diversity in DNA sequences psbA- *trnH* intergenic region was revealed.

Five different chloroplast haplotypes were found (Figure 22). Among offspring of ten maternal plants, only two of them (Kov42 and Kov54) matched exactly the sequences (CE5 and CE51, respectively) determined in Kovary Meadows by the previous screen (Štorchová and Olson, 2004). Kov44 sequence was identical to EF094093 (USA). The haplotypes Kov41 and Kov52 did not match previous sequences in genbank as shown in Table (9).

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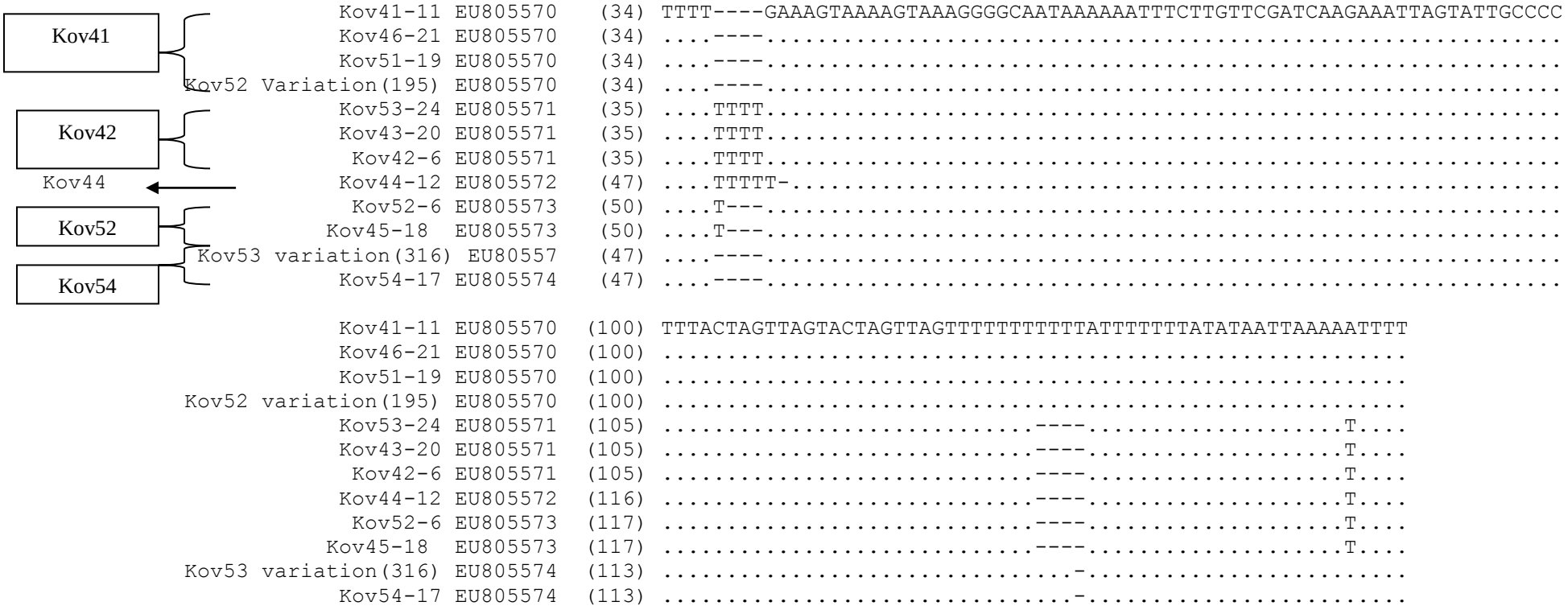


Figure (22): **The alignment of five haplotypes of Chloroplast *psbA-trnH* fragment of the representatives of each family, 195 &316 of *S. vulgaris* population**.

Table (9): **The *psbA-trnH* region in *S. vulgaris* Kovary compared to European and American populations**. Kov42 and Kov54 sequences matched exactly the sequences CE5 and CE51 respectively, determined in Kovary Meadows by the previous screen (Štorchová and Olson 2004). Kov44 sequence was identical to EF094093 (USA). The haplotypes Kov41 and Kov52 didn't match previous records in Genbank.

Chloroplast haplotype and accession No.	Matching sequence from Europe (Location and accession No.)	Matching sequence from N. America (Location and accession No.)
Kov54 EU805574	EuropeCE51 AY629290	N.America EF094076
Kov44 EU805572	-	N.America EF094093
Kov42 EU805571	Europe CE5 AY629289	N.America EF094082
Kov52 EU805573	-	-
Kov41 EU805570	-	-

5.1.1.5. The association between mtDNA haplotypes and cpDNA haplotypes

Associations between mtDNA haplotypes and cpDNA haplotypes across individuals are shown in Figure (23). The *cox1* PCR-RFLP haplotype A was always associated with *atp1* haplotype A; the *cox1* haplotype B was associated with either *atp1* haplotype B, or C. Each coding region haplotype (*cox1* or *atp1*) was associated with one to four different RFLP haplotypes; although the opposite was not true: each RFLP haplotype was associated with just one coding region haplotype. Each cpDNA haplotype was associated with one mtDNA RFLP haplotype, except for the cpDNA haplotype Kov52, which was associated with two different mtDNA RFLP haplotypes (BB and BF*). Two plants 195 and 316 carried different mtDNA haplotypes than their siblings. Both organellar haplotypes matched the haplotypes of other families (Kov41 for the plant 195 and Kov54 for the plant 316), which suggested co-transmission of the two organelles. Another explanation is an error in labeling the plants in the greenhouse. However, enormous attention was paid to the cultivation of plants, excluding all individuals suspected from confusion.

This observation is in agreement with the observed linkage between mtDNA haplotypes and cpDNA haplotypes as shown in previous Figures.

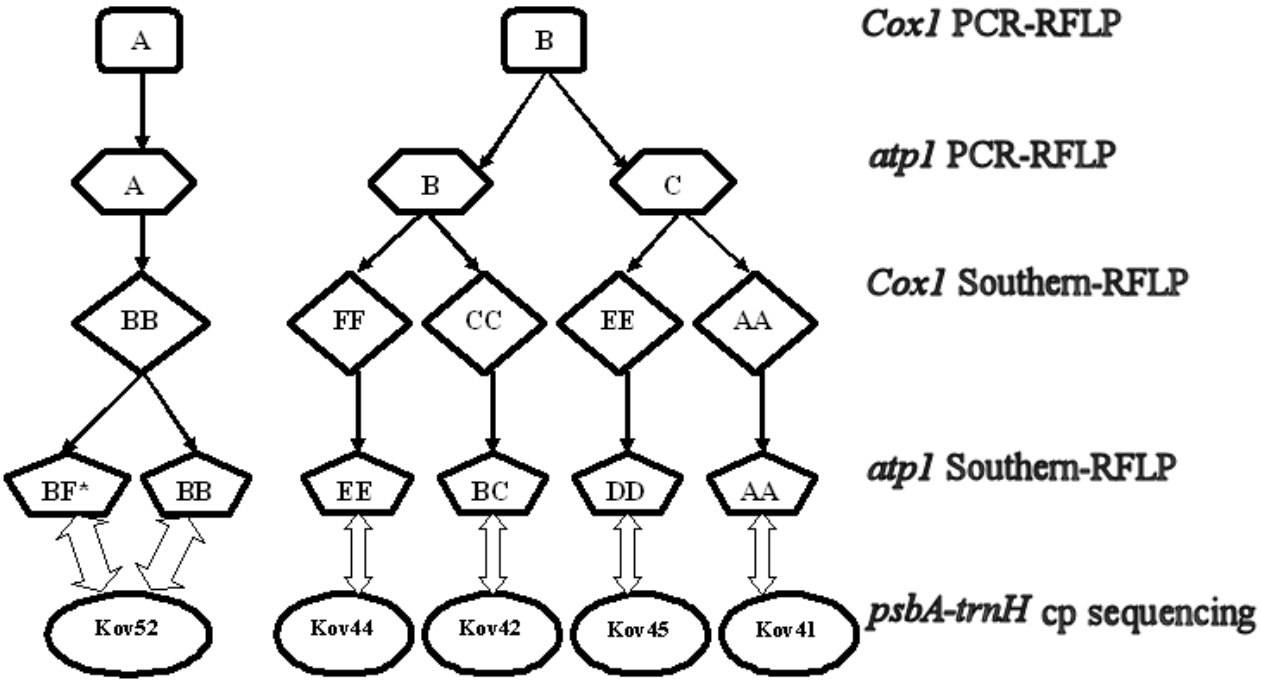


Figure (23): **Relationships among mitochondrial *atp1* and *cox1* coding regions and RFLP haplotypes and chloroplast *psbA-trnH* haplotypes as revealed in *S. vulgaris* from Kovary population.**

5.1.2. Variation of Southern-RFLP pattern in atp1 *flanking region in two families at individual and within-individual levels*

In case of strict maternal inheritance of organellar genomes, all offspring share DNA markers with the mother and among siblings. Here, different picture was revealed. It was found that two kinds of deviations from strict maternal inheritance. First, three individuals showed major changes in Southern-RFLP in one or several mt or cpDNA markers, most likely attributable to paternal inheritance, as described in previous chapters. Second, variability affecting exclusively the banding pattern of HindIII digested flanking region of the *atp1* gene was observed in two sibships.

5.1.2.1. Within family variation in families Kov52 and Kov45

Families Kov52 and Kov45 were the most variable families in the study. They showed variable Southern-RFLP patterns among progeny in *atp1* flanking region digested with HindIII. The extra bands in these two families were quite strong in family Kov52 (Figure 24). We can see multiple unique bands for each individual accompanied by a major band specific for the family and additional fainter band found in 20 individuals of the family. In the Kov45 family four individuals with an extra band nearly as intensive as the major bands (5300, 4600 and 4100 nt) were found among 39 siblings (Figure 25).

These complex patterns are very difficult to explain by paternal transmission of mtDNA. We have to consider additional phenomena as heteroplasmy, lineage sorting and de novo origin of mtDNA haplotype by recombination (stoichiometric shifting), as discussed in detail in Discussion.

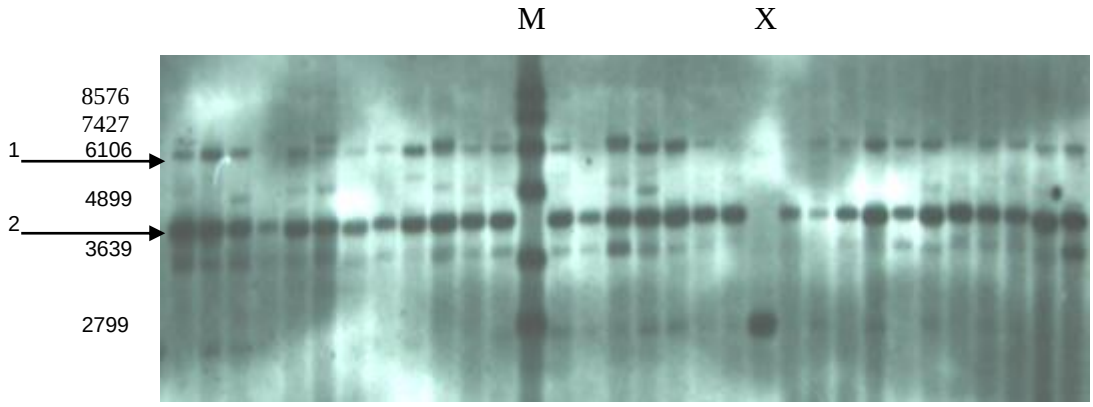


Figure (24): **The highly variable family southern pattern**. Arrowhead 1 points one of the minor bands, arrowhead 2 points the major band of the family, X belonging to the individual (195) of the family Kov52 with a different banding pattern obtained most likely due to paternal inheritance. M) Is DNA molecular size marker (bp)

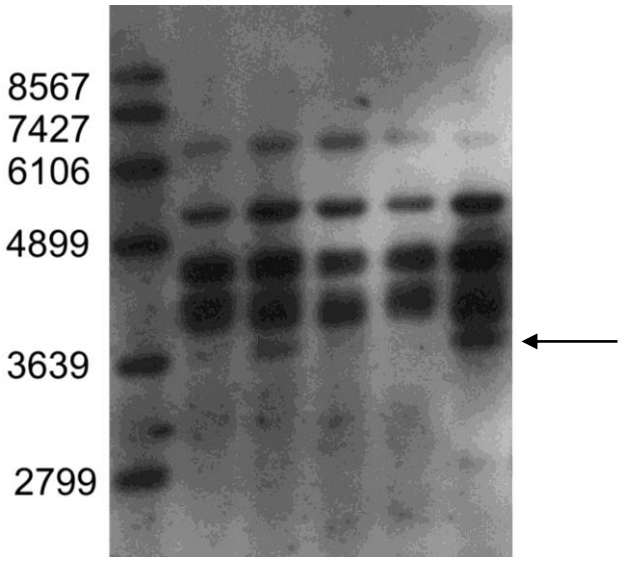


Figure (25): **Variation in mitochondrial Southern-RFLP patterns within the family Kov45**. DNA was digested with HindIII and hybridized with DIG labeled *atp1* probe. Arrowhead points to the additional band present in some siblings. M) DNA molecular size marker (bp)

Families other than Kov45 and Kov52 generally contained no variation among individuals of each family, except bands much fainter than major bands (Figure 26).

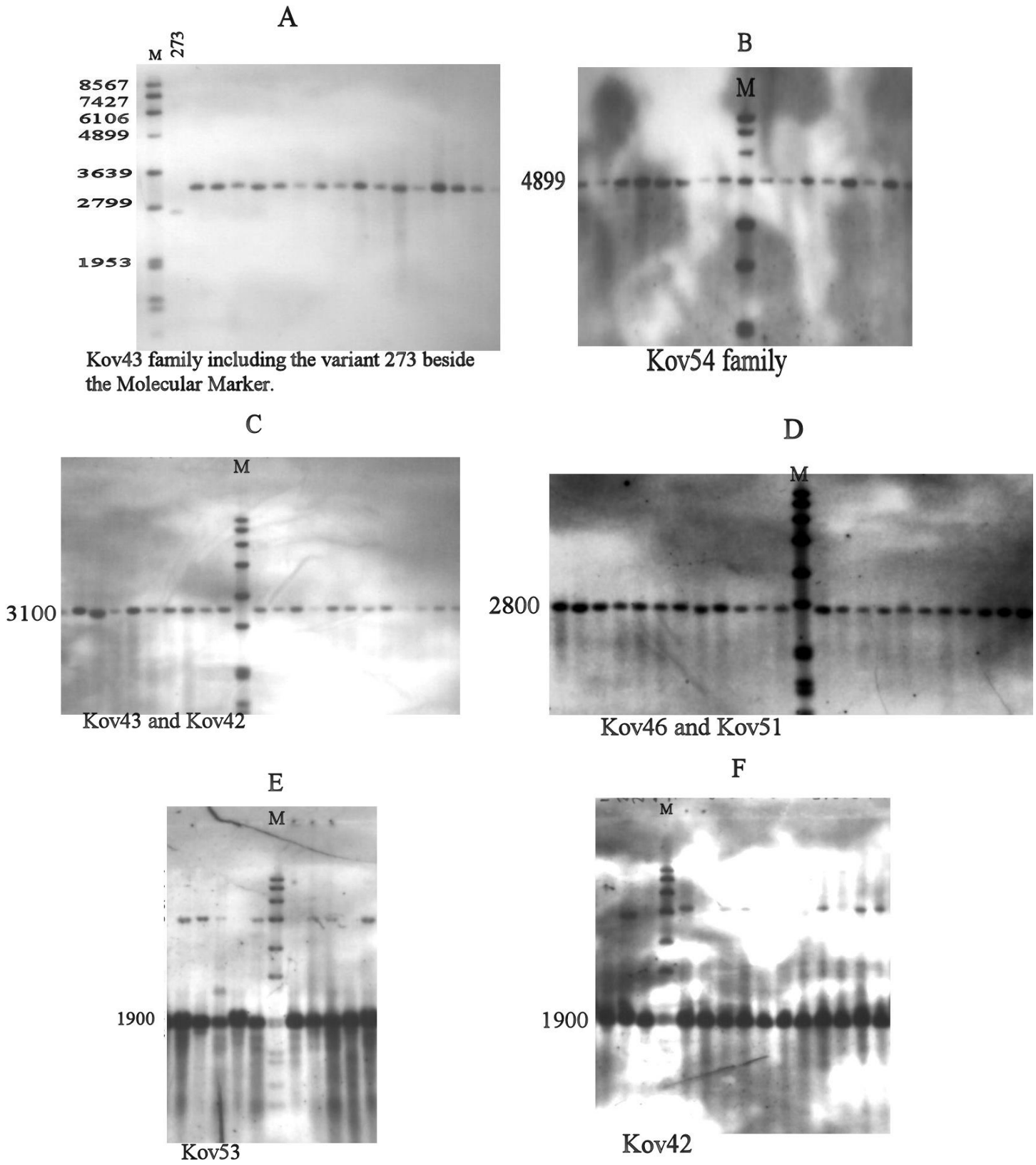


Figure (26): The uniformity of most of *S. vulgaris* Kovary families. In A, B, C, and D the DNA digested with HindIII enzyme and hybridized with *atp1* probe. In E and F the DNA was digested with EcoRI and hybridized with *cox1* probe. M) is Molecular size marker (bp).

5.1.3. Change in Southern-RFLP markers in the course of one year of vegetative growth

The change in the pattern of the minor band in some families has been documented for example in the EcoRI digestion of the representatives hybridized with *atp1* probe as shown in Figure (27) there's missing band in family Kov51.

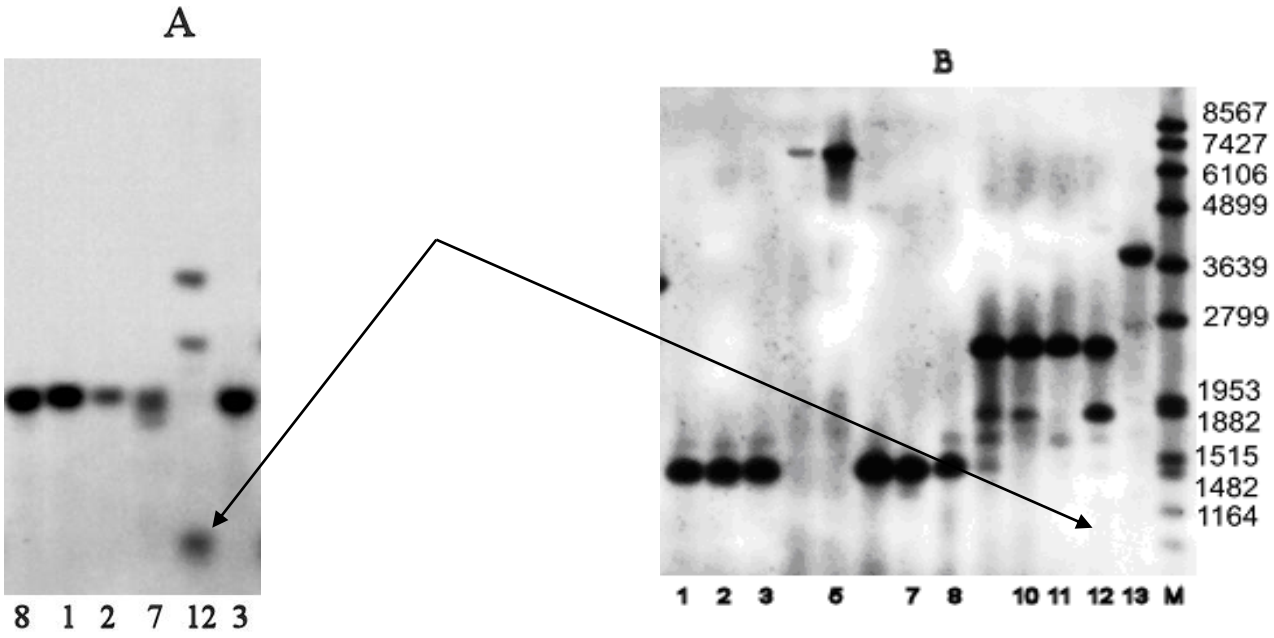


Figure (27): **The change in the pattern in mitochondrial RFLP pattern among representatives of ten families from the *S. vulgaris* population**. A) is the pattern of EcoRI digestion with *atp1* probe in January 2007, B) is the pattern of DNA of the same individual extracted in November 2008, 1) Kov42, 2) Kov53, 3) Kov43, 5) Kov54, 6)Kov53, 7) Kov45, 8)Kov52, 10) Kov41, 11) Kov46, 12)Kov51, 13)Kov44. There's missing band in family Kov51.M) is Molecular size marker (bp).

5.1.4. Within-individual variation using Southern-RFLP

To study within-individual variation using Southern-RFLP patterns, two different stems on the same individual from the Kov52 sibship were analyzed. Figure (28) compares members of the Kov52 family, each of them represented by DNA from two different branches. The within-individual variation in plants number 13 and 33 is clearly visible. For example, the intensity of band 2 differs seven fold between two branches of the plant number 13 and there was a >2 fold difference in bands intensity for band 1 on different stems from plant 34. Within-individual variation in was found in 5 plants of 34 from this family. These data are interpreted by Sorting of mitochondrial genomes as a major force which causes changes in mitochondrial genomes copy number in different stems of the same plant. It was noticed that these stems carry the same flower gender.

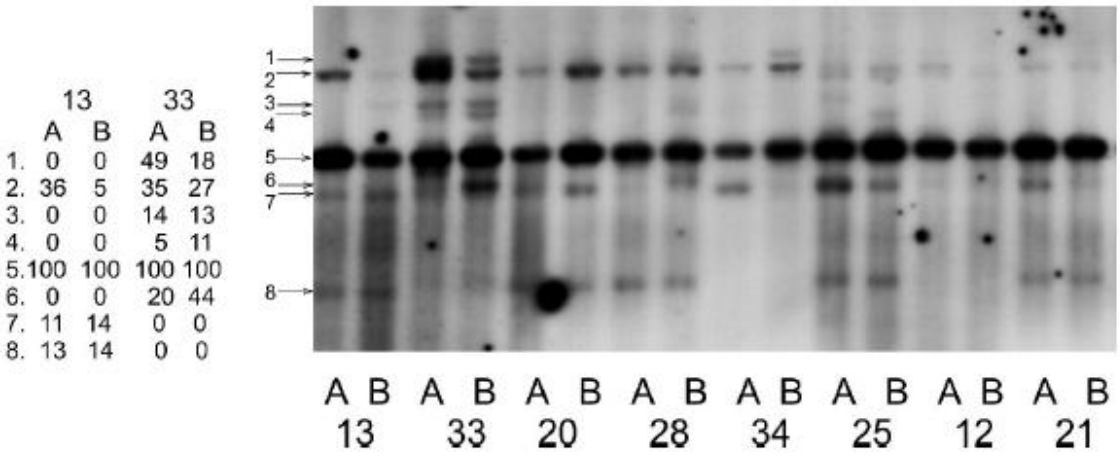


Figure (28): **Within-individual variation in the mitochondrial Southern-RFLP patterns in the family Kov52, with HindIII & *atp1* probe**. A and B denote two different branches from the same individual identified by the number. Arrowheads point to the bands which intensities were quantified. The values of band intensities related to the strongest band (number 5) and expressed in % are shown at left (intensity values were measured by Karel Muller using Photoimager FLA7000).

5.1.5. Sex and haplotypes

Most of the plants possessed the flowers of one gender at the same time, and a different gender appeared after cutting and regrowing so two types of females were found among *S. vulgaris* Kovary population. The first type is females maintain the gender during two years growing period while in the second type is females shifting sometimes to hermaphrodites after cutting and regrowing as shown in Table (10). Twenty plants from various families possessed both female and hermaphrodite flowers at the same time which is termed gynomonecious individuals (Andersson, 1999; McCauley and Olson, 2008). One gynomonecious plant (Kov44-4) was analyzed by Southern-RFLP to examine if there is association between mitochondrial haplotype and the sex of the branch. DNA samples were extracted once from the female branch and once from the hermaphrodite branch of the same plant, and there was no relationship between mitochondrial Southern-RFLP pattern and the sex of the branch as shown in Figure (29). The banding pattern of DNA from all branches was the same.

The average hermaphrodite frequency across the families was 59% (Table 10). All the families with the *atp1* coding region haplotype KovC exhibited progeny hermaphrodite frequencies < 50%, whereas those with A or B haplotypes always exhibited progeny haplotype frequencies > 50% (Figure 30). The KovC families produced significantly fewer hermaphrodites than the families carrying KovA and KovB haplotypes ($F_{2, 7} = 4.94$, $P = 0.046$).

Table (10): **Proportion of hermaphrodites (H), females (F) and shifting females (ShiftF) in each family of *S. vulgaris* Kovary**.

	Number of individuals of each family	**H (%)**	**F (%)**	**Shift F (%)**
Kov 41	**18**	**39**	**17**	**44**
Kov 46	**32**	**34**	**41**	**25**
Kov 51	**26**	**42**	**46**	**12**
Kov 54	**38**	**21**	**47**	**32**
Kov 42	**32**	**56**	**22**	**22**
Kov 43	**38**	**87**	**0**	**13**
Kov 53	**38**	**50**	**21**	**29**
Kov 44	**36**	**92**	**3**	**5**
Kov 45	**39**	**64**	**18**	**18**
Kov 52	**34**	**85**	**6**	**9**

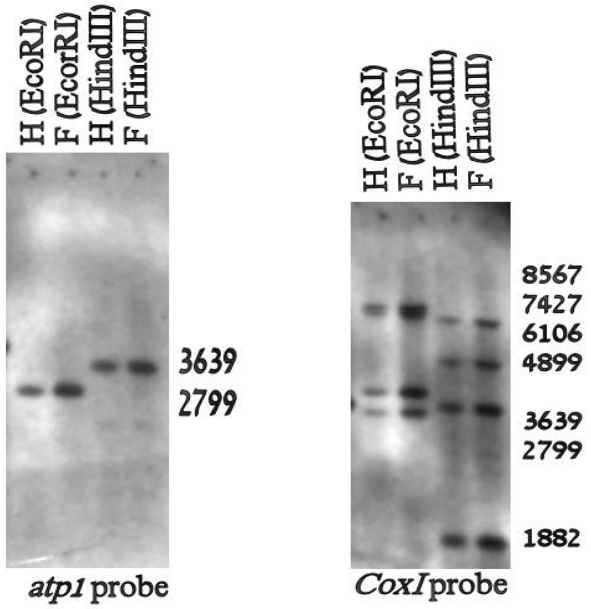


Figure (29): **No variation between two branches of the same plant (gynomonecious individual) one of them is hermaphrodite (H) and one of them is female (F)**. Once the DNA was digested with EcoRI and once with HindIII enzymes and hybridized with *atp1* and *cox1* probes.

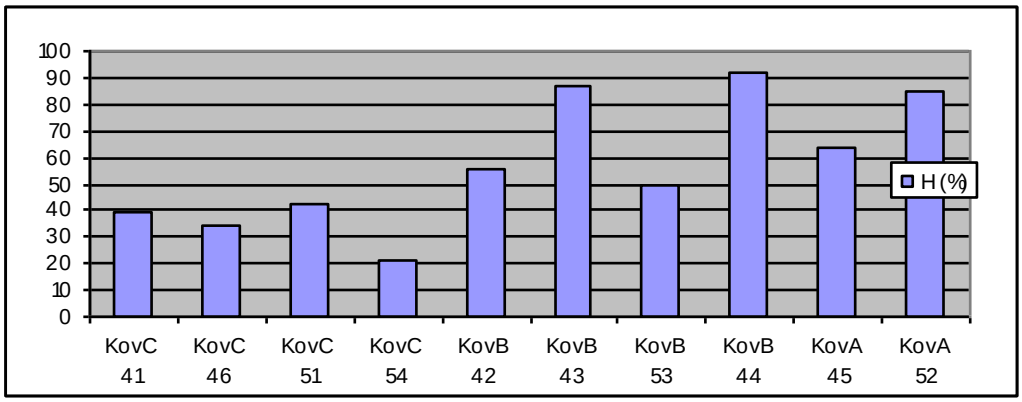


Figure (30) **Percentage of hermaphrodites associated with 3 mtDNA haplotypes A, B and C**.

5.1.5. Association between mtDNA variation & transcription profiles

To investigate whether high variation in mtDNA markers in *S. vulgaris* was accompanied by variation in transcription profiles, K.Muller performed Northern hybridization at the same time of Southern hybridization by H.Elansary and in the same time in an associated experiment. Total genomic DNA from leaves (by H.Elansary) and total RNA from buds of various sizes (1 to 4 mm) were extracted (by K.Muller) from eleven individuals of Kov52 family from the same branches used for DNA extraction, including the plant carrying different organellar haplotypes due to the paternal transmission, and hybridized with the *atp1* probe. As shown in (Figure 31A) all the individuals shared the same transcription profile, except for the plant 316 (which is labeled as 23*) that shared the same pattern with a Kov41 individual. The same observation was made after rehybridization of the same membrane with *cox1* probe (Figure 31B).

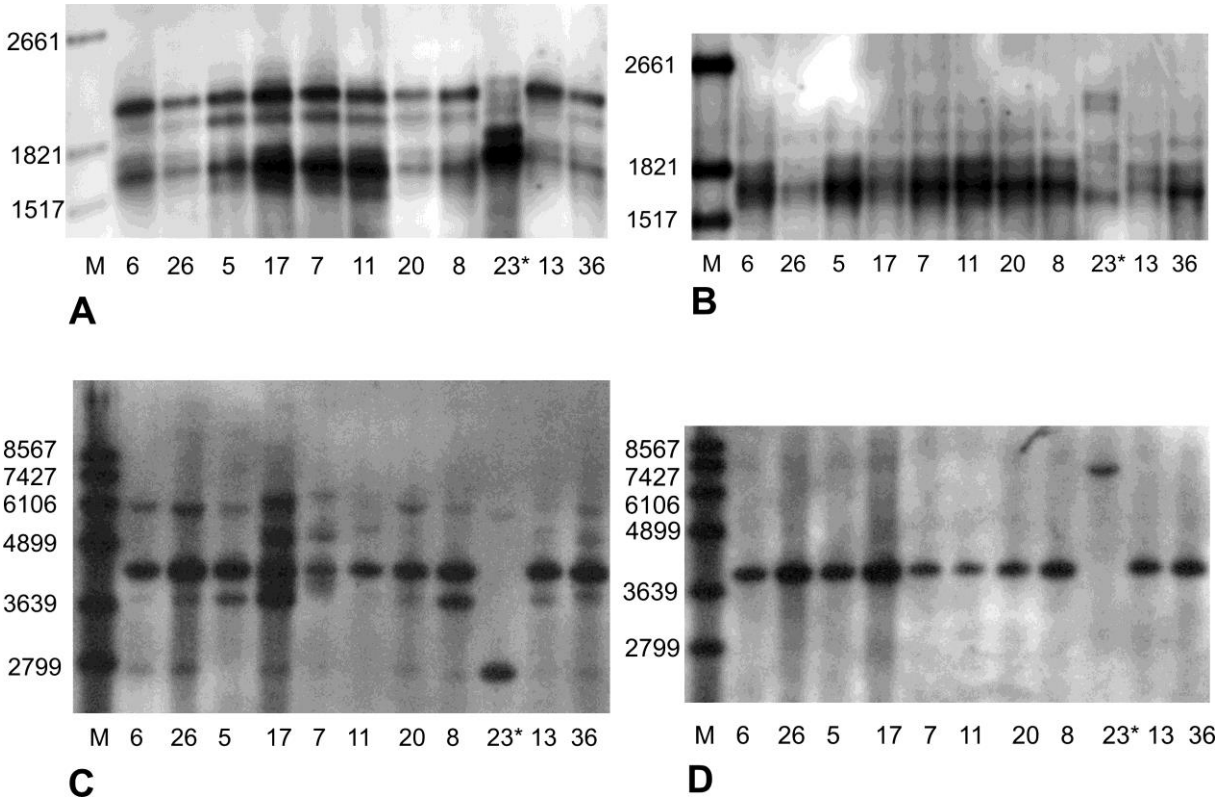


Figure (31): **The comparison of transcription profiles and mitochondrial RFLP patterns among siblings from the family Kov52**. Total RNA was transferred to the membrane and hybridized with DIG labeled *atp1* (A) or *cox1* (B) probes. DNA was digested with HindIII and hybridized with DIG labeled *atp1*(C) or *cox1* (D) probes. The numbers denote individual plants. The plant 23*(316) shows both different RFLP pattern and transcription profile from its siblings. M) DNA or RNA molecular size marker (bp).

5.2. Controlled crosses of **S. vulgaris** *between females from Kovary population and hermaphrodites from the same as well as from distant populations*

The previous chapter focused on the progeny of open pollinated plants which didn't allow the investigation of the pollen donor (father). Therefore controlled crosses were performed between Kovary population families and pollen donors from the same as well as from distant populations. The hermaphrodites from geographically distant populations carry characteristic chimeric genes in their mt genomes. These regions allow designing primers capable to track even very low level of paternally transmitted mtDNA genomes. Crosses with fathers bearing chimeric genes permit to clearly distinguish paternal transmission from other phenomena influencing mt transmission. The focus here in this chapter is on the crosses among geographically distant populations.

5.2.1. PCR screening of controlled crosses progeny with aim to detect paternal transmission of mitochondrial genome and heteroplasmic individual

Controlled crosses were done as shown in Table (11). PCR amplification using primers specific for the mt DNA of each father was used to detect paternal transmission. Owing to the specificity of the primers for chimeric genes present in pollen donors only, even tiny amount of paternal mtDNA in the progeny could be easily detected.

In the case of Krasnojarsk as a father, the first primer pair was (BobKr_188For-BobKr_393Rev) which amplify unique chimeric region of mtDNA (PCR fragment of about 300bp) was used. The PCR product was found in almost all the offspring of the crosses. It's sequencing revealed identity to the paternal mtDNA. To examine if this fragment originated from the nucleus or mitochondria, another two primer pairs were used to screen the offspring of all KrnXKov crosses. If low level paternal leakage was taking place, other primers specifically targeting paternal mt DNA should confirm this transmission. The second primer pair (putORF atpA 56F2- Cob443R) amplified larger fragment (1050bp) containing unknown ORF, which was cotranscribed with the *Cob* gene (unpublished results). The screen with those primers revealed no PCR fragment in more than 631 plants. The third primer pair (InvBobAtp_For1- InvBobAtp_Rev2) was used and gave negative results again. We interpret the results as an absence of paternal transmission of mtDNA albeit in trace amount. The

positive results produced with one primer pairs only may be explained by the transfer of a corresponding portion of mtDNA to the nucleus in father and than to the progeny via pollen.

In case of crosses with a pollen donor from MV population (Virginia, USA) the primer pair (putORF atpA 56F2-putORFatpA 279R2) which amplifies chimeric gene found in MV plants only was used. There were no PCR products in 376 progeny of these crosses. In conclusion, no evidence for paternal transmission was found among 1007 progeny from 29 Crosses.

The sex ratio is being calculated for all the offspring of the controlled crosses to compare it with the mother's families.

Table (11): **The different controlled crosses done and percentage of females, hermaphrodites, and gender shifting (if any).**

Mother	father	Symbol	Number of individuals	Number of H	Number of F	Shift if any	H (%)	F (%)
Kov52-30	Krn7	X1	47	20	27	-	42.5	57.44
Kov45-26	Krn7	X3	14	2	12	-	14.29	85.71
Kov46-20	Krn7	X4	12	10	2	-	83.34	16.66
Kov52-30	Krn52	X5	52	30	22	-	57.7	42.3
Kov45-26	Krn7	X12	53	0	53	-	0	100
Kov44-13	Krn7	X13	71	1	70	-	1.4	98.6
Kov52-30	Krn7	X14	78	1	77	-	1.28	98.72
Kov54-27	Krn52	X15	87	0	87	-	0	100
Kov53-21	Krn52	X16	34	0	34	-	0	100
Kov41-3	Krn7	X17	42	0	42	-	0	100
Kov41-8	Krn7	X18	41	2	39	-	4.87	95.13
Kov41-3	Krn7	X19	13	0	13	-	0	100
Kov41-8	Krn7	X20	3	0	3	-	0	100
Kov54-27	Krn7	X21	3	0	3	-	0	100
Kov45-26	Krn7	X22	8	0	8	-	0	100
Kov53-21	Krn7	X23	22	1	21	-	4.54	95.46
Kov41-3	Krn7	X24	17	2	15	-	11.76	86.4
Kov45-26	Krn7	X25	18	0	18	-	0	100
Kov45-26	Krn7	X26	8	0	8	-	0	100
Kov41-8	Krn7	X27	3	0	3	-	0	100
Kov52-6	Krn52	X28	5	0	5	-	0	100
Kov52-30	MV 3-1	X10	75	71	4	-	94.7	5.3
Kov44-13	MV 3-1	X11	75	70	5	-	93.4	6.6
Kov53-21	MV 3-1	X36	40	-	-	-	-	-
Kov51-14	MV 3-1	X37	50	-	-	-	-	-
Kov41-8	MV 3-1	X38	44	-	-	-	-	-
Kov41-11	MV 3-1	X39	37	-	-	-	-	-
Kov51-22	MV 3-1	X40	50	-	-	-	-	-
Kov53-11	MV 3-1	X41	5	-	-	-	-	-

5.2.2. Southern-RFLP of **cox1** *and* **atp1** *gene flanking regions of controlled crosses*

Southern-RFLP using *atp1* and *cox1* probes were also used to analyze the offspring of the controlled crosses as shown in Figures 32 and 33. The figures show different crosses made between Krn52 (father) and Kov52-30 (labeled as x5) as a mother, Krn7 (father) and each of Kov52-30 (labeled as x1), Kov45-26 (labeled as x3) and Kov46-20 (labeled as x4) as mothers, all the offspring carried the same RFLP pattern as its mother and there were no evidence of paternal transmission or mitochondrial rearrangements. In the other hand in Figure (33), the offspring of the cross X3 between Krn7 (father) and Kov45-26 (mother) using both *atp1* & *cox1* probes, and it was visible that there's additional band on the member X3-4 with *atp1* probe which dose not correspond to both parents. As paternal transmission of mtDNA was excluded by PCR screen, other interpretation of mtDNA variation should be taken into account, as will be commented in discussion.

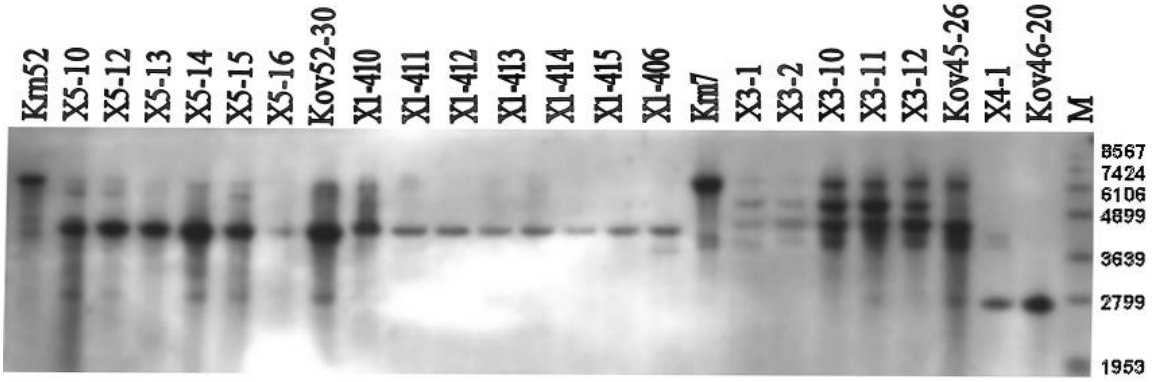


Figure (32): ***atp1*** **Southern-RFLP of X1, X3, X4 and X5 crosses**. All the offspring matched the maternal mtDNA haplotypes. The crosses made between Krn52 (father) and Kov52-30 (labeled as x5) as a mother, KrnII7 (father) and each of Kov52-30 (labeled as x1), Kov45-26 (labeled as x3) and Kov46-20 (labeled as x4) as mothers, M) DNA molecular size marker (bp).

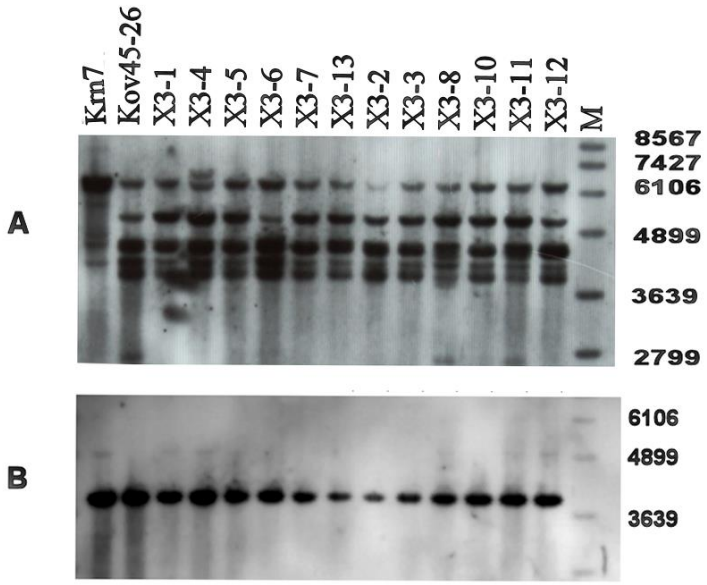


Figure (33): **The offspring of the cross X3 between Krn7 (father) and Kov45-26 (mother) using *atp1* probe (A) *cox1* probe (B)**. It's visible that there's additional band on the member X3-4 with *atp1* probe which does not correspond to both parents, M) DNA molecular size marker (bp).

5.3. Variation in mitochondrial and chloroplast genomes in Silene latifolia

The subject of this chapter focuses on another plant from the same genus *Silene* but with different reproduction system to compare the level of diversity in mt and cpDNA between *S. vulgaris* and *S. latifolia* and to analyze the association between chloroplast and mitochondrial genomes in this species. Southern-RFLP markers were used to reveal variation in mitochondrial gene flanking regions, which was higher than in coding regions.

5.3.1. Sequencing of cox1 *and* atp1 *genes of* S. latifolia

PCR-RFLP using EcoRI digestion of *cox1* gene PCR product found no variation in the *cox1* region of eight representatives of each population in *S. latifolia* (Figure 34), scattered around Europe (Table 15)

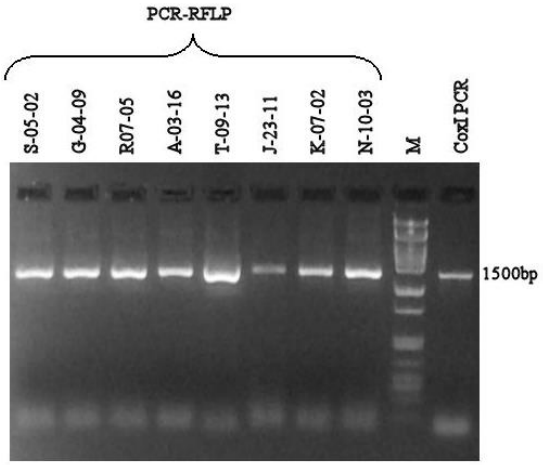


Figure (34): **No variation in PCR-RFLP of *cox1* gene of 8 representatives of *S. latifolia* using EcoRI, compared to normal *cox1* PCR product of *S. latifolia***. M: Molecular Size Marker.

Sequencing of the *atp1*(772) and *cox1* (608bp)genes in plants from different populations revealed that there was only one haplotype in the coding regions of both of *atp1* and *cox1* genes (Appendix C).The sequences of the *atp1* coding regions matched exactly the previous records in GenBank, originating in Europe (Accession No. DQ841781) by Houliston and Olson (2006) also *cox1* sequences matched those previously screened in Europe (Accession No. EU883283) by Touzet and Delph (2009).

5.3.2. Southern-RFLP variation in mitochondrial gene flanking region

Southern-RFLP of flanking regions of the mitochondrial genes *atp1, cob and cox1* was determined for the representatives from 8 European populations of *S. latifolia*. Southern hybridization revealed no variation among the flanking regions of *atp1* and *cob*, digested with EcoRI and HindIII) among the 8 populations.

On the other hand five (J, K, A, S1 and S2) different RFLP haplotypes in the *cox1* flanking regions were found among *S. latifolia* populations as shown in Table (12). Additional faint bands were observed in all *cox1* RFLP patterns (Figure 35). One individual belonging to T (Tolkamer) Netherlands group showed different pattern than other members of its population and carried haplotype K (Figure 36). In general, the polymorphism was only detected in a *cox1* flanking regions, not around the *cob* and *atp1* genes. In contrast to flanking region, the *cox1* coding region was uniform, and could not be used for study of population structure and association between chloroplast and mitochondrial genomes. The F_{ST} value for the mtDNA haplotypes was 0.84 which suggests high population structure.

Table (12): **Southern-RFLP patterns in *S. latifolia* using combinations of probes (*atp1, cob* and *cox1*) and restriction enzymes (HindIII and EcoRI)**. No variation in the flanking regions was observed among populations using *atp1* and *cob* probes, while *cox1* &EcoRI detected 5 mtDNA haplotypes and *cox1* & HindIII detected 4 mtDNA haplotypes. Molecular weights are shown below each haplotype in Kb.

Population	**Southern-RFLP patterns with combinations of probes and enzymes**					
	***atp1* probe&EcoRI**	***atp1* probe&HindIII**	***cob* probe&EcoRI**	***cob* probe& HindIII**	***cox1* probe & HindIII**	***cox1* probe & EcoRI**
A. (Århus)Denmark	**No Variation** 1.5	**No Variation** 3.65	**No Variation** 1.5	**No Variation** 2.2 & 1.76	**K** 6.1	**A** 5.5 &4.9
T.(Tolkamer) Netherlands					**K**	**A**
K. (Kåseberga 1)Sweden					**K**	**K** 8.5 &4.9
G. (Köpinge) Sweden					**K**	**K**
R (Renkum)Netherlands					**K**	**K**
N.(Norra Åsum)Sweden					**K**	**K**
J. (Jönköping)Sweden					**J** 5.3 & 2.8	**J** 8 &4.9
S (Stechendorf 2). Germany					**S1** 6.15	**S1** 8.8&4.9
					S2 4.5	**S2** 8.7&4.9

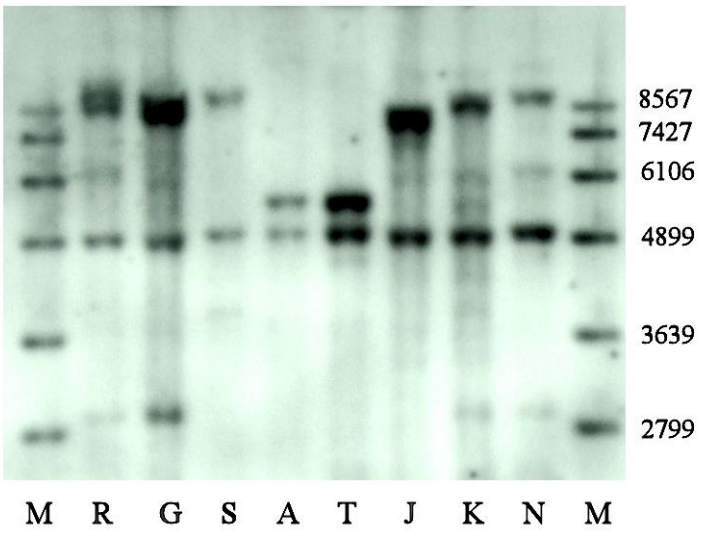


Figure (35): **Variation in mitochondrial Southern-RFLP patterns among representatives of eight populations of *S. latifolia* from different parts of Europe**. Total DNA was digested with EcoRI and hybridized with DIG labeled *cox1* probe. The individual plants belong to the following populations: R) Netherlands, G) Sweden, S) Germany, A) Denmark, T) Netherlands, J) (Jönköping) Sweden, K) (Kåseberga) Sweden, N) (Norra Åsum) Sweden and M) is DNA molecular size marker (bp).

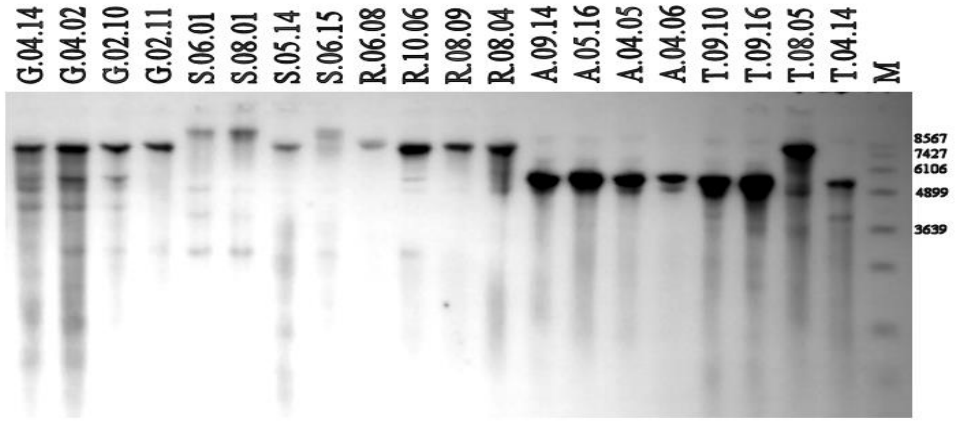


Figure (36): **The individual T.08.05 matched haplotypes occurring in populations G and R.**

5.3.3. Chloroplast haplotype comparison among populations

Chloroplast haplotypes were determined in all European populations by the sequencing of the chloroplast *psbA-trnH* intergenic region. Five different chloroplast haplotypes were found (Figure 37). A-05-16, A-04-05, J-23-11 and K-04-13 were identical for the American and European sequences in previous screens whereas the haplotype T-09-10 differed from the closest European and American counterparts and A-04-05 by the presence of one extra T

(Table13). The plant (T.03.05) matched K-04-13 haplotype. The FST value was 0.687 for cpDNA haplotypes.

```
                  105                                                                 174
(D)J.23.11  (105) TATAATTTATTTTTTTTTTATTCTTTACTAGAAAATCCTAAAAAAAAATAAAACGACTTTACAAAAGTCT
(E)K.04.13  (105) ..................-.....................................................
(C)A.04.05  (105) ..G...............-.............................-......................
(A)A.05.16  (105) ..G.............................................-......................
(B)T.09.10  (105) ..G.............................................-......................
                  175                                                   231
   J.23.11  (175) TATTATGTCAATTCGTCTTTTCTTGTTTTTTAATTAAGATAAGAAGTCGTTCGTTTT
   K.04.13  (174) ..................................................
   A.04.05  (173) ..............................................A..----...
   A.05.16  (174) ..............................................A..----...
   T.09.10  (174) ..............................................A..----...
```

Figure (37): ***psbA-trnH* chloroplast haplotypes found in *S. latifolia* European populations**.
T-03-05 matched (K) mitochondrial and chloroplast haplotypes.

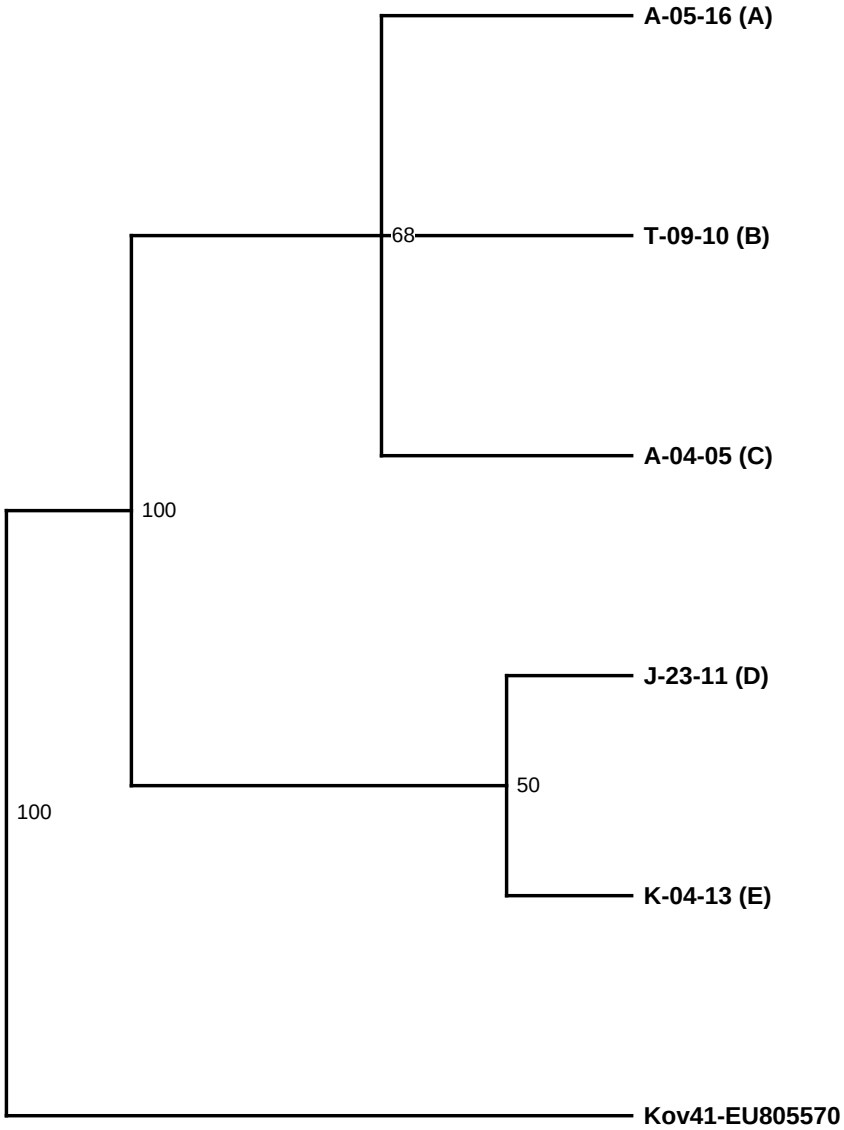
Table (13): **The *psbA-trnH* chloroplast intergenic region in *S. latifolia* European populations compared to matching sequences from GenBank in European and American populations**.

Chloroplast haplotype	Matching sequence from Europe (Location and accession No.)	Matching sequence from N. America (Location and accession No.)	Notes
A-05-16	Scotland AF518927	N.America EF091514	
A-04-05	France AF518913	N.America EF091502	
T-09-10	France AF518913	N.America EF091502	Differed than these sequences by the presence of one extra T
J-23-11	Hungary AF518912	N.America EF091505	
K-04-13	Portugal AF518926	N.America EF091509	

5.3.4. The association between mitochondrial haplotypes and chloroplast haplotypes

Each mtDNA Southern-RFLP haplotype was associated with specific cpDNA haplotype, except for A RFLP haplotype which was associated with 3 different cpDNA haplotypes (Figure 38). Each cpDNA haplotype was associated with one or two mtDNA haplotypes. This pattern could be explained by faster evolution of chloroplast intergenic region versus mtDNA haplotype A or faster evolution of mtDNA versus cpDNA haplotypes D or F. Maximum Parsimony tree showed close relatedness of three cpDNA haplotypes which were associated with mtDNA haplotype A., This observation favors the explanations of the

observed pattern (Figure 38).There was 2 private alleles (private alleles are found in one population only) in cpDNA and 3 private alleles in mtDNA as shown in Table (14). Combination of cpDNA and mtDNA haplotypes or Fisher Exact Test (all four possibilities – AA, AB, BA, BB) (Weir, 1996), which would suggest combination of chloroplast and mitochondrial genomes, was not found in this survey of *S. latifolia* organellar markers.



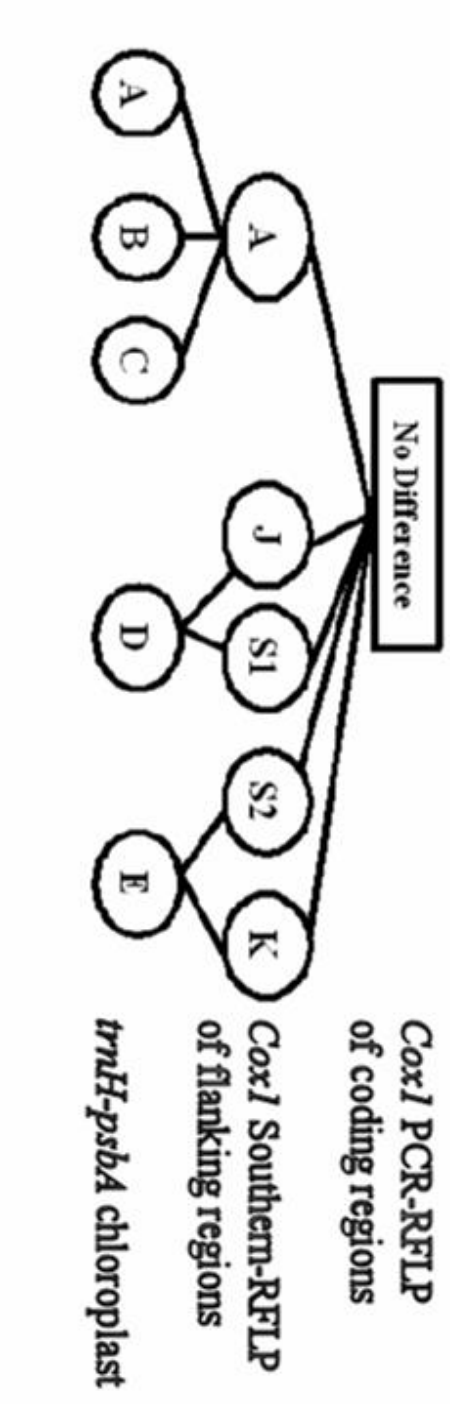


Figure (38): **Maximum Parsimony tree based on the *psbA- trnH* sequences of *S. latifolia* (On the left) compared to the association between mt and cpDNA haplotypes (On the right).** Single nt substitution and one additional A in a 10A repeat separate haplotype A and C. Single nt substitution (A instead of T) separates haplotypes B and C. *S. vulgaris* ***psbA-trnH*** (EU805570) was used as an out-group. Numbers at branching points are bootstrap values from 10000 replicates.

Table (14): **Association between cpDNA (columns) and mtDNA (rows) within 48 individuals from eight populations**. Letters in the leftmost column indicate the populations. Letters in the second column refer to mtDNA haplotypes. Numbers in cells refer to the number of individuals possessing the respective combination of cpDNA and mtDNA haplotypes. There was 2 private allele in cpDNA (marked with yellow) and 3 private alleles in mtDNA (marked with green).

Populations	mtDNA haplotype	cpDNA haplotypes				
		1	2	2	4	5
A	A	5				
	A		1			
T	A		3	2		
	K					1
J	J					6
S	S1					3
	S2				3	
K	K					6
G	K					6
R	K					6
N	K					6

5.3.5. Comparison of the diversity of mt and cpDNA markers between S. vulgaris *and* S. latifolia

Table (15) abstracts a comparison between *S. vulgaris* Kovary population and *S. latifolia* European populations. The nucleotide diversities, the average number of nucleotide differences per site between a pair of randomly chosen sequences (Nei, 1987), achieved in the chloroplast *psbA-trnH* intergenic region was higher 3x in *S. vulgaris* (1.895 %) than *S. latifolia* (0.635 %).This result is in the same trend as a previous screen compared between *S. vulgaris* and *S. latifolia* (Sloan *et al*., 2008). There was uniformity in *S. latifolia* populations coding regions sequenced in *cox1* and *atp1* mitochondrial genes which gave nucleotide diversity equals zero. In the other hand, there was nucleotide diversity in the same regions in *S. vulgaris* and the values were 1.123, 0.329 in *atp1* and *cox1* mitochondrial genes, respectively. The number of segregation sites was also higher 3x in *S. vulgaris* than *S. latifolia*. In the same trend we can see the *atp1* flanking regions were we got here 6 haplotypes in *S. vulgaris* from one population (the offspring of 10 mother plants) from small geographic area compared to 8 populations sampled from large geographic area in Europe. In the case of *cox1* the number of Southern-RFLP haplotypes was equal in both cases (5 haplotypes).

The diversity within a single European population of *S. vulgaris* Kovary was higher than in a set of 8 European populations of *S. latifolia* as revealed by total number of haplotypes,

number of private haplotypes (Table 16) using the same PCR-RFLP and Southern-RFLP markers. The comparison in Table (16) shows clearly that *S. vulgaris* plants collected in much smaller area have five times more haplotypes and private alleles on the both levels of mt and cpDNA than *S. latifolia*.

Table (15): **Polymorphism comparison between *S. vulgaris* and *S. latifolia* by gene or intergenic spacer based on sequencing and on Southern-RFLP.**

Based on Sequencing						
	***S. vulgaris* (one population, 12 individuals)**			***S. latifolia* (eight populations, 48 individuals)**		
Organellar region	**No. of haplotypes**	**Nucleotide Diversity at individual sites (Pi)%**	**Segregation sites**	**No. of haplotypes**	**Nucleotide Diversity at individual sites (Pi)%**	**Segregation sites**
Cp *psbA-trnH*(332 bp)	**5**	**1.895**	**28**	**5**	**0.635**	**9**
mt *cox1* (608 bp)	**2**	**0. 329**	**2**	**1**	**0**	**0**
mt *atp1* (772 bp)	**3**	**1.123**	**13**	**1**	**0**	**0**
Based on Southern-RFLP (flanking regions)						
	No. of haplotypes			**No. of haplotypes**		
atp1	**6**			**1**		
cox1	**5**			**5**		

Table (16): **Comparing the number of haplotypes and private alleles between *S. latifolia* in the current study and *S. vulgaris* from previous study by Štorchová and Olson (2004)**. The geographic area from which *S. latifolia* populations were sampled was about five times larger (Northern and Central Europe), than area of *S. vulgaris* populations (a part of Central Europe)

	***S. vulgaris* (8 populations, 86 individuals) (Štorchová and Olson, 2004)**		***S. latifolia* (8 populations, 48 individuals)**	
	No. of haplotypes	**No. of private haplotypes**	**No. of haplotypes**	**No. of private haplotypes**
cox1	30	28	5	3
psbA-trnH	24	22	5	2

5.4. Uniformity of mitochondrial and chloroplast DNA of **Aldrovanda vesiculosa** *populations across populations from four continents*

If a high variation in *S. vulgaris* mt and cpDNA represents one extreme, *A. vesiculosa* with nearly uniform organellar DNA is another extreme. Whereas gynodioecious reproduction system and balancing selection is responsible for *S. vulgaris* organellar diversity (Touzet and Delph 2009), the reason for uniformity of *A. vesiculosa* is not fully understood.

5.4.1. Sequence uniformity of cpDNA in **A. vesiculosa**

DNA sequence analysis of the six chloroplast intergenic regions, totally 3680 nt, revealed extreme genetic uniformity, considering the huge sampling area of *A. vesiculosa* spanning four continents. Only two indels were found, the presence/absence of one T in a T array in the intergenic regions *rpl20-rps12* and *atpB-rbcL* (Table 17). The two polymorphisms divided the set of *A. vesiculosa* samples into two subsets – European and non-European accessions (Australian, African and Asian). There were two exceptions. The plant from Tokyo (Japan) which was identical to European accessions and the plant from Romania (Europe) which was identical to non-European accessions.

The variation found here is very little compared to the variation found in *Silene* in one intergenic region *psbA-trnH*. Five cpDNA haplotypes were found in each of *S. vulgaris* (from one Meadow) and *S. latifolia* (from Northern Europe) compared to two haplotypes only In *A. vesiculosa* found from 15 accessions from four continents.

Table (17): **Sequence variation among chloroplast intergenic regions of *A. vesiculosa* accessions from four continents**. The only difference was the number of T (10 vs. 11) in two intergenic regions.

Abbreviation	Geographic origin	*trnS-trnG*	*trnP-trnW*	*Cpa-Cpb*	*psbA-trnH*	*rpl20--rps12*	*atpB-rbcL*
EP	Europe - East Poland	IDENTICAL	IDENTICAL	IDENTICAL	IDENTICAL	11T	10T
UK	Europe - North Ukraine					11T	10T
HU	Europe - Hungary					11T	10T
LI	Europe - Lithuania					11T	10T
SW	Europe - Switzerland					11T	10T
NR	Europe - North Russia					11T	10T
JPT	Japan - Tokyo					11T	10T
RO	Europe- Romania					10T	11T
SEA	SE Australia					10T	11T
NA	North Australia					10T	11T
SWA	SW Australia					10T	11T
KA	Australia Katherine					10T	11T
AR	Australia - Armidale					10T	11T
JPK	Japan - Kyoto					10T	11T
AF	Africa - Botswana					10T	11T

5.4.2. Uniformity of flanking regions of mitochondrial genes of A. vesiculosa

Southern hybridization analysis using the probes derived from the genes *atp1* and *cox1* revealed no differences within RFLP of flanking regions of these genes in *A. vesiculosa* accessions from four continents as shown in Figure (39), the Figure showing a comparison between the accessions and the offspring of one mother of *S. vulgaris* Kovary. Sequencing of partial *atp1* of *A. vesiculosa* coding region was identical within all the accessions. However, there was short unique sequence in *atp1* coding region in *A. vesiculosa*, not present in any other angiosperm species including the closest relatives *Drosera regia* and *Dionaea muscipula*.

There is no within-species variation in *atp1* and *cox1* mtDNA flanking regions in *A. vesiculosa* compared to the large variation described so far in *S. vulgaris.* 12 mtDNA haplotypes in *cox1* by Olson and McCauley (2000), 30 mtDNA haplotypes in *cox1* by Štorchová and Olson (2004), 6, 5 mtDNA haplotypes in *atp1* and *cox1*, respectively, discovered in this study also in *S. latifolia*, 5 mtDNA haplotypes in *cox1* were found. The

atp1 coding region was monomorphic in *A. vesiculosa* as well as in *S. latifolia* but polymorphic in *S. vulgaris*.

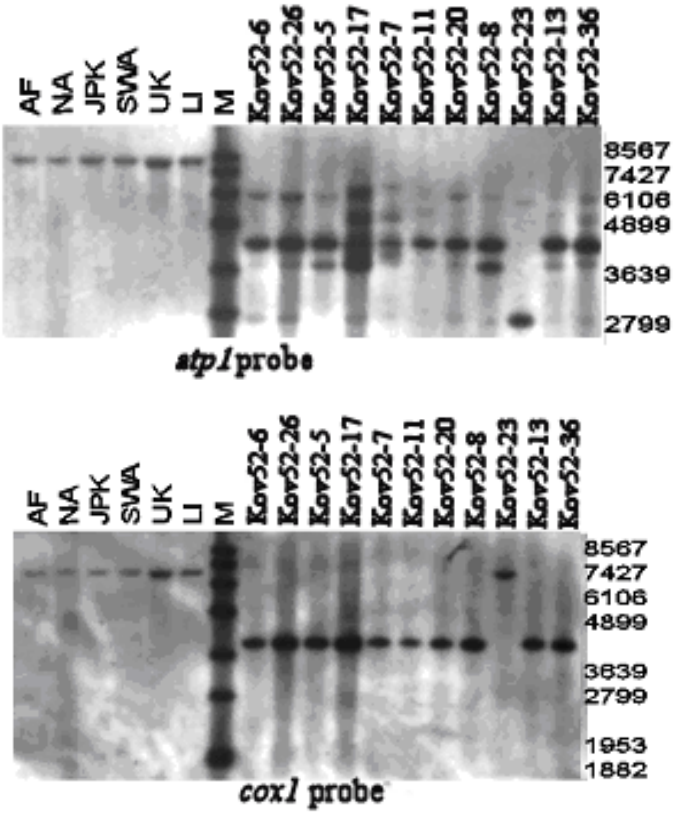


Figure (39): **Comparison of mitochondrial RFLP pattern between *A. vesiculosa* accessions from different continents and offspring of one mother (Kov52) of *S. vulgaris*.** There are no differences among mitochondrial flanking regions of *atp1* and *cox1* genes in *A. vesiculosa* accessions using both probes (*atp1* and *cox1*) compared to high variation in *S. vulgaris* using *atp1* probe. (AF) Africa, (NA) North Australia, (JPK) Japan Kyoto, (SWA) South West Australia, (UK) Ukraine, (LI) Lithuania and M – DNA molecular size marker.

5.4.3. The atp1 *gene in* A. vesiculosa *contains unique substitution*

The mitochondria ATP synthase subunit alpha (ATP1) is a conserved essential enzyme among organisms required for ATP synthesis. It is the fundamental center of cell energy production (Ackerman and Tzagoloff, 2005; Devenish *et al*., 2008). The alignment of the sequence of this gene in *A. vesiculosa* with representative *atp1* sequences retrieved in GenBank revealed a unique substitution of 12 nt (Figure 40).

The alignment of *A. vesiculosa* Atp1 amino acid sequence with orthologous proteins across the plant kingdom and also with Atp1 in yeast showed a unique motif of four amino acids (TGWS) was found at the positions 73-76 of partial *A. vesiculosa* Atp1 amino acid sequence, which corresponded to the positions 121-124 of yeast Atp1 (Figure 41).

The sequence of this region may vary at the broadest taxonomic scale (Figure 41) but the consensus amino acid sequence LSDH, from which some species differ in one or two substitutions, was found in angiosperms. The TGWS motif, present in *A. vesiculosa* Atp1, was exceptional not only among plants, but also among all living organisms, as was documented by BLAST search (Appendix F). Atp1 proteins of closely related *D. regia* and *D. muscipula* contained the angiosperm consensus LSDH (Figure 41) at corresponding positions.

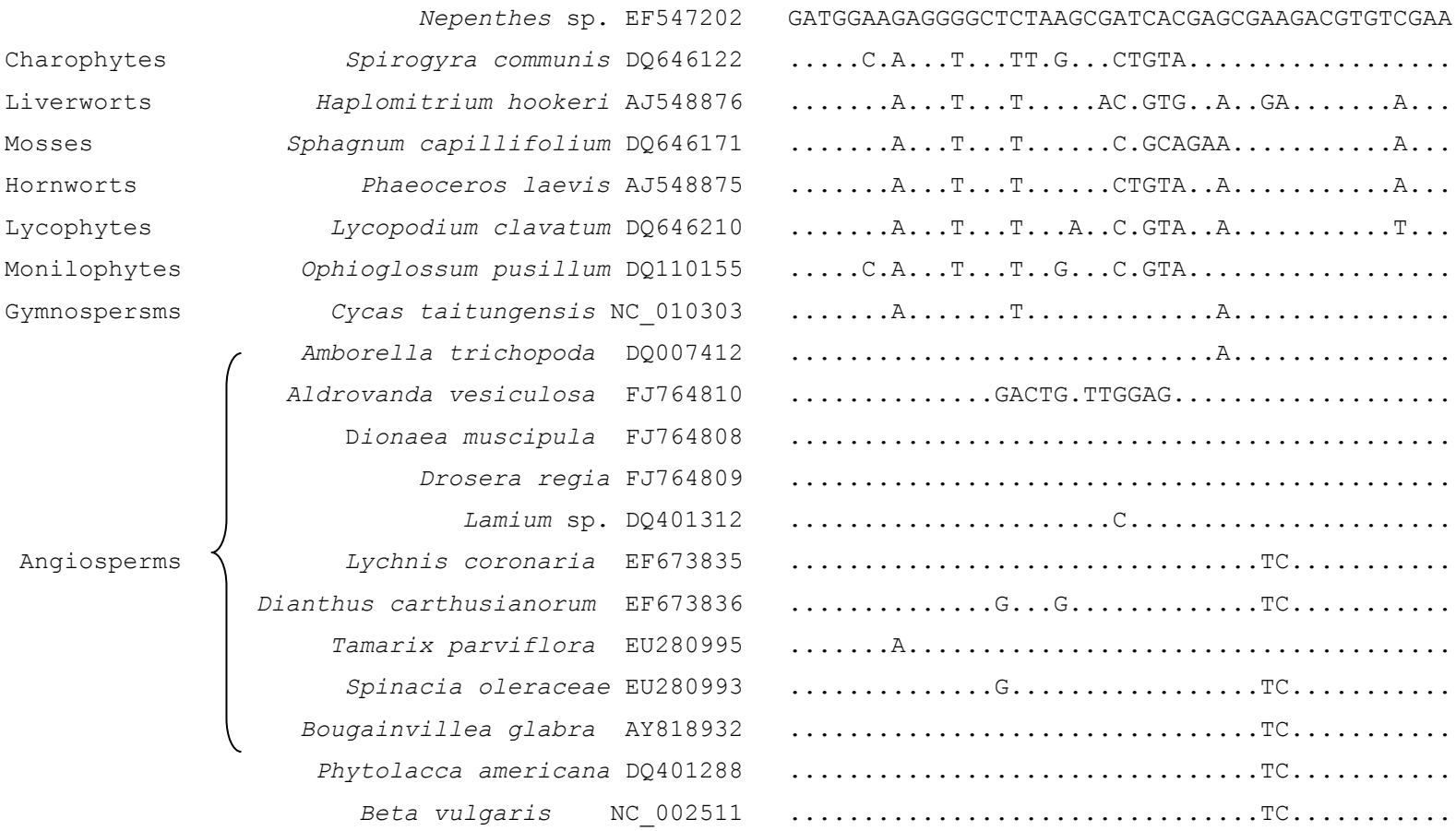


Figure (40): **The unique substitution in *A. vesiculosa* compared to Angiosperms and other groups.** Taxonomic groups are according for Qiu *et al*. (2006).

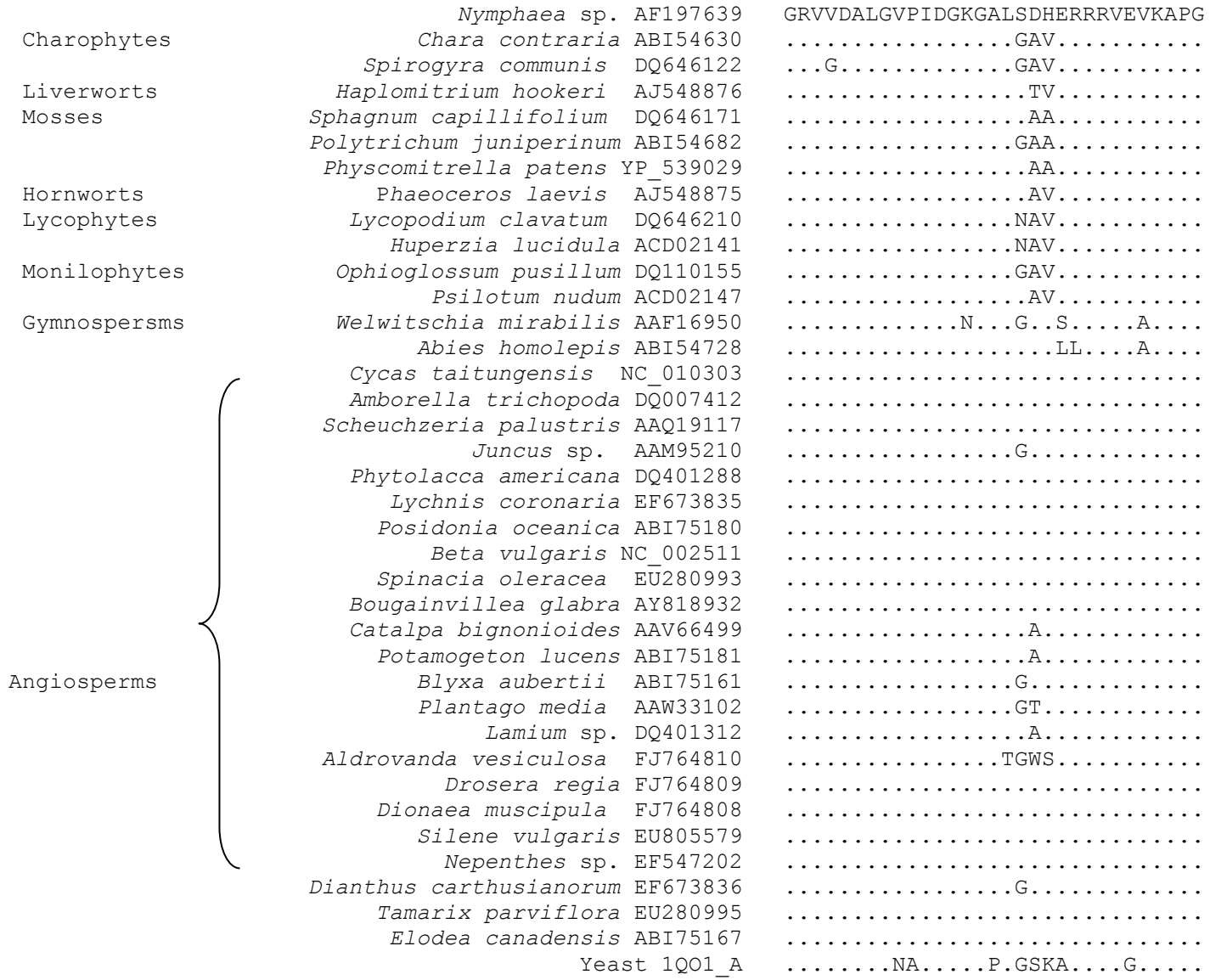


Figure (41): **The alignment of the unique TGWS motif compared to different organisms.** Taxonomic groups are according for Qiu *et al*.(2006

According to the 3D model of yeast Atp1 (1QO1_1), the unique motif (TGWS) ortholog is placed on the surface of the central domain of Atp1, beyond the catalytic sites (Stock *et al*., 1999) as shown in Figure (42A). A three dimentional model was developed of the Atp1 protein sequence showing TGWS motif, also alignment of the same molecule with Yeast Atp1 protein was developed, which shows the conservative nature of this molecule and also showing the three dimentional differences within the TGWS motif (Figure 42B&C).

Interestingly, the only other difference outside this region between Atp1 proteins of *A. vesiculosa* and *D. regia* or *D. muscipula* was one substitution in a 400 amino acid long alignment.

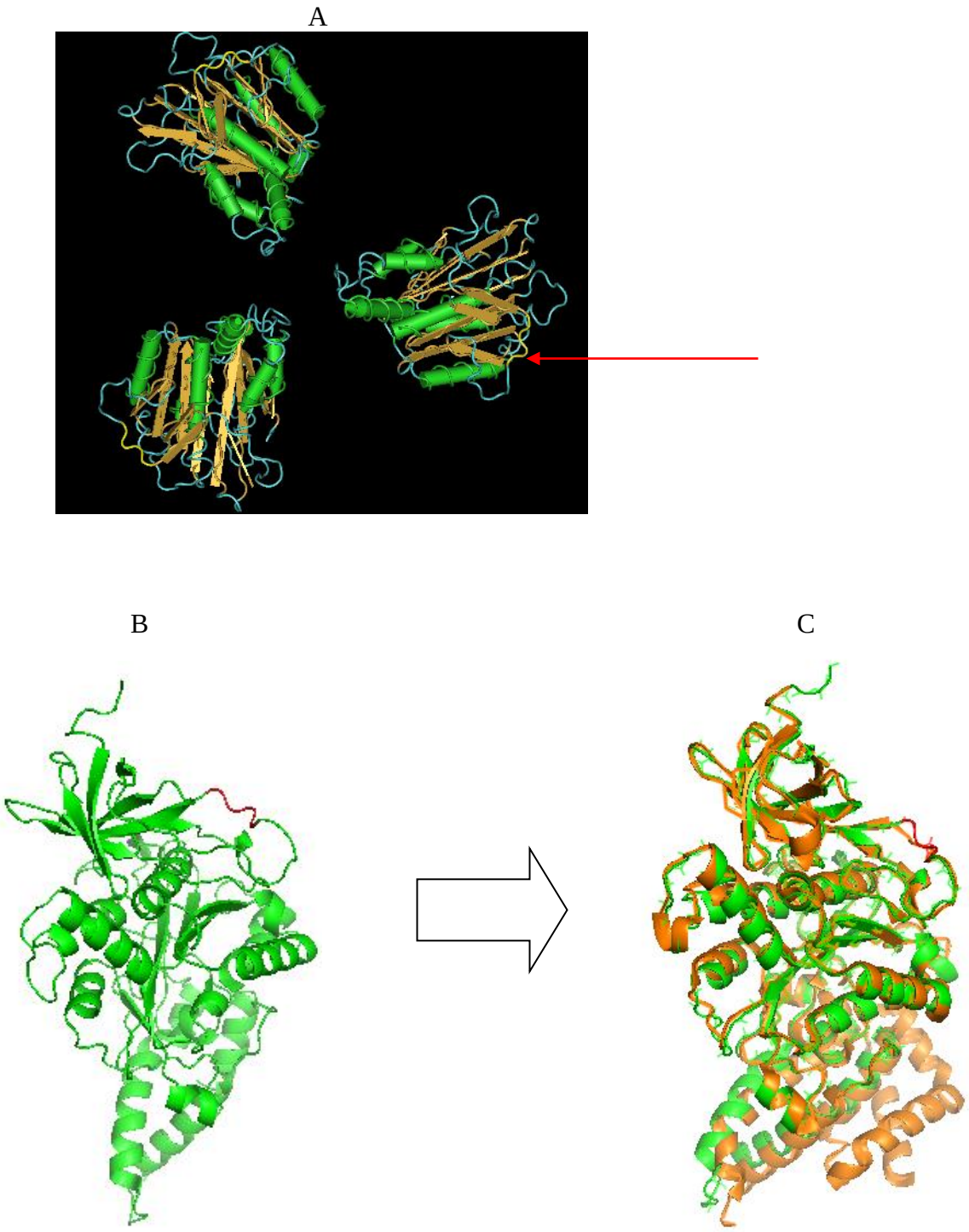


Figure (42): **Three dimentional models of Atp1 protein of Yeast and *A. vesiculosa*.** A) The position of the four amino acids in the Yeast AtpA which is marked by the arrow head and the yellow color. B)The three dimentional structure of *A. vesiculosa* Atp1 protein C) Three dimentional alignment of both of *A. vesiculosa* Atp1 (green) and Yeast Atp1 (Orange). The three dimentional structure was inferred using MODWEB and the alignment was done using PYMOL.The red color marking the unique protein motif TGWS.

5.4.4. Genetic distances

Chloroplast intergenic regions of *D. regia* and *D. muscipula* were sequenced to provide outgroups for expected tree construction, based on chloroplast sequences from various *A. vesiculosa* accessions. Owing to extremely low variation of chloroplast intergenic regions in *A. vesiculosa*, the tree could not be generated. However, the chloroplast sequences of *D. muscipula* and *D. regia* was used to derive relationship among three species.

Genetic distances among *A. vesiculosa*, *D. muscipula* and *D. regia* were calculated using Maximum likelihood, HKY nucleotide substitution model, PAUP 4 as shown in Table (17). The analysis revealed that *A. vesiculosa* was closer to *D. regia* than *D. muscipula* based on five of six analyzed genes.

Table **(**17): **Genetic distances calculated from nt substitutions in six chloroplast intergenic regions of *A. vesiculosa, D. muscipula* and *D. regia***. Empirical nt frequencies and a substitution model HKY85 were applied.

Chloroplast region	*A. vesiculosa – D. muscipula*	*A. vesiculosa - D. regia*	*D. muscipula – D. regia*
atpB-rbcL	0.12916	0.07999	0.10471
cpa-cpb	0.16493	0.15485	0.13024
psbA-trnH	0.14824	0.16078	0.15353
trnS-trnG	0.13837	0.13775	0.08475
trnP-trnW	0.09017	0.07574	0.06902
rpl20-rps12	0.09966	0.06080	0.06796
Combined data	0.12218	0.09869	0.09667

The sequences of the same chloroplast regions in *D. muscipula* and *D. regia*, intended to be used as outgroups. Five chloroplast regions produced reliable alignment between *A. vesiculosa*, *D. muscipula* and *D. regia*, whereas only 570 nt from the *cpa-cpb* could be unambiguously aligned. Genetic distances were calculated based on various nt substitution models. They were in agreement with genetic distances calculated using HKY model (Table17). Five chloroplast intergenic regions, as well as the combined data set suggested shorter genetic distance between *A. vesiculosa* and *D. regia* than between *A. vesiculosa* and *D. muscipula*. The intergenic region *psbA-trnH* was the only one showing the opposite result.

However, the species under study differentiated by indels rather than by nt substitutions in this last region. *D. muscipula* and *D. regia*, but not *A. vesiculosa*, possessed 80 nt insertion similar (75%) to chloroplast *trnY* from *Nepenthes vieillardii* in the *psbA-trnH* intergenic region. The shorter distance between *D. regia* and *A. vesiculosa* cpDNA than between *D. muscipula* and *A. vesiculosa* cpDNA doesn't mean that *D. regia* is the closest relative of *A. vesiculosa*. It merely suggests that the chloroplast genome of *D. regia* is closely related to chloroplast genome of *A. vesiculosa*. The evolution of chloroplast genome doesn't always follows the evolution of nuclear sequences.

Discussion

6. Discussion

6.1. Silene vulgaris

The firs part of the thesis (the natural population and the controlled crosses) investigated the inheritance and the diversity of plant organelles. *S. vulgaris* was chosen for this study because it has high variation in organellar DNA markers (Olson and McCauley, 2002; Štorchová and Olson, 2004; Houliston and Olson, 2006; Barr *et al*., 2007; Touzet and Delph, 2009).

Several techniques were adopted, like DNA sequencing, PCR-RFLP and Southern RFLP markers. The latest technique is traditional, hard and expensive but the results achieved with it gave us insight into a world rarely understood.

In this study the mitochondrial marker based techniques were used to track paternal inheritance and vegetative sorting/substoichiometric shifting in mtDNA in a natural population of gynodioecious *S. vulgaris* and further more to distinguish between them if possible. Although it is difficult to disentangle these processes, this study provided evidence that both affect mitochondrial variation in natural populations of *S. vulgaris*.

6.1.1. Mitochondrial and chloroplast variation in S. vulgaris

In *a* natural population of *S. vulgaris* from Kovary Meadows, PCR-RFLP revealed three haplotypes in *atp1* and two haplotypes in *cox1* coding regions. This PCR-RFLP result is comparable to two or three haplotypes of *atp1* and *cox1*, respectively, found before in several European populations of *S. vulgaris* by McCauley *et al*. (2005) or the 5, 6 haplotypes of *atp1* and *cox1*, respectively, found in eastern USA populations by McCauley and Ellis (2008).

Southern-RFLP analysis revealed six haplotypes in the same number of progeny of Kovary mothers, which confirms the sensitivity of this method as a tool for studying intraspecific polymorphism (Štorchová and Olson, 2004). Higher variation in the flanking regions of mitochondrial genes than the coding regions is due to the fact that they are under lower selection pressure, prone to frequent recombination and consequently evolve faster (Satoh *et al*., 2006; Kubo and Mikami, 2007). It was shown that each PCR-RFLP haplotype was associated with a specific set of Southern-RFLP haplotypes. The sets of Southern haplotypes didn't overlap which could be interpreted by that the diversification in mitochondrial gene

flanking regions occurred after the sequence variation of the specific gene had been established.

The five different cpDNA haplotypes found associated with mtDNA flanking regions. This association may suggest co-evolution of both kinds of markers at least for the time necessary for the accumulation of nucleotide substitutions and indels, which differentiate particular chloroplast haplotypes. This observation may suggest that the processes responsible for variation in mitochondrial genomes (particularly various types of recombination) have comparable rate with the sequence changes in chloroplast intergenic regions.

6.1.2. Paternal transmission of mitochondrial genome

In the natural population study, the father was unknown, so three assumptions were adopted to assumed that paternal inheritance was responsible for the mtDNA haplotype of a particular plant if (1) the mtDNA markers of this individual differed from its siblings, (2) its mitochondrial PCR-RFLP haplotype was not present in maternal plant even in trace amount, and (3) if it differed in all mt markers analyzed, which made paternal inheritance more parsimonious explanation than vegetative sorting and de novo origin of mtDNA haplotype by recombination (substoichiometric shifting), similar assumptions were adopted in previous studies of the inheritance of mt and cpDNA in *S. vulgaris* (McCauley *et al*., 2005; McCauley *et al*., 2007).

The “Knock back” approach revealed that Kov52 mother plant was homoplasmic; therefore one of the three haplotype variants (316) carried paternally transmitted mtDNA, which is in agreement with the criteria mentioned above. On the other hand, it wasn’t possible neither to confirm nor to exclude paternal transmission in the remaining two variants (195 and 273) plants because of the lack of suitable restriction sites which prevented similar test in the remaining two plants. The decrease of the heteroplasmy in the offspring of the heteroplasmic Kov45 mother and the increase of the heteroplasmy in the offspring of the homoplasmic mother Kov52 achieved by the knock back approach was in agreement with the heteroplasmy changes found before in *S. vulgaris* by Pearl *et al*. (2009) which occurred mainly due to the vegetative sorting of mtDNA.

In conclusion, three cases of paternal transmission were identified (two of them putative) in the natural population, two of them carried different mt and cpDNA haplotypes than their siblings but otherwise found in some members of the same population. These two cases are similar to the cases identified by McCauley *et al*. (2005) in the mtDNA and McCauley *et*

al.(2007) in cpDNA. The third variant (273) carried unique *cox1* flanking region and identical chloroplast haplotype to its siblings. The occurrence of 1-3 cases (about 0.3- 1.0 %) of paternal transmission among 331 offspring is comparable to 4% of non maternal offspring revealed among 318 *S. vulgari*s plants by McCauley *et al*. (2005) and the 4% paternal inheritance (complete or partial replacement of the maternal genome counted together) found in the homoplasmic mothers offspring in *S. vulgaris* by Pearl *et al*.(2009). The co-transmission of both organelles cannot be excluded because of the rare paternal inheritance (1.9%) of cpDNA found before in a natural population of *S. vulgaris* (McCauley *et al*., 2007).

6.1.3. Vegetative sorting of mtDNA

The Southern RFLP profiles sometimes contained multiple bands, which might have been derived from the additional gene copies, partial non-functional duplications, or from chimeric genes (Aksyonova *et al*., 2005; Kmiec *et al*., 2006). In two families (Kov45 and Kov52), Southern-RFLP of *atp1* flanking regions showed multiple bands with variable intensities. These families displayed among-individual variation and also within individual variation. Similar among-individual variation in Southern-RFLP patterns, as observed in the Kov52 and Kov45 families, was described in a P2 line of Maize (Kuzmin *et al*., 2005).They found large number of differences between sibling mtDNA, both qualitative (novel mtDNA arrangements) and quantitative (different abundance of the same arrangement). They also found some mtDNA regions are lost in the progeny, whereas novel arrangements appear or increase in abundance. Interestingly, they found that changes in mtDNA profiles lead to changes in profiles of mitochondrial transcripts as we found in this study (Figure 31). They explained this mitochondrial genome destabilization by recessive nuclear mutations. These alleles alter copy number control of mitochondrial subgenomes and disrupt normal transfer of mitochondrial genomic components to progeny, resulting in differences in mtDNA profiles among sibling. The among-individual variation in Southern-RFLP *atp1* in the families Kov52 and Kov45 may arose from sorting of mitochondrial genomes during vegetative growth of heteroplasmic individual (Arrieta-Montiel *et al*. 2001; McCauley *et al*., 2008; Pearl *et al*., 2009). Multiple paternities were discovered in *S. latifolia* (Teixeira and Bernasconi, 2007). We cannot exclude the rule of multiple paternities effect on the among-individual variation in family Kov52.

Differences in Southern-RFLP banding patterns between the branches of the same plant (within-individual variation) were expressed as dramatic differences in band intensities. It

was suggested that such variation in frequencies of different markers may arose from sorting of mitochondrial genomes during vegetative growth which may cause changes in the copy number of mitochondrial genomes in different stems of the same plant (Arrieta-Montiel *et al*., 2001; McCauley and Olson, 2008). Interestingly, the branches differing in Southern-RFLP profiles produced flowers of the same gender. Indeed, vegetative sorting of mtDNA is the proper explanation which could be applied to the changes in the Southern-RFLP band intensities after one year of vegetative growth in the greenhouse.

Using the greenhouse cross between the Kov45 female and a geographically distant hermaphrodite Krn in the X3 cross, it was possible to detect additional chimeric *atp1* (truncated or full gene) in *atp1* Southern-RFLP pattern which might be caused by de novo origin of mitochondrial haplotype by recombination followed by substoichiometric shifting because the paternal transmission in this case was excluded by PCR screening and no band of the same size was observed in maternal DNA. This description corresponds to the definition of substoichiometric shifting (Small *et al*., 1987).

The offspring (1007) from controlled crosses (29) with Krasnojarsk (Siberia) or MV (Virginia, USA) fathers so far analyzed revealed no paternal transmission of mitochondrial genome. This finding may disagree with the limited or rare paternal transmission found in *S. vulgaris* local populations (McCauley *et al*., 2005; Pearl *et al*., 2009). The strict maternal inheritance may be interpreted by the block of paternal transmission that could happen at any stages of Gametogenesis, Fertilization and Postfertilization (Birky, 2001; Barr *et al*., 2005; White *et al*., 2009). It may also reflect the large geographic and presumably genetic distance between the nuclear genomes of both parents. Further investigation of specific populations and also of nuclear genes in *S. vulgaris* will shed light on the factors controlling the transmission of organelles in this species.

6.1.4. Association between mtDNA haplotype and sex ratio

The statistical significant association between *atp1* type KovC and higher frequency of hermaphrodites may be caused by the presence of different CMS gene in KovC type than in the remaining two *atp1* haplotypes. This suggestion is in agreement with non-random association of sex and mtDNA haplotype observed in *S. vulgaris* (Olson and McCauley, 2002; Štorchová and Olson, 2004). It was proposed that this putative CMS gene could be less efficiently restored by the set of restorer genes present in the pollen of Kovary Meadow

population and that at least two different CMS types could be present in the population under study.

The high proportion of shifting females could be related to the high level of heteroplasmy observed in *S. vulgaris* (Welch *et al*., 2006; Pearl *et al*., 2009). Those plants which possessed both female and hermaphrodite flowers at the same time could be explained by that different parts of the same plant carry significantly different mt populations that include different forms of CMS (Andersson, 1999). However, no differences were detected in mtDNA markers between the branches of the same individual bearing female or hermaphrodite flowers. This lack of evidence for mtDNA variation among the branches of different gender could mean either that restoration may work well in some flowers rather than others owing to the different gene expression. It is also possible that other DNA markers should be applied to track genetic variation.

6.2. Silene latifolia

Both of *S. vulgaris* and S. *latifolia* are strongly related but they have different reproduction system as mentioned before, so it was nice opportunity to compare variation of mtDNA markers in plants differing in reproduction system. As *S. latifolia* is monomorphic for most of mitochondrial genes in terms of their coding DNA sequences (Touzet and Delph, 2009), so the focusing here is on mtDNA genes flanking regions to estimate variation of mtDNA. Polymorphism in gene flanking regions was detected, which enabled variation and population structure study based on organellar markers between both species of interest. Southern-RFLP polymorphism was also useful in determining association between cp and mtDNA haplotypes in *S. latifolia.*

6.2.1. Variation among S. latifolia *populations*

The variation revealed in *S. latifolia* population was in the mtDNA *cox1* Southern-RFLP flanking regions (5 haplotypes) and in the mtDNA *psbA-trnH* intergenic spacer (5 haplotypes). The mtDNA variation in the flanking regions achieved in this study is higher than that achieved by Touzet and Delph (2009) in the coding regions and emphasizes the importance of the use of mtDNA flanking regions for the study of within and among populations variation. The variation achieved here is in agreement with previous studies conducted on *S. latifolia* populations (Taylor and Keller, 2007; Sloan et al., 2008; Joulivet

and Bernasconi, 2007). F*ST* values achieved here were 0.687, 0.84 for cp and mtDNA, respectively, suggested high populations structure which is in agreement with previous studies on *S. latifolia* in USA (McCauley, 1994; Richards *et al*., 2003).

Among populations variation could be explained by the joint effect of gene flow and genetic drift. Pollen and seed movement contribute significantly to gene flow because of the obligate outcrossing of dioecious plants. Both processes of gene flow and genetic drift vary among populations were some populations increase in genetic diversity like T or S and show subpopulation structure, while others are more uniform (Slatkin, 1985; Hagen and Hamrick, 1998; Richards *et al*., 1999) and beside those processes there are several factors including variable mutation rate (Sloan *et al*., 2008), colonization history (Barr *et al*., 2007) and introgressive hybridization of cpDNA with closely related species like *S. dioica* (Minder *et al*., 2007).

6.2.2. Variation in S. latifolia *populations compared to* S. vulgaris

The nucleotide diversities found in this study in the chloroplast intergenic region *psbA-trnH* of a single population of *S. vulgaris* (1.895 %) was higher than in a population set of *S. latifolia* (0.635 %). Both values were slightly higher than other chloroplast genes in the previous studies in the same species (Houliston and Olson, 2006; Sloan *et al.*, 2008). The number of segregation sites also followed the same trend as nucleotide diversity. In the comparison between the set of *S. latifolia* populations used in this study and a previous screen done on *S. vulgaris* populations from much smaller area (Štorchová and Olson, 2004) as shown before (Table 16), there were 2, 3 private alleles and 5, 5 haplotypes in cpDNA and mtDNA, respectively, in *S. latifolia* compared to 22, 38 private alleles and 24, 30 haplotypes in cpDNA and mtDNA, respectively, in *S. vulgaris.*

The higher polymorphism found in *S. vulgaris* than *S. latifolia* may be explained by the balancing selection which acts on CMS factors in gynodioecious *S. vulgaris*. *S. vulgaris* harbors many old haplotypes compared to dioecious species *S. latifolia*. These ancient haplotypes resulted from long term balancing selection (Houliston and Olson, 2006; Touzet and Delph, 2009). In addition, mtDNA polymorphism and divergence in *Silene* vulgaris could be further increased also by mutation rate heterogeneity which is a characteristic of mtDNA (Sloan *et al.*, 2008).

6.2.3. CpDNA-mtDNA association in S. vulgaris *and* S. latifolia

In *S. vulgaris*, each Southern-RFLP mitochondrial haplotype was associated with specific cpDNA haplotype except for 2 mtDNA haplotypes (BF* & BB) which both of them nested under one cpDNA haplotype (Figure 23). In *S. latifolia*, the mtDNA haplotype A associated with three cpDNA haplotypes (A,B and C) as shown before in Figure (38). Single nt substitution and one additional A in a 10A repeat separate haplotype A and C. Single nt substitution (A instead of T) separates haplotypes B and C. An indel of 4 nt and several substitutions differed cpDNA haplotypes D and E than A,B and C.

The association between cpDNA-mtDNA observed in this study in both species is in agreement with previous studies of *S. vulgaris* in which mtDNA haplotypes were nested within cpDNA haplotypes (Olson and McCauley, 2000) or cpDNA haplotypes were nested within mtDNA haplotypes. (Štorchova and Olson, 2004). Similarly to previous studies, the structure of data corresponding to free combination of chloroplast and mitochondrial markers (the four-gamete test: AA, AB, BA, BB or Fisher Exact Test) has not been found.

Further investigation could find more combinations of cp and mtDNA haplotypes in *S. latifolia*. The larger data may give a broader idea concerning how strong is linkage disequilibrium between cp and mtDNA in *S. latifolia*.

6.3. Aldrovanda vesiculosa

Above mentioned results dealt with the genus *Silene*, sitting on a fast evolving branch of angiosperm in phylogenetic trees built on the basis of mt genes (Mower *et al*., 2006). There was a question, how high could mt and cpDNA polymorphism in a plant species with completely different reproduction system and life strategy? *Aldrovanda vesiculosa* was chosen because it has very low genetic variation in the nuclear genome (Maldonado San Martín *et al*., 2003; Hoshi *et al*., 2006). Extremely low genetic variation was found in cp and mtDNA among *A. vesiculosa* plants collected in four continents. Here we will discuss these results in the light of the current literature.

6.3.1. mt and cpDNA uniformity in A. vesiculosa *populations*

The two indels found were present by the presence/absence of one T in the T array. The variation in the number of T is common feature of non-coding regions of organellar DNA (Hamilton *et al*., 2003) and may vary within the same population (Lia *et al*., 2007). This T separates the European and non European which could be related with morphological and physiological characters. There are four main differences between the European and the Australian accessions, they are the color (Australians have anthocyanin), overwintering characteristics (Australians have more dormant turion and not well adapted for cold), axillary buds characters (Australians have long and frequently formed buds), and Boron sensitivities (Australians are more sensitive for deficiency) as reported by Adamec (1999). The difference in physiological and morphological characters could be caused by rare point mutations leading to the loss of anthocyanin production or turion formation. The same explanation may be applied for the red Hungarian plants (Adamec, 2005) also a recent observation that some Australian *A. vesiculosa* plants are able to produce turions (Adamec, unpubl.) favors this suggest.

The uniformity of chloroplast intergenic regions and mitochondrial flanking regions among populations are in agreement with the documented low genetic variability of *A. vesiculosa* study carried out by Hoshi *et al*. (2006) where they found homologue sequences among six populations in the internal transcribed spacer of *45S* rDNA. Maldonado San Martín *et al*. (2003) required 151 RAPD primers to obtain 21 polymorphic primers in most of our accessions, also Allozymes analysis used among plants from NE Poland, east Poland, and south Germany at 15 loci of seven enzymatic systems tested showed no difference (Adamec and Tichý, 1997).

This uniformity contrasts with variation revealed in chloroplast genomes of other aquatic plant species (Koga *et al*., 2008; Tan *et al*., 2008; Madeira *et al*., 2007) .This uniformity could be explained by, that *A. vesiculosa* might be a newly formed species (Hoshi *et al*., 2006) and the hypothesis of a recent and severe bottleneck followed by long distance dispersal by migratory birds (Maldonado San Martín *et al*., 2003).

The two exceptions found which are Romania (non-European Homologue) and Tokyo (European homologue) could be explained by the highly unequal in time and space post-glacial spread of *A. vesiculosa* along the migration routes of water birds. Characteristics of the Romania population was found to be different than its European sisters in a previous

study carried by Adamec (1999b) comparing European populations grown under comparable conditions found that Romanian plants are more robust, flower more frequently and produce capsules has different shape. Phenetic relationships analysis of Japan Tokyo accession studied by Maldonado San Martín *et al*. (2003) showed that it was closer to the Australian accessions; also they found that NW Australia accession was genetically most differentiated from all other accessions and recombinant, while no difference was detected among all Australian accessions. However, the difference in a number of Ts in T array is a character bearing very low phylogenetic information value. It can arise repeatedly within the same population or even individual (Hamilton *et al*., 2003; Lia *et al*., 2007).

A. vesiculosa is clearly here the opposite case of *S. vulgaris* or *S. latifolia*, were there is no variation in the flanking regions of the mtDNA and also in the coding regions. The variation found in the cpDNA is a minor variation compared to *Silene*.

6.3.2. Unique atp1 sequence in A. vesiculosa

It's now well known that some carnivorous plants like *Utricularia* and *Genlisea* maintain high rate of mt and cpDNA nucleotide substitution in several genes (Jobson and Albert, 2002; Müller *et al*., 2004; Ellison and Gotelli, 2009). It had been hypothesized that these carnivorous plant species possess unique molecular adaptations associated with the active trapping of prey. For example, Jobson *et al*. (2004) found that the mitochondrial enzyme cytochrome *c* oxidase (*COX1*) in *Utricularia* (Lentibulariaceae) has two unusual amino acid substitutions (Cys-113 and Cys-114) which could contribute to the increased respiratory capacity needed for extensive active ion pumping in the traps. Finally, energetics hypothesis had been concluded by Ellison and Gotelli (2009) showing that the combination of a unique molecular mutation in a key metabolic pathway and the relaxed morphological requirements of aquatic and epiphytic habitats in the *Utricularia* is the driver of morphological diversity in this genus. The unique substitution of four amino acids (TGWS) which was found in *A. vesiculosa* in this study are variable at the highest taxonomic level, but conserved among angiosperms as shown before, we cannot predict whether the substitution revealed in *A. vesiculosa* may affect the functioning of ATP1, a subunit of a key complex responsible for energy conversion in mitochondria. However, the substitution was unique, not found in ATP1 of any other organism. As nt alignment (Figure 40) suggested, replacement of 12 nt in the *atp1* gene of *A. vesiculo*sa rather than gradual changes of four neighboring codons were

responsible for this amino acids substitution. It's not possible to identify the origin of this stretch because it too short. It may be derived from mitochondria genome, but the possibility of horizontal gene transfer from different species, frequent in mtDNA of some plants (Bergthorsson *et al*., 2003, 2004; Richardson and Palmer, 2007) cannot be excluded.

6.3.3. Phylogenetic relationship among A. vesiculosa, D. regia *and* D. muscipula

The uniformity within *A. vesiculosa* didn't allow the use of *Drosera regia* and *Dionaea muscipula* as outgroups to generate a tree reflecting phylogenetic relationships among A. vesiculosa accessions from four continents as mentioned in the introduction. Instead, the cpDNA sequence was used to infer the problematic relations among them.

Although, that it's well established that *D. muscipula* is a sister *A. vesiculosa*, and this pair is sister to *D. regia* (Cameron *et al.*, 2002; Rivadavia *et al*., 2003), these results were based mainly on nuclear sequences and that if cpDNA alone was considered, the tree was either not resolved, or *A. vesiculosa* was closer to *Drosera regia.* The results presented in this study seems to be contradictory for the previous studies but the facts that (1) both of *18S* and *atpB* genes analysis had an alternative topology in which *Aldrovanda* alone is sister to *Drosera* (Cameron *et al*., 2002) and (2) that the phylogenetic relationships among *D. arctturi*, *D. regia*, *Dionaea* and *Aldrovanda* were not well resolved in the *rbcL* tree with high bootstrap support (Rivadavia *et al*., 2003) which support the finding in this study. The problematic phylogenetic positioning of *D. regia* is accompanied by unique morphological characters within the *Drosera* (Takahashi and Sohma, 1982; Williams *et al*., 1994) and it has a unique chromosome number among *Drosera* species of 2n=34 which is similar to those of *Dionaea* (2n=30 or 32) and *Aldrovanda* (2n=38) (Rivadavia *et al.*, 2003).

The inconsistencies between gene trees based on nuclear and cytoplasmic markers could be explained by chloroplast capture, the introgression of a chloroplast from one species into another (Tsitrone *et al.*, 2003) which has been found in several plant species (Wolfe and Elisens, 1995; VanRaamsdonck *et al.*, 1997; Jackson *et al.* 1999; Barrett and Case, 2006; Gaskin and Wilson, 2007; Liston *et al*., 2007).

7. Conclusions

7. Conclusions

The purpose of this Ph.D. work was oriented towards two points.

1. The analysis of organellar diversity among three plant species (*Silene vulgaris, Silene latifolia and Aldrovanda vesiculosa*). The three plant species had different morphological and physiological characters. The focus here was on two physiological points.

1.1. The reproduction system which is directly correlated with the mitochondrial DNA (in the case of CMS). This point was presented in the comparison between *Silene vulgaris* and *Silene latifolia*.

1.2. The different life strategy which is directly correlated with the organellar DNA diversity. This point was presented by the comparison between the two *Silene* species from one side and *Aldrovanda vesiculosa* from the other side. The later usually propagate vegetatively and live in different media (water), it present different life strategy under the umbrella of angiosperms.

2. The analysis of the inheritance of mitochondrial and chloroplast genome in *Silene vulgaris*. The study of this inheritance was facilitated by the high polymorphism available in the organellar DNA. This polymorphism could be detected by the different molecular markers used in this study. These molecular markers included either, gene coding regions markers previously used by D.E.McCauley and his team (McCauley *et al*., 2005; McCauley *et al*., 2007; Pearl *et al*., 2009) or non-coding regions markers like Southern-RFLP. These variable markers gave us larger insight into the inheritance of organellar DNA.

Indeed the evidence on rare paternal inheritance of organellar genomes was confirmed in *S. vulgaris* as found in previous studies. The high mtDNA variation was visible among individuals and within individual. The within individual variation achieved by Southern-RFLP is novel and had not been found before using this method. The large sampling size and the types of mitochondrial markers (*atp1 and cox1*) in Southern-RFLP were not applied before. Only *cox1* was analyzed by Southern-RFLP in previous studies in *S. vulgaris* (Olson and McCauley, 2002; Štorchová and Olson, 2004).The limited paternal inheritance of mitochondrial genomes in *S. vulgaris* alone does not explain the high diversity achieved here, which required consideration of other processes like mitochondrial sorting and substoichiometric shifting to understand this phenomenon.

The core conclusions of this Ph.D. study could be summarized in two points:

1. Organellar DNA diversity vary among species of angiosperms

a. The comparison between organellar diversities of *S. vulgaris* and *S. latifolia* confirmed that the later has higher organellar diversity in both of coding and non-coding regions. *S. vulgaris* is gynodioecious plant whereas *S. latifolia* is a dioecious plant species. This study gave evidence that the breeding system is predictive of organellar DNA polymorphism. The higher polymorphism found in *S. vulgaris* than *S. latifolia* was explained by the balancing selection which acts on CMS factors in gynodioecious *S. vulgaris*. *S. vulgaris* harbors many old haplotypes compared to dioecious species *S. latifolia*. These ancient haplotypes resulted from long term balancing selection. In addition, mtDNA polymorphism and divergence in *Silene* vulgaris could be further increased also by mutation rate heterogeneity which is a characteristic of mtDNA.

b. Organellar DNA uniformity was found among accessions of aquatic carnivorous plant *A. vesiculosa* from four continents. The tiny variation in cpDNA discovered in *A. vesiculosa* in this study is novel, that it represents the first documented sequence variation among *A. vesiculosa* accessions. *A. vesiculosa* belongs to Caryophyllales, and is therefore distantly related to *Silene*. It was proposed that long-distance dispersal, frequent vegetative

propagation and maybe also recent bottleneck and slow mutation rate contribute to genetic uniformity observed in this specie.

***2. Rare paternal inheritance and other processes like mitochondrial sorting and substoichiometric shifting may explain the high variation in mtDNA in* S. vulgaris**

a. The rare paternal inheritance of mtDNA was confirmed in this study in *S. vulgaris* natural population. There was no paternal inheritance detected in the controlled crosses between geographically distant populations which could be interpreted by the block of paternal transmission that could happen at any stages of Gametogenesis, Fertilization and Postfertilization. It may also reflect the large geographic and presumably genetic distance between the nuclear genomes of both parents.

b. The among and within individual variation could be explained by two point of views:

i. Mitochondrial sorting which exist in heteroplasmic individuals like *S. vulgaris*. The process includes random transfer of different numbers of copies of each allele from one mitochondria and cell to the next. The final result of it is a change in the frequency of mitochondrial alleles within different plant tissues.

ii. Substoichiometric shifting which include changes in the relative frequencies of different sublimons in the mtDNA. These sublimons could have rapid and dramatic changes in relative copy number of portions of the mitochondrial genome over the time of one generation and usually involves only a single subgenomic DNA molecule. This process could change the plant phenotype.

Paternal inheritance cannot be excluded because it could happen in parallel with these processes. Further investigation could enhance our understanding of these phenomena.

Next step in the current project of Helena Štorchová as suggested by her is to understand the forces which shape genetic variation in mitochondrial genomes of *S. vulgaris* by determination of sequences corresponding to the bands, observed in complex RFLP patterns of *atp1* flanking regions in several families. This task is facilitated by the knowledge of the complete sequence of mitochondrial genome in *S. vulgaris*, which will be soon available. H.Štorchová will search the sequence of complete genome for repeats and estimate, whether recombination in these repeats could be responsible for creation of new fragments flanking the *atp1* gene. Then, probes derived from these repeats will be prepared and the plants showing within-individual variation will be tested again.

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8.APPENDIX

Appendix A: pGEM-T Easy Vector sequence

GGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGA
T*ATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAA
CGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTG
GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCAC
AATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG
CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACG
CGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCA
CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAA
GAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAA
AATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA
CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCC
TGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCG
CTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCG
CTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCG
CCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTA
TCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGT
AGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTA
GAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA
AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTC
AAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAA
AACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC
CTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATAT
ATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCT
ATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGT
CGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTG
CAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATA
AACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATC
CGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTT
CGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTG
GTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACG
ATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCT
CCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCA
CTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT
AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAAT
AGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTC
TTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA
TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACC
AGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGG
AATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAAT
ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTT
GAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCG
AAAAGTGCCACCTGATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGA
AAATACCGCATCAGGAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG
TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGG
CAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTG
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AAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATC
ACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGA
ACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAAC
GTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCT
GGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTA
ATGCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAA
GGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCA
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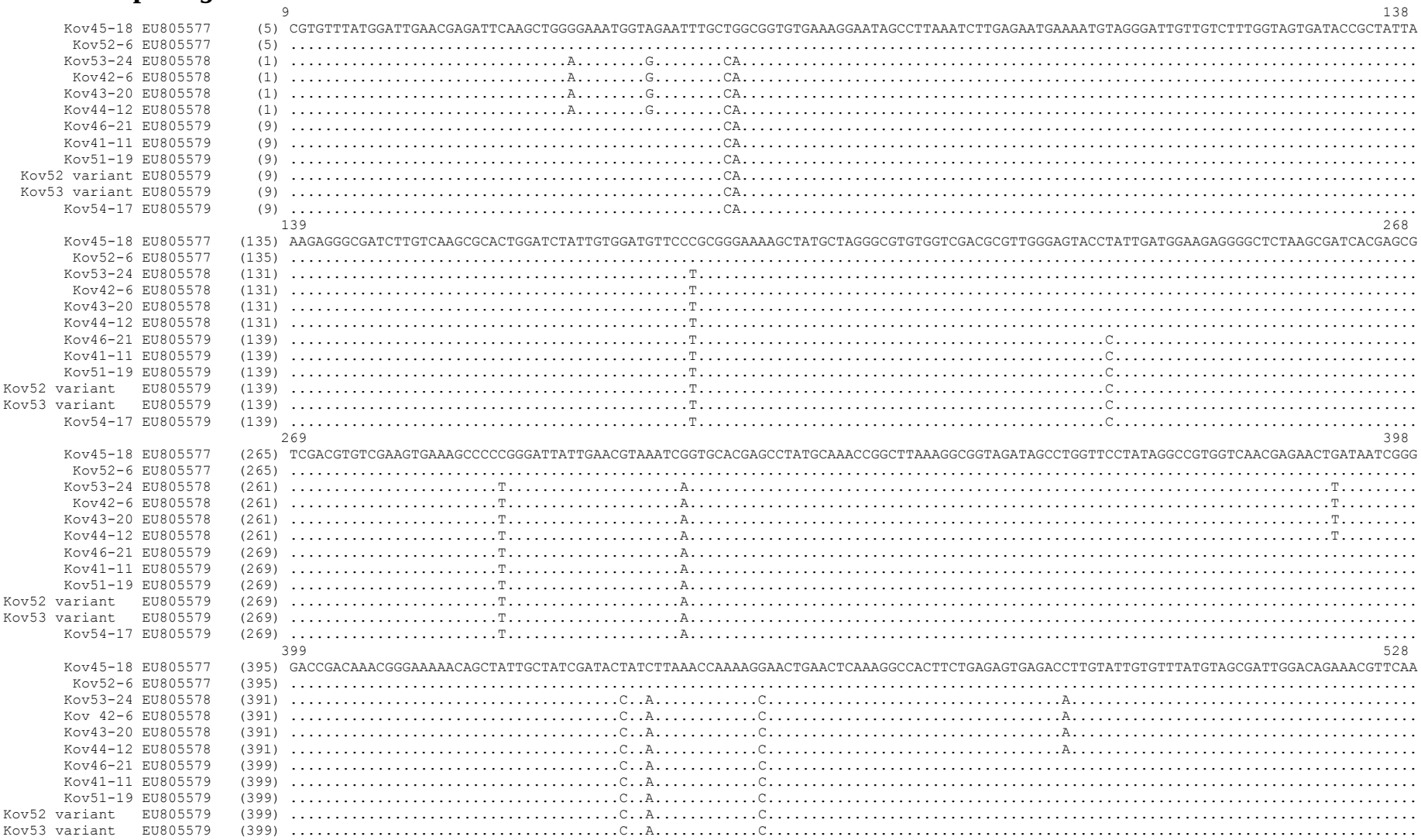
APPENDIX B: S. Vulgaris *alignments*

1. atp1 *alignment*



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     Kov54-17 EU805579   (399) .....................................C..A...........C.....................................................................................
                              529                                                                                                                          658
     Kov45-18 EU805577   (525) CCGTGGCACAATTAGTTCAAATTCTTTCAGAAGCTGATGCTTTGGAATATTCCATTCTTGTAGCAGCCACTGCTTCGGATCCTGCTCCTCTTCAATTTCTGGCCCCATATTCTGGATGTGCTATGGGAGA
      Kov52-6 EU805577   (525) .....................................................................................................................................
     Kov53-24 EU805578   (521) .T.................................GA.....................................................................................
      Kov42-6 EU805578   (521) .T.................................GA.....................................................................................
     Kov43-20 EU805578   (521) .T.................................GA.....................................................................................
     Kov44-12 EU805578   (521) .T.................................GA.....................................................................................
     Kov46-21 EU805579   (529) ................G..................A.....................................................................................
     Kov41-11 EU805579   (529) ................G..................A.....................................................................................
     Kov51-19 EU805579   (529) ................G..................A.....................................................................................
Kov52 variant EU805579   (529) ................G..................A.....................................................................................
Kov53 variant EU805579   (529) ................G..................A.....................................................................................
     Kov54-17 EU805579   (529) ................G..................A.....................................................................................
                              659                                                                                                                          788
     Kov45-18 EU805577   (655) ATATTTCCGCGATAATGGAATGCACGCATTAATAATCTATGATGATCTTAGTAAACAGGCAGTGGCATATCGACAAATGTCATTATTGTTACGCCGACCACCAGGCCGTGAGGCTTTCCCAGGCGACGTT
      Kov52-6 EU805577   (655) .....................................................................................................................................
     Kov53-24 EU805578   (651) ...........................................................G.........................................................................
       Kov42-6 EU805578  (651) ...........................................................G.........................................................................
     Kov43-20 EU805578   (651) ...........................................................G.........................................................................
     Kov44-12 EU805578   (651) ...........................................................G.........................................................................
     Kov46-21 EU805579   (659) ...........................................................G.........................................................................
     Kov41-11 EU805579   (659) ...........................................................G.........................................................................
     Kov51-19 EU805579   (659) ...........................................................G.........................................................................
Kov52 variant EU805579   (659) ...........................................................G.........................................................................
Kov53 variant EU805579   (659) ...........................................................G.........................................................................
     Kov54-17 EU805579   (659) ...........................................................G.........................................................................
                              789                                                                                                                          918
     Kov45-18 EU805577   (785) TTCTATTTACATTCTCGTCTCTTAGAAAGAGCCGCTAAACGATCGGACCAGACAGGTGCAGGTAGCTTGACCGCCTTACCCGTCATTGAAACACAAGCTGGAGACGTATCAGCCTATATTCCCACCAATG
      Kov52-6 EU805577   (785) .....................................................................................................................................
     Kov53-24 EU805578   (781) .....................................................................................................................................
      Kov42-6 EU805578   (781) .....................................................................................................................................
     Kov43-20 EU805578   (781) .....................................................................................................................................
     Kov44-12 EU805578   (781) .....................................................................................................................................
     Kov46-21 EU805579   (789) .....................................................................................................................................
     Kov41-11 EU805579   (789) .....................................................................................................................................
     Kov51-19 EU805579   (789) .....................................................................................................................................
Kov52 variant EU805579   (789) .....................................................................................................................................
Kov53 variant EU805579   (789) .....................................................................................................................................
     Kov54-17 EU805579   (789) .....................................................................................................................................
                              919                                                                                                                         1048
     Kov45-18 EU805577   (915) TGATCTCCATTACTGATGGACAAATCTGTTTGGAAACAGAGCTCTTTTATCGCGGAATTAGACCTGCTATTAACGTCGGCTTATCTGTCAGTCGCGTAGGGTCTGCCGCTCAGTTGAAAGCTATGAAACA
      Kov52-6 EU805577   (915) .....................................................................................................................................
     Kov53-24 EU805578   (911) .....................................................................................................................C.......................
      Kov42-6 EU805578   (911) .....................................................................................................................C.......................
     Kov43-20 EU805578   (911) .....................................................................................................................C.......................
     Kov44-12 EU805578   (911) .....................................................................................................................C.......................
     Kov46-21 EU805579   (919) .....................................................................................................................C.......................
     Kov41-11 EU805579   (919) .....................................................................................................................C.......................
     Kov51-19 EU805579   (919) .....................................................................................................................C.......................
Kov52 variant EU805579   (919) .....................................................................................................................C.......................
Kov53 variant EU805579   (919) .....................................................................................................................C.......................
     Kov54-17 EU805579   (919) .....................................................................................................................C.......................
                             1049                                                                                                                         1178
      Kov45-18 EU805577 (1045) AGTTTGCGGGAGTCCAAAACTGGAATTGGCACAATATCGCGAAGTGGCCGCCTTTGCTCAATTTGGCTCAGACCTTGATGCTGCGACTCAGGCATTACTCAATAGAGGTGCAAGGCTTACAGAAGTACCG
      Kov52-6 EU805577  (1045) .....................................................................................................................................
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     Kov53-24 EU805578  (1041) ...C.....................................................................................................................
      Kov42-6 EU805578  (1041) ...C.....................................................................................................................
     Kov43-20 EU805578  (1041) ...C.....................................................................................................................
     Kov44-12 EU805578  (1041) ...C.....................................................................................................................
      Kov46-21 EU805579 (1049) ...C.....................................................................................................................
     Kov41-11 EU805579  (1049) ...C.....................................................................................................................
     Kov51-19 EU805579  (1049) ...C.....................................................................................................................
Kov52  variant EU805579 (1049) ...C.....................................................................................................................
Kov53 variant EU805579  (1049) ...C.....................................................................................................................
     Kov54-17 EU805579  (1049) ...C.....................................................................................................................
                            1179                                    1218
     Kov45-18 EU805577  (1175) AAACAACAACAATACGCACCACTTCCAATTGAAAAACAAA
      Kov52-6 EU805577  (1175) ........................................
     Kov53-24 EU805578  (1171) ..............T.........................
     Kov42-6 EU8055788  (1171) ..............T.........................
     Kov43-20 EU805578  (1171) ..............T.........................
     Kov44-12 EU805578  (1171) ..............T.........................
     Kov46-21 EU805579  (1179) ..............T.....G...................
     Kov41-11 EU805579  (1179) ..............T.....G...................
      Kov51-19 EU805579 (1179) ..............T.....G...................
Kov52  variant EU805579 (1179) ..............T.....G...................
Kov53 variant EU805579  (1179) ..............T.....G...................
     Kov54-17 EU805579  (1179) ..............T.....G...................
```

2. *cox1* alignment

```
                               1                                                                                                                              130
      Kov45-18 EU805575    (1) CCGCGGAGTGATGGGCACATGCTTCTCAGTACTGATTCGTATGGAATTAGCACGCCCAGGCGATCAAATTCTTGGCGGAAATCATCAACTTTATAACGTTTTAATAACGGCTCACGCCTTTTTAATGATC
       Kov42-6 EU805575    (1) ..................................................................................................................................
       Kov52-6 EU805575    (1) ..................................................................................................................................
      Kov44-12 EU805575    (1) ..................................................................................................................................
      Kov53-24 EU805575    (1) ..................................................................................................................................
      Kov43-20 EU805575    (1) ..................................................................................................................................
      Kov46-21 EU805576    (1) .........................................................C..T.....................................................................
      Kov51-19 EU805576    (1) .........................................................C..T.....................................................................
      Kov54-17 EU805576    (1) .........................................................C..T.....................................................................
      Kov41-11 EU805576    (1) .........................................................C..T.....................................................................
 Kov52 variant EU805576    (1) .........................................................C..T.....................................................................
 Kov53 variant EU805576    (1) .........................................................C..T.....................................................................
                               131                                                                                                                            260
      Kov45-18 EU805575  (131) TTTTTTATGGTTATGCCTGCGATGATAGGCGGATTTGGTAATTGGTTTGTTCCTATTCTGATAGGTGCACCTGACATGGCATTTCCACGATTAAATAACATTTCATTCTGGTTGTTGCCCCCTTCTCTCT
       Kov42-6 EU805575  (131) ..................................................................................................................................
       Kov52-6 EU805575  (131) ..................................................................................................................................
      Kov44-12 EU805575  (131) ..................................................................................................................................
      Kov53-24 EU805575  (131) ..................................................................................................................................
      Kov43-20 EU805575  (131) ..................................................................................................................................
      Kov46-21 EU805576  (131) ..................................................................................................................................
      Kov51-19 EU805576  (131) ..................................................................................................................................
      Kov54-17 EU805576  (131) ..................................................................................................................................
      Kov41-11 EU805576  (131) ..................................................................................................................................
 Kov52 variant EU805576  (131) ..................................................................................................................................
 Kov53 variant EU805576  (131) ..................................................................................................................................
                               261                                                                                                                            390
      Kov45-18 EU805575  (261) TGCTCCTCTTAAGCTCAGCCTTAGTAGAAGTGGGTAGCGGGACTGGGTGGACGGTCTATCCGCCCTTAAGTGGTATTACCAGCCATTCTGGAGGAGCAGTTGATTTAGCCATTTTTAGTCTTCATCTCTC
       Kov42-6 EU805575  (261) ..................................................................................................................................
       Kov52-6 EU805575  (261) ..................................................................................................................................
      Kov44-12 EU805575  (261) ..................................................................................................................................
      Kov53-24 EU805575  (261) ..................................................................................................................................
      Kov43-20 EU805575  (261) ..................................................................................................................................
      Kov46-21 EU805576  (261) ..................................................................................................................................
      Kov51-19 EU805576  (261) ..................................................................................................................................
      Kov54-17 EU805576  (261) ..................................................................................................................................
      Kov41-11 EU805576  (261) ..................................................................................................................................
 Kov52 variant EU805576  (261) ..................................................................................................................................
 Kov53 variant EU805576  (261) ..................................................................................................................................

                               391                                                                                                                            520
      Kov45-18 EU805575  (391) TGGTGTTTCATCCATTTTAGGTTCTATCAATTTTATAACAACTATCTTCAACATGCGTGGACCTGGAATGACTATGCATAGATTACCCTTATTTGTGTGGTCCGTTCTAGTAACAGCATTCCTACTTTTA
       Kov42-6 EU805575  (391) ..................................................................................................................................
       Kov52-6 EU805575  (391) ..................................................................................................................................
      Kov44-12 EU805575  (391) ..................................................................................................................................
      Kov53-24 EU805575  (391) ..................................................................................................................................
      Kov43-20 EU805575  (391) ..................................................................................................................................
      Kov46-21 EU805576  (391) ..................................................................................................................................
      Kov51-19 EU805576  (391) ..................................................................................................................................
      Kov54-17 EU805576  (391) ..................................................................................................................................
      Kov41-11 EU805576  (391) ..................................................................................................................................
 Kov52 variant EU805576  (391) ..................................................................................................................................
 Kov53 variant EU805576  (391) ..................................................................................................................................
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                          521                                                                                                650
     Kov45-18 EU805575   (521) TTATCACTTCCGGTACTGGCGGGGGCAATTACCATGTTATTAACCGATCGAAACTTTAATACAACCTTTTTTGATCCGGCTGGGGGAGGAGACCCCATATTATATCAGCATCTTTTTTGGTTTTTCGGTC
      Kov42-6 EU805575   (521) ................................................................................................................
      Kov52-6 EU805575   (521) ................................................................................................................
     Kov44-12 EU805575   (521) ................................................................................................................
     Kov53-24 EU805575   (521) ................................................................................................................
     Kov43-20 EU805575   (521) ................................................................................................................
     Kov46-21 EU805576   (521) ................................................................................................................
     Kov51-19 EU805576   (521) ................................................................................................................
     Kov54-17 EU805576   (521) ................................................................................................................
     Kov41-11 EU805576   (521) ................................................................................................................
Kov52 variant EU805576   (521) ................................................................................................................
Kov53 variant EU805576   (521) ................................................................................................................

                          651                                                                                                780
     Kov45-18 EU805575   (651) ATCCAGAGGTCTATATTCTCATTCTGCCTGGATTCGGTATCATAAGTCATATCGTTTCTACTTTTTCTGGAAAACCGGTCTTCGGGTATCTAGGCATGGTTTATGCCATGATCAGTATAGGTGTTCTTGG
      Kov42-6 EU805575   (651) ................................................................................................................
      Kov52-6 EU805575   (651) ................................................................................................................
     Kov44-12 EU805575   (651) ................................................................................................................
     Kov53-24 EU805575   (651) ................................................................................................................
     Kov43-20 EU805575   (651) ................................................................................................................
     Kov46-21 EU805576   (651) ................................................................................................................
     Kov51-19 EU805576   (651) ................................................................................................................
     Kov54-17 EU805576   (651) ................................................................................................................
     Kov41-11 EU805576   (651) ................................................................................................................
Kov52 variant EU805576   (651) ................................................................................................................
Kov53 variant EU805576   (651) ................................................................................................................

                          781                                                                                                910
     Kov45-18 EU805575   (781) ATTTCTTGTTTGGGCTCATCATATGTTTACTGTGGGCTTAGACGTTGATACCCGCGCTTACTTCACCGCAGCTACCATGATCATAGCGGTCCCCACTGGAATTAAAATCTTTAGTTGGATCGCTACCATG
      Kov42-6 EU805575   (781) ................................................................................................................
      Kov52-6 EU805575   (781) ................................................................................................................
     Kov44-12 EU805575   (781) ................................................................................................................
     Kov53-24 EU805575   (781) ................................................................................................................
     Kov43-20 EU805575   (781) ................................................................................................................
     Kov46-21 EU805576   (781) ................................................................................................................
     Kov51-19 EU805576   (781) ................................................................................................................
     Kov54-17 EU805576   (781) ................................................................................................................
     Kov41-11 EU805576   (781) ................................................................................................................
Kov52 variant EU805576   (781) ................................................................................................................
Kov53 variant EU805576   (781) ................................................................................................................

                          911                                                                                               1040
     Kov45-18 EU805575   (911) TGGGGGGGTTCGATACAATACAAAACACCCATGTTATTTGCTGTAGGGTTCATCTTTTTGTTCACCGTGGGAGGACTCACTGGAATAGTCCTGGCAAATTCTGGGCTAGACATTGCTCTACATGACACTT
      Kov42-6 EU805575   (911) ................................................................................................................
      Kov52-6 EU805575   (911) ................................................................................................................
     Kov44-12 EU805575   (911) ................................................................................................................
     Kov53-24 EU805575   (911) ................................................................................................................
     Kov43-20 EU805575   (911) ................................................................................................................
     Kov46-21 EU805576   (911) ................................................................................................................
     Kov51-19 EU805576   (911) ................................................................................................................
     Kov54-17 EU805576   (911) ................................................................................................................
     Kov41-11 EU805576   (911) ................................................................................................................
Kov52 variant EU805576   (911) ................................................................................................................
Kov53 variant EU805576   (911) ................................................................................................................
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                                1041                                                                                                                1170
      Kov45-18 EU805575  (1041) ATTATGTGGTTGCACATTTCCATTATGTACTTTCTATGGGAGCCGTTTTTGCTTTATTTGCAGGATTTTACTATTGGGTGGGAAAAATCTTTGGTCGAACATACCCTGAAACTTTAGGTCAAATCCATTT
       Kov42-6 EU805575  (1041) ....................................................................................................................
       Kov52-6 EU805575  (1041) ....................................................................................................................
      Kov44-12 EU805575  (1041) ....................................................................................................................
      Kov53-24 EU805575  (1041) ....................................................................................................................
      Kov43-20 EU805575  (1041) ....................................................................................................................
      Kov46-21 EU805576  (1041) ....................................................................................................................
      Kov51-19 EU805576  (1041) ....................................................................................................................
      Kov54-17 EU805576  (1041) ....................................................................................................................
      Kov41-11 EU805576  (1041) ....................................................................................................................
  Kov52 variant EU805576  (1041) ....................................................................................................................
  Kov53 variant EU805576  (1041) ....................................................................................................................

                                1171                                                                                                                1300
      Kov45-18 EU805575  (1171) TTGGATCACTTTTTTCGGGGTTAATCTTACCTTCTTCCCGATGCATTTTTTAGGGCTTTCGGGTATGCCACGTCGCATTCCAGATTATCCAGATGCTTACGCAGGCTGGAATGCCCTTAGCAGTTTTGGC
       Kov42-6 EU805575  (1171) ....................................................................................................................
       Kov52-6 EU805575  (1171) ....................................................................................................................
      Kov44-12 EU805575  (1171) ....................................................................................................................
      Kov53-24 EU805575  (1171) ....................................................................................................................
      Kov43-20 EU805575  (1171) ....................................................................................................................
      Kov46-21 EU805576  (1171) ....................................................................................................................
      Kov51-19 EU805576  (1171) ....................................................................................................................
      Kov54-17 EU805576  (1171) ....................................................................................................................
      Kov41-11 EU805576  (1171) ....................................................................................................................
  Kov52 variant EU805576  (1171) ....................................................................................................................
  Kov53 variant EU805576  (1171) ....................................................................................................................

                                1301                                                                                                                1430
      Kov45-18 EU805575  (1301) TCTTATATCTCCGTAGTTGGGATTTGTTGTTTCTTCGTGATCGTAACAATCACTTTAAGCAGTGGAAAGAACAAAAGATGTGCTCCAAGTCCTTGGGCTGTTGAAGAGAATTCACCCACACTGGAATGGA
       Kov42-6 EU805575  (1301) ....................................................................................................................
       Kov52-6 EU805575  (1301) ....................................................................................................................
      Kov44-12 EU805575  (1301) ....................................................................................................................
      Kov53-24 EU805575  (1301) ....................................................................................................................
      Kov43-20 EU805575  (1301) ....................................................................................................................
      Kov46-21 EU805576  (1301) ....................................................................................................................
      Kov51-19 EU805576  (1301) ....................................................................................................................
      Kov54-17 EU805576  (1301) ....................................................................................................................
      Kov41-11 EU805576  (1301) ....................................................................................................................
  Kov52 variant EU805576  (1301) ....................................................................................................................
  Kov53 variant EU805576  (1301) ....................................................................................................................

                                1431             1452
      Kov45-18 EU805575  (1431) TGGTACAAAGCCCTCCAGCCTT
       Kov42-6 EU805575  (1431) ......................
       Kov52-6 EU805575  (1431) ......................
      Kov44-12 EU805575  (1431) ......................
      Kov53-24 EU805575  (1431) ......................
      Kov43-20 EU805575  (1431) ......................
      Kov46-21 EU805576  (1431) ......................
      Kov51-19 EU805576  (1431) ......................
      Kov54-17 EU805576  (1431) ......................
      Kov41-11 EU805576  (1431) ......................
Kov52 variant   EU805576  (1431) ......................
  Kov53 variant EU805576  (1431) ......................
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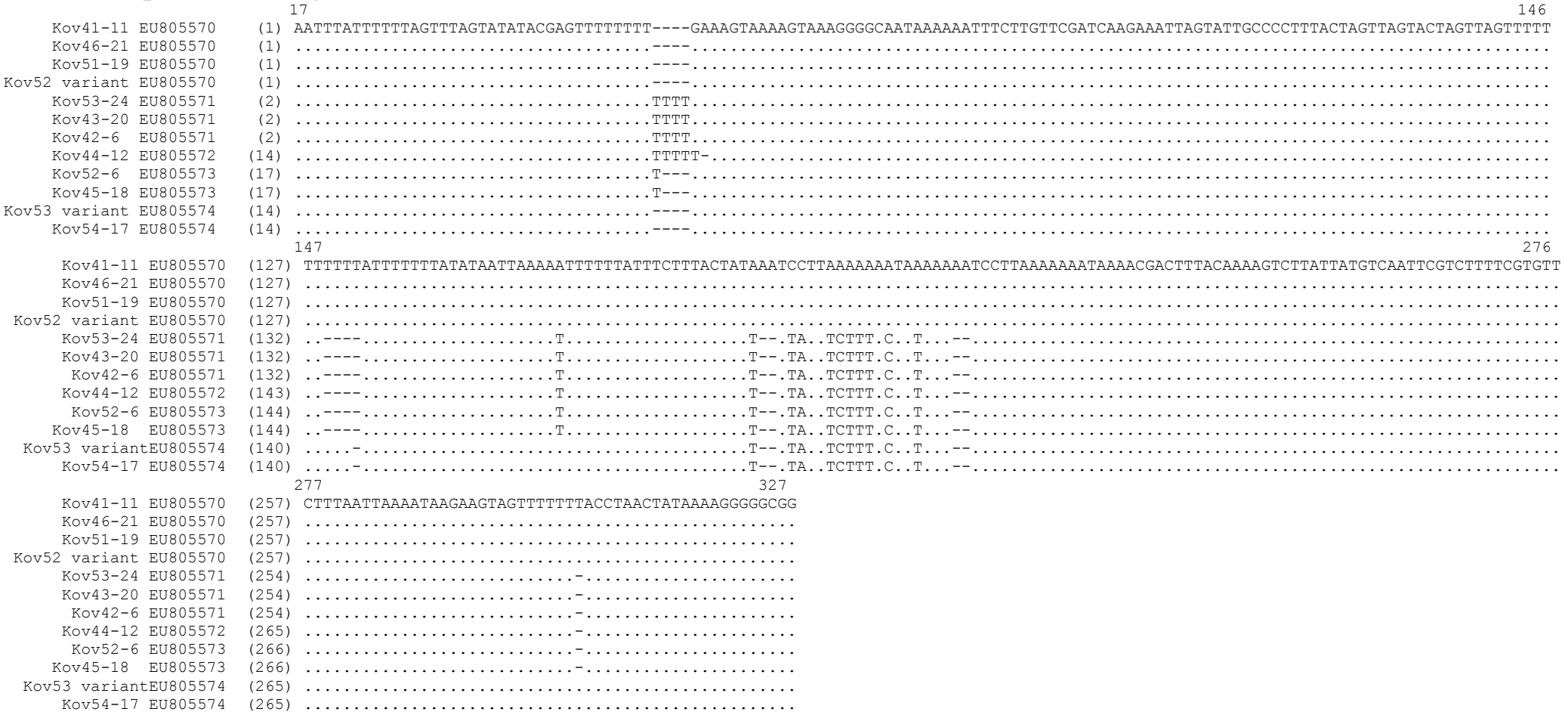
3. psbA-trnH *alignment*



Appendix C: S. latifolia *alignments*

1. psbA-trnH

```
                1                                                                   70
J.23.11     (1) ATAAAATTTCTTTTTTAGTTTAGTATATAAAAGTAAAGGGGCAATAAAAAATTTCTTGTTCGATCAAGAA
K.04.13     (1) ............................................................................
A.04.05     (1) ............................................................................
A.05.16     (1) ...........................................................T................
T.09.10     (1) ............................................................................
                71                                                                  140
J.23.11    (71) ACTAGTATTGCCCCTTTACTAGTTCGTTTTTTTATATAATTTATTTTTTTTTTTATTCTTTACTAGAAAAT
K.04.13    (71) ..............................................-.............................
A.04.05    (71) ..................................G.............-.............................
A.05.16    (71) ..................................G............................................
T.09.10    (71) ..................................G............................................
                141                                                                 210
J.23.11   (141) CCTAAAAAAAAATAAAACGACTTTACAAAAGTCTTATTATGTCAATTCGTCTTTTCTTGTTTTTTAATTA
K.04.13   (140) ............................................................................
A.04.05   (140) ...........-............................................................
A.05.16   (141) ...........-............................................................
T.09.10   (141) ...........-............................................................
                211                                             261
J.23.11   (211) AGATAAGAAGTCGTTCGTTTTTTTACGTAACTATAAAAGGGGGCGGATGTA
K.04.13   (210) ...........................................
A.04.05   (209) ...........A..----.........................
A.05.16   (210) ...........A..----.........................
T.09.10   (210) ...........A..----.........................
```

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2. *CoxI*

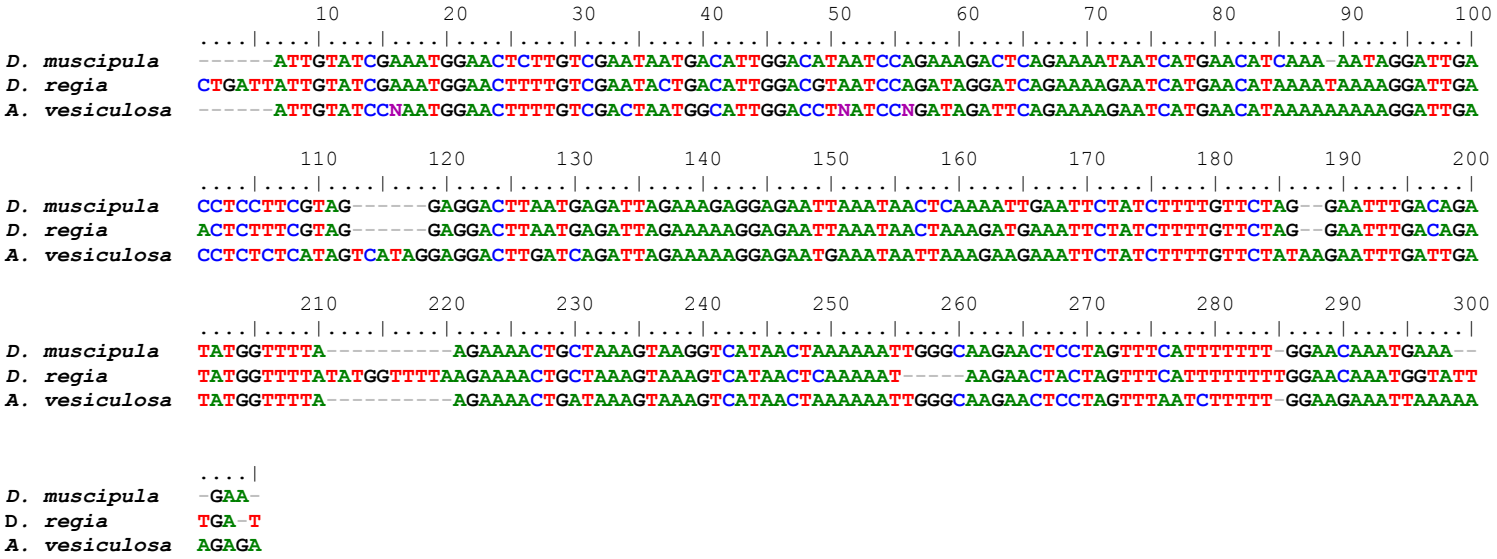
```
                 1                                                                                                                               130
A.09.14    (1)   CAGTACTGATTCGTATGGAATTAGCACGCCCCGGCGATCAAATTCTTGGTGGAAATCATCAACTTTATAATGTTTTAATAACGGCTCACGCTTTTTTAATGATCTTTTTTATGGTTATGCCTGCGATGAT
J.19.12    (1)   ..................................................................................................................................
K.07.02    (1)   ..................................................................................................................................
S.06.01    (1)   ..................................................................................................................................
                 131                                                                                                                             260
A.09.14  (131)   AGGTGGATTTGGTAATTGGTTTGTTCCGATTCTGATAGGTGCACCTGACATGGCATTTCCCCGATTAAATAATATTTCATTCTGGTTGTTGCCCCCAAGTCTCTTGCTCCTATTAAGCTCAGCCTTAGTA
J.19.12  (131)   ..................................................................................................................................
K.07.02  (131)   ..................................................................................................................................
S.06.01  (131)   ..................................................................................................................................
                 261                                                                                                                             390
A.09.14  (261)   GAAGTGGGTAGCGGGACTGGGTGGACGGTCTATCCGCCCTTAAGTGGTATTACCAGCCATTCTGGAGGAGCAGTTGATTTAGCCATTTTTAGTCTTCATCTCTCTGGTGTTTCATCCATTTTAGGTTCTA
J.19.12  (261)   ..................................................................................................................................
K.07.02  (261)   ..................................................................................................................................
S.06.01  (261)   ..................................................................................................................................
                 391                                                                                                                             520
A.09.14  (391)   TCAATTTTATAACAACTATCTTCAACATGCGTGGACCTGGAATGACTATGCATAGATTACCCTTATTTGTGTGGTCCGTTCTAGTAACAGCATTCCTACTTTTATTATCACTTCCGGTACTGGCGGGGGC
J.19.12  (391)   ..................................................................................................................................
K.07.02  (391)   ..................................................................................................................................
S.06.01  (391)   ..................................................................................................................................
                 521                                                                                     608
A.09.14  (521)   AATTACCATGTTATTAACCGATCGAAACTTTAATACAACCTTTTTTGATCCGGCTGGGGGAGGAGACCCCATATTATATCAGCATCTT
J.19.12  (521)   ........................................................................................
K.07.02  (521)   .......................................................................................
S.06.01  (521)   .......................................................................................
```

3. *atp1*

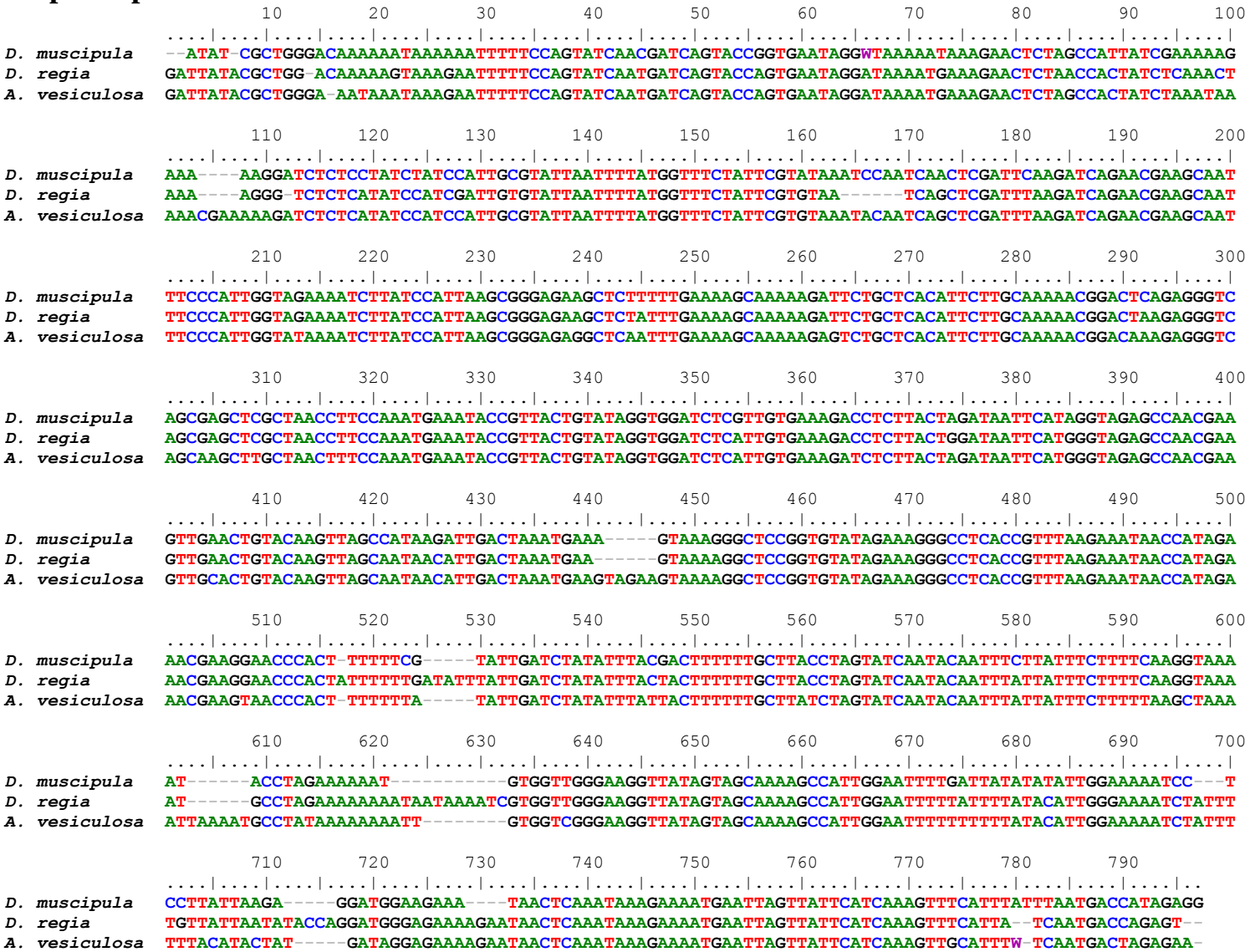
```
                1                                                                                                                                  130
J.19.12     (1) AACGTAAATCAGTGCACGAGCCTATGCAAACCGGCTTAAAGGCGGTAGATAGCCTGGTTCCTATAGGCCGTGGTCAACGAGAACTTATAATCGGGGACCGACAAACGGGAAAAACAGCTATTGCTATCGA
k.07.02     (1) ....................................................................................................................................
S.06.01     (1) ....................................................................................................................................
A.09.14     (1) ....................................................................................................................................
              131                                                                                                                                  260
J.19.12   (131) TACCATATTAAACCAAAAGCAACTGAACTCAAAGGCCACTTCTGAGAGTGAGACATTGTATTGTGTTTATGTAGCGATTGGACAGAAACGTTCAACTGTGGCACAATTAGTTCAAATTCTTTCAGAAGCG
k.07.02   (131) ....................................................................................................................................
S.06.01   (131) ....................................................................................................................................
A.09.14   (131) ....................................................................................................................................
              261                                                                                                                                  390
J.19.12   (261) AATGCTTTGGAATATTCCATTCTTGTAGCAGCCACTGCTTCGGATCCTGCTCCTCTTCAATTTCTGGCCCCATATTCTGGATGTGCTATGGGAGAATATTTCCGCGATAATGGAATGCACGCATTAATAA
k.07.02   (261) ....................................................................................................................................
S.06.01   (261) ....................................................................................................................................
A.09.14   (261) ....................................................................................................................................
              391                                                                                                                                  520
J.19.12   (391) TCTATGATGATCTTAGTAAACAGGCGGTGGCATATCGACAAATGTCATTATTGTTACGCCGACCACCAGGCCGTGAGGCTTTCCCAGGCGACGTTTTCTATTTACATTCTCGTCTCTTAGAAAGAGCCGC
k.07.02   (391) ....................................................................................................................................
S.06.01   (391) ....................................................................................................................................
A.09.14   (391) ....................................................................................................................................
              521                                                                                                                                  650
J.19.12   (521) TAAACGATCGGACCAGACAGGTGCAGGTAGCTTGACCGCCTTACCCGTCATTGAAACACAAGCTGGAGACGTATCAGCCTATATTCCCACCAATGTGATCTCCATTACTGATGGACAAATCTGTTTGGAA
k.07.02   (521) ....................................................................................................................................
S.06.01   (521) ....................................................................................................................................
A.09.14   (521) ....................................................................................................................................
              651                                                                                                                              772
J.19.12   (651) ACAGAGCTCTTTTATCGCGGAATTAGACCTGCTATTAACGTCGGCTTATCTGTCAGTCGCGTCGGGTCTGCCGCTCAGTTGAAAGCTATGAAACAAGTCTGCGGGAGTCCAAAACTTGAATT
k.07.02   (651) ....................................................................................................................................
S.06.01   (651) ....................................................................................................................................
A.09.14   (651) ....................................................................................................................................
```

APPENDIX D: A. vesiculosa, D. regia and D. muscipula *alignments*

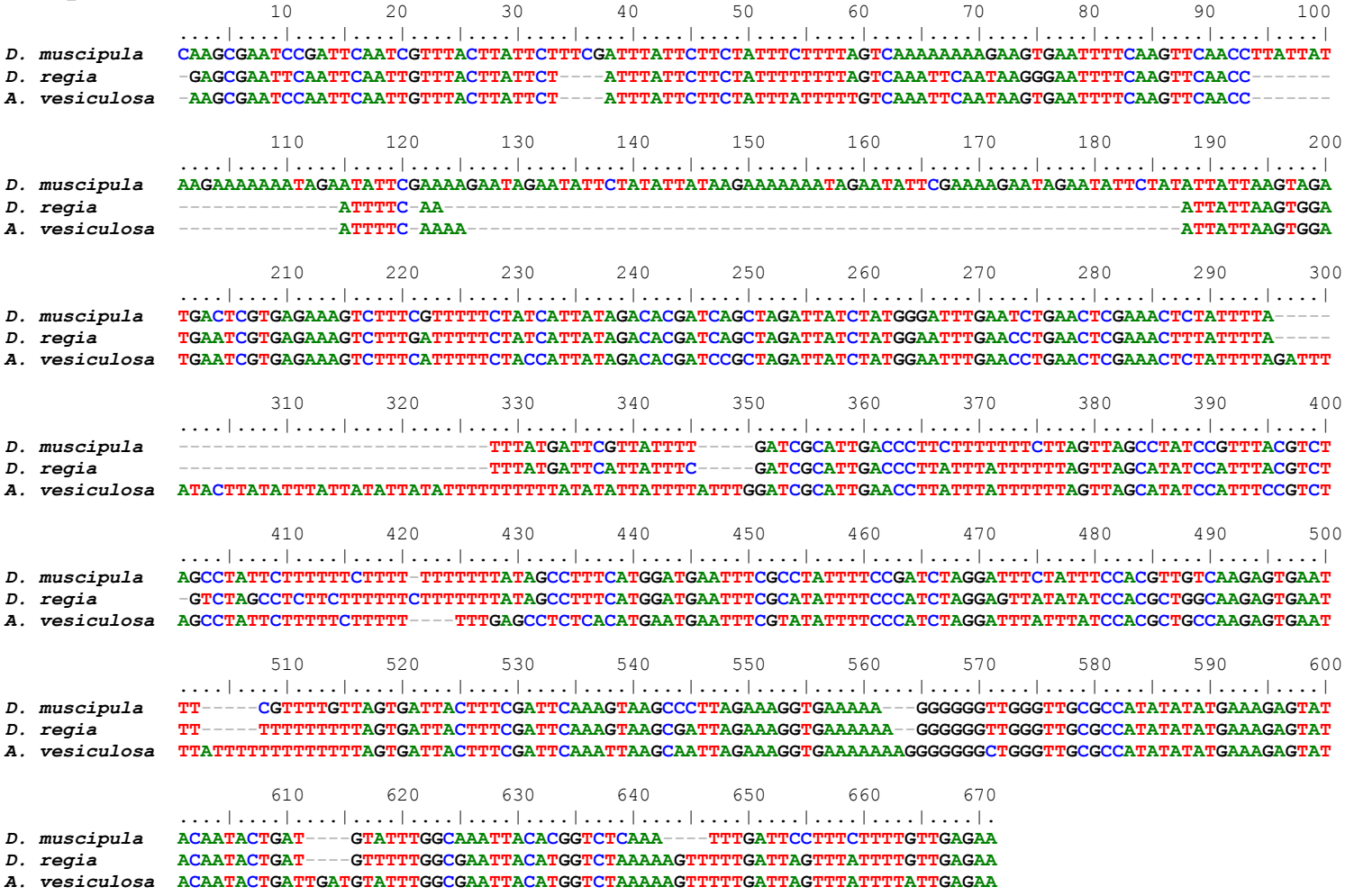
1. trnS – trnG



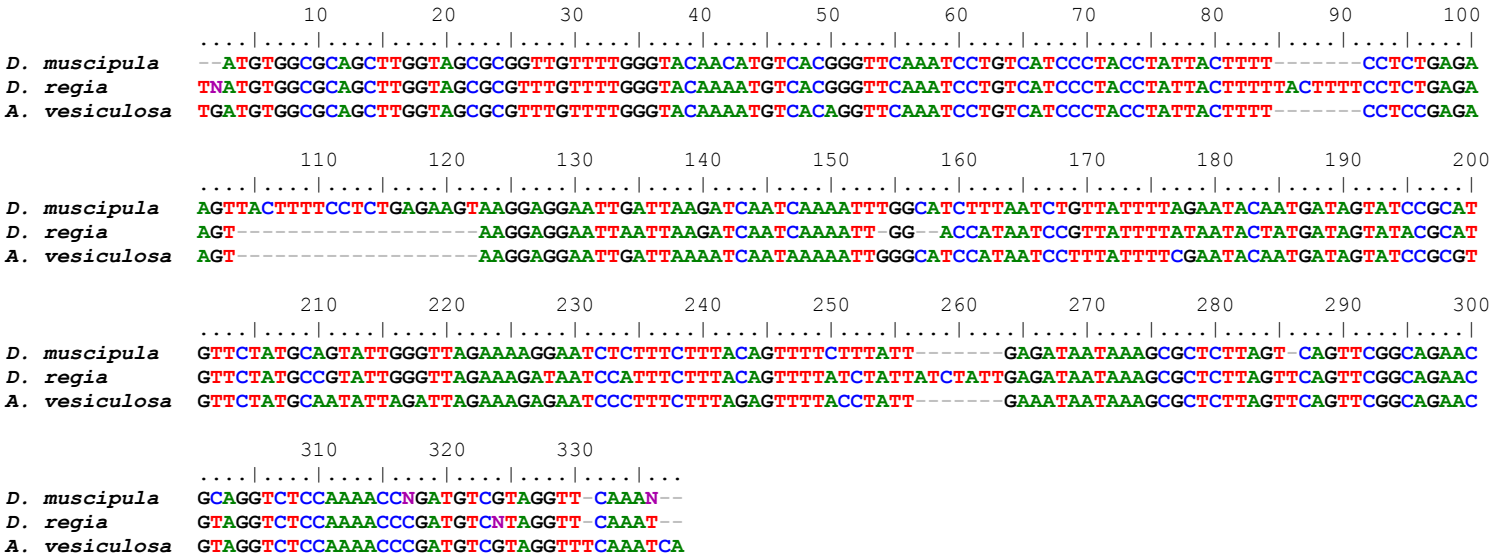
2. rpl20-rps12



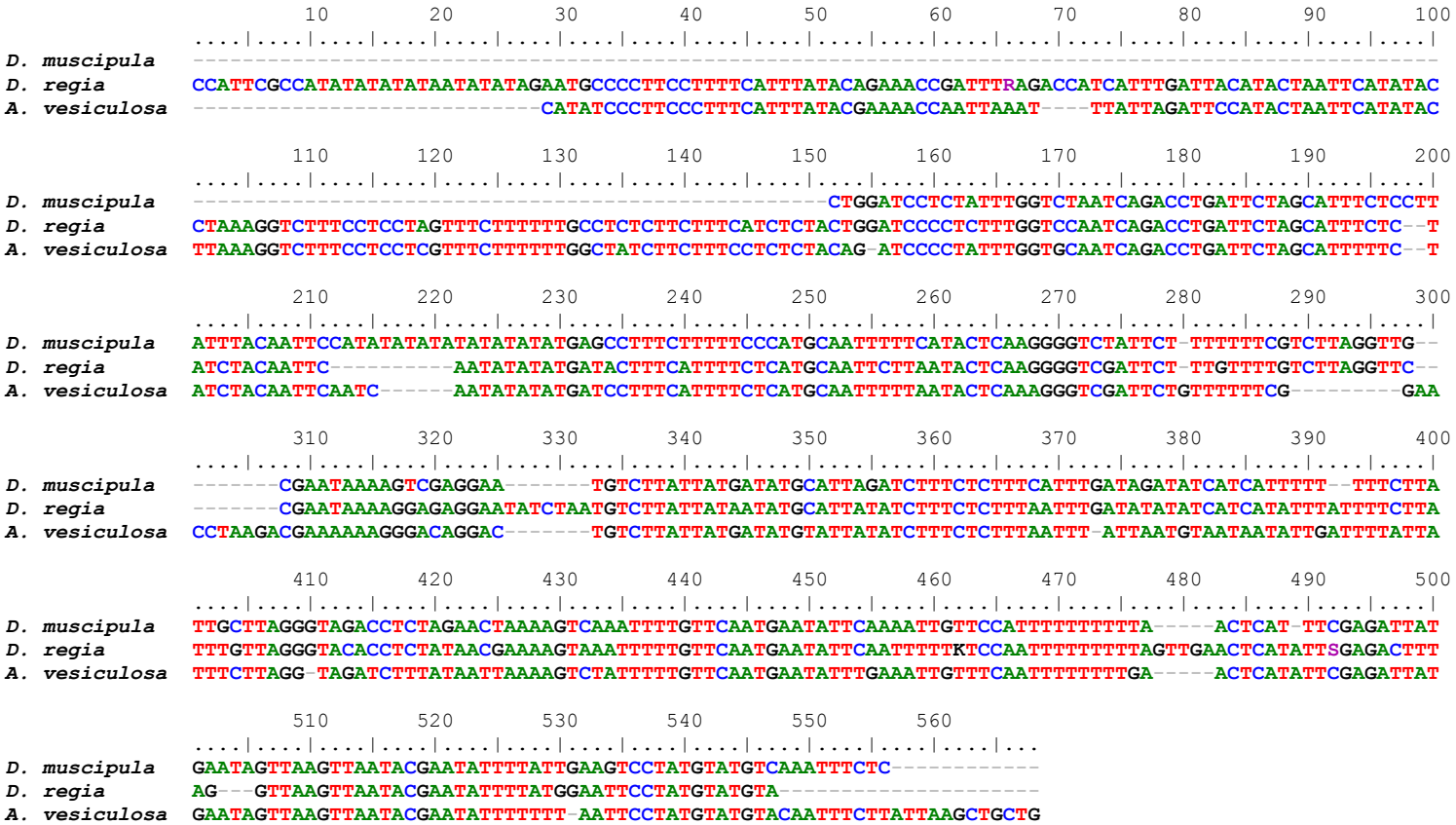
3. atpB-rbcL


10 20 30 40 50 60 70 80 90 100
D. muscipula CAAGCGAATCCGATTCAATCGTTTACTTATTCTTTCGATTTATTCTTCTATTTCTTTTAGTCAAAAAAAAGAAGTGAATTTTCAAGTTCAACCTTATTAT
D. regia -GAGCGAATTCAATTCAATTGTTTACTTATTCT----ATTTATTCTTCTATTTTTTTTTAGTCAAATTCAATAAGGGAATTTTCAAGTTCAACC-------
A. vesiculosa -AAGCGAATCCAATTCAATTGTTTACTTATTCT----ATTTATTCTTCTATTTATTTTTGTCAAATTCAATAAGTGAATTTTCAAGTTCAACC-------
110 120 130 140 150 160 170 180 190 200
D. muscipula AAGAAAAAAATAGAATATTCGAAAAGAATAGAATATTCTATATTATAAGAAAAAAATAGAATATTCGAAAAGAATAGAATATTCTATATTATTAAGTAGA
D. regia --------------ATTTTC-AA----------ATTATTAAGTGGA
A. vesiculosa --------------ATTTTC-AAAA----------ATTATTAAGTGGA
210 220 230 240 250 260 270 280 290 300
D. muscipula TGACTCGTGAGAAAGTCTTTCGTTTTTCTATCATTATAGACACGATCAGCTAGATTATCTATGGGATTTGAATCTGAACTCGAAACTCTATTTTA-----
D. regia TGAATCGTGAGAAAGTCTTTGATTTTTCTATCATTATAGACACGATCAGCTAGATTATCTATGGAATTTGAACCTGAACTCGAAACTTTATTTTA-----
A. vesiculosa TGAATCGTGAGAAAGTCTTTCATTTTTCTACCATTATAGACACGATCCGCTAGATTATCTATGGAATTTGAACCTGAACTCGAAACTCTATTTTAGATTT
310 320 330 340 350 360 370 380 390 400
D. muscipula ----------TTTATGATTCGTTATTTT-----GATCGCATTGACCCTTCTTTTTTTCTTAGTTAGCCTATCCGTTTACGTCT
D. regia ----------TTTATGATTCATTATTTC-----GATCGCATTGACCCTTATTTATTTTTTTAGTTAGCATATCCATTTACGTCT
A. vesiculosa ATACTTATATTTATTATATTATATTTTTTTTTTTATATATTATTTTATTTGGATCGCATTGAACCTTATTTATTTTTTAGTTAGCATATCCATTTCCGTCT
410 420 430 440 450 460 470 480 490 500
D. muscipula AGCCTATTCTTTTTTCTTTT-TTTTTTTATAGCCTTTCATGGATGAATTTCGCCTATTTTCCGATCTAGGATTTCTATTTCCACGTTGTCAAGAGTGAAT
D. regia -GTCTAGCCTCTTCTTTTTTTCTTTTTTTTATAGCCTTTCATGGATGAATTTCGCATATTTTCCCATCTAGGAGTTATATATCCACGCTGGCAAGAGTGAAT
A. vesiculosa AGCCTATTCTTTTTCTTTTT----TTTGAGCCTCTCACATGAATGAATTTCGTATATTTTCCCATCTAGGATTTATTTATCCACGCTGCCAAGAGTGAAT
510 520 530 540 550 560 570 580 590 600
D. muscipula TT-----CGTTTTGTTAGTGATTACTTTCGATTCAAAGTAAGCCCTTAGAAAGGTGAAAAA---GGGGGGTTGGGTTGCGCCATATATATGAAAGAGTAT
D. regia TT-----TTTTTTTTTAGTGATTACTTTCGATTCAAAGTAAGCGATTAGAAAGGTGAAAAAA--GGGGGGTTGGGTTGCGCCATATATATGAAAGAGTAT
A. vesiculosa TTATTTTTTTTTTTTTTAGTGATTACTTTCGATTCAAATTAAGCAATTAGAAAGGTGAAAAAAAGGGGGGGCTGGGTTGCGCCATATATATGAAAGAGTAT
610 620 630 640 650 660 670
D. muscipula ACAATACTGAT----GTATTTGGCAAATTACACGGTCTCAAA----TTTGATTCCTTTCTTTTGTTGAGAA
D. regia ACAATACTGAT----GTTTTTGGCGAATTACATGGTCTAAAAAGTTTTTGATTAGTTTATTTTGTTGAGAA
A. vesiculosa ACAATACTGATTGATGTATTTGGCGAATTACATGGTCTAAAAAGTTTTTGATTAGTTTATTTTATTGAGAA

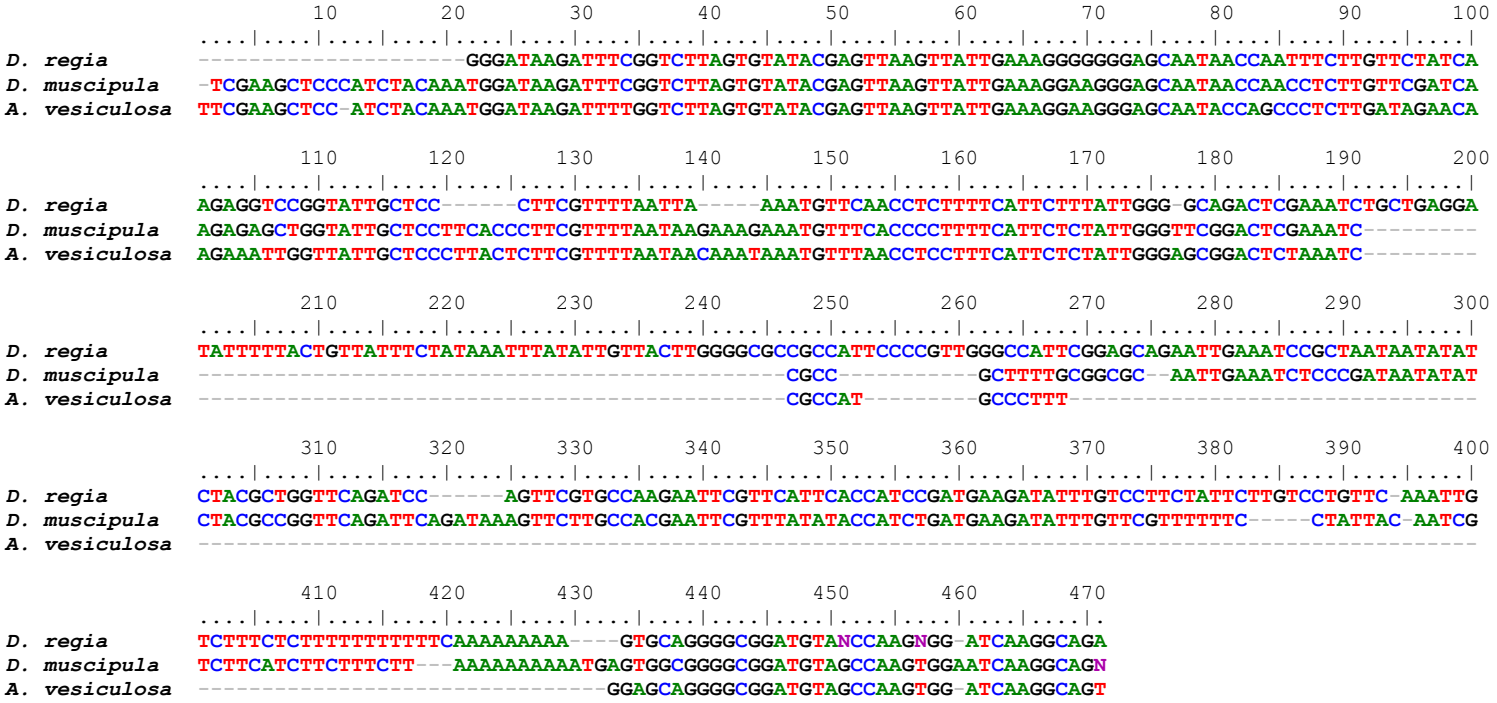
4. trnP-trnW


10 20 30 40 50 60 70 80 90 100
D. muscipula --ATGTGGCGCAGCTTGGTAGCGCGGTTGTTTTGGGTACAACATGTCACGGGTTCAAATCCTGTCATCCCTACCTATTACTTTT-------CCTCTGAGA
D. regia TNATGTGGCGCAGCTTGGTAGCGCGTTTGTTTTGGGTACAAAATGTCACGGGTTCAAATCCTGTCATCCCTACCTATTACTTTTTACTTTTCCTCTGAGA
A. vesiculosa TGATGTGGCGCAGCTTGGTAGCGCGTTTGTTTTGGGTACAAAATGTCACAGGTTCAAATCCTGTCATCCCTACCTATTACTTTT-------CCTCCGAGA
110 120 130 140 150 160 170 180 190 200
D. muscipula AGTTACTTTTCCTCTGAGAAGTAAGGAGGAATTGATTAAGATCAATCAAAATTTGGCATCTTTAATCTGTTATTTTAGAATACAATGATAGTATCCGCAT
D. regia AGT------------------AAGGAGGAATTAATTAAGATCAATCAAAATT-GG--ACCATAATCCGTTATTTTATAATACTATGATAGTATACGCAT
A. vesiculosa AGT------------------AAGGAGGAATTGATTAAAATCAATAAAAATTGGGCATCCATAATCCTTTATTTTCGAATACAATGATAGTATCCGCGT
210 220 230 240 250 260 270 280 290 300
D. muscipula GTTCTATGCAGTATTGGGTTAGAAAAGGAATCTCTTTCTTTACAGTTTTCTTTATT-------GAGATAATAAAGCGCTCTTAGT-CAGTTCGGCAGAAC
D. regia GTTCTATGCCGTATTGGGTTAGAAAGATAATCCATTTCTTTACAGTTTTATCTATTATCTATTGAGATAATAAAGCGCTCTTAGTTCAGTTCGGCAGAAC
A. vesiculosa GTTCTATGCAATATTAGATTAGAAAGAGAATCCCTTTCTTTAGAGTTTTACCTATT-------GAAATAATAAAGCGCTCTTAGTTCAGTTCGGCAGAAC
310 320 330
D. muscipula GCAGGTCTCCAAAACCNGATGTCGTAGGTT-CAAAN--
D. regia GTAGGTCTCCAAAACCCGATGTCNTAGGTT-CAAAT--
A. vesiculosa GTAGGTCTCCAAAACCCGATGTCGTAGGTTTCAAATCA

5. cpa-apb


10 20 30 40 50 60 70 80 90 100
D. muscipula
D. regia
A. vesiculosa
110 120 130 140 150 160 170 180 190 200
D. muscipula
D. regia
A. vesiculosa
210 220 230 240 250 260 270 280 290 300
D. muscipula
D. regia
A. vesiculosa
310 320 330 340 350 360 370 380 390 400
D. muscipula
D. regia
A. vesiculosa
410 420 430 440 450 460 470 480 490 500
D. muscipula
D. regia
A. vesiculosa
510 520 530 540 550 560
D. muscipula
D. regia
A. vesiculosa

6. psbA-trnH


10 20 30 40 50 60 70 80 90 100
D. regia ----------GGGATAAGATTTCGGTCTTAGTGTATACGAGTTAAGTTATTGAAAGGGGGGGAGCAATAACCAATTTCTTGTTCTATCA
D. muscipula -TCGAAGCTCCCATCTACAAATGGATAAGATTTCGGTCTTAGTGTATACGAGTTAAGTTATTGAAAGGAAGGGAGCAATAACCAACCTCTTGTTCGATCA
A. vesiculosa TTCGAAGCTCC-ATCTACAAATGGATAAGATTTTGGTCTTAGTGTATACGAGTTAAGTTATTGAAAGGAAGGGAGCAATACCAGCCCTCTTGATAGAACA
110 120 130 140 150 160 170 180 190 200
D. regia AGAGGTCCGGTATTGCTCC------CTTCGTTTTAATTA-----AAATGTTCAACCTCTTTTCATTCTTTATTGGG-GCAGACTCGAAATCTGCTGAGGA
D. muscipula AGAGAGCTGGTATTGCTCCTTCACCCTTCGTTTTAATAAGAAAGAAATGTTTCACCCCTTTTCATTCTCTATTGGGTTCGGACTCGAAATC---------
A. vesiculosa AGAAATTGGTTATTGCTCCCTTACTCTTCGTTTTAATAACAAATAAATGTTTAACCTCCTTTCATTCTCTATTGGGAGCGGACTCTAAATC---------
210 220 230 240 250 260 270 280 290 300
D. regia TATTTTTACTGTTATTTCTATAAATTTATATTGTTACTTGGGGCGCCGCCATTCCCCGTTGGGCCATTCGGAGCAGAATTGAAATCCGCTAATAATATAT
D. muscipula ----------CGCC----------GCTTTTGCGGCGC--AATTGAAATCTCCCGATAATATAT
A. vesiculosa ----------CGCCAT---------GCCCTTT----------
310 320 330 340 350 360 370 380 390 400
D. regia CTACGCTGGTTCAGATCC------AGTTCGTGCCAAGAATTCGTTCATTCACCATCCGATGAAGATATTTGTCCTTCTATTCTTGTCCTGTTC-AAATTG
D. muscipula CTACGCCGGTTCAGATTCAGATAAAGTTCTTGCCACGAATTCGTTTATATACCATCTGATGAAGATATTTGTTCGTTTTTTC-----CTATTAC-AATCG
A. vesiculosa ----------
410 420 430 440 450 460 470
D. regia TCTTTCTCTTTTTTTTTTTCAAAAAAAAA----GTGCAGGGGCGGATGTANCCAAGNGG-ATCAAGGCAGA
D. muscipula TCTTCATCTTCTTTCTT---AAAAAAAAAATGAGTGGCGGGGCGGATGTAGCCAAGTGGAATCAAGGCAGN
A. vesiculosa ----------GGAGCAGGGGCGGATGTAGCCAAGTGG-ATCAAGGCAGT

Appendix E: Southern Hybridization Buffers

The preparation of the antibody is as following:

Spin the Dig antibody concentrated stock from Roche Company for 5 min on 10000 rpm. Then take 5 uL (for big membrane) and dilute them in 160 uL of 1xblocking buffer and mix.

The preparation of the blocking buffer and the detection is acourding for Roche Company

To prepare 5 x Malic acid buffer:

23.2 g Malic acid +17.6 g NaCl and adjust the pH to 7.5 by NaOH (solid), notice that you should add the NaOH slowly not all the amount because it heat producing reaction.

To prepare 10x blocking buffer

10 gm powder blocking +90ml Malic buffer 1x

To prepare 1x blocking buffer

10 ml 10xblocking buffer +90ml Malic buffer 1x

To prepare detection buffer:

0.1 M Tris, 0.1M NaCl in water and adjust the pH to 9.5

To prepare 20X SSC:

3M NaCl 175.3g +

0.3M Na3Citrat.2H2O 88.2 g /Liter

To prepare Denaturation buffer:

1.5 M NaCl 87.5g

0.5M NaOH 20.0 g /Liter

To prepare Neutralization buffer:

1.5 M NaCl 87.5g

0.5M Tris 60g and adjust the pH to 7.5 with HCl

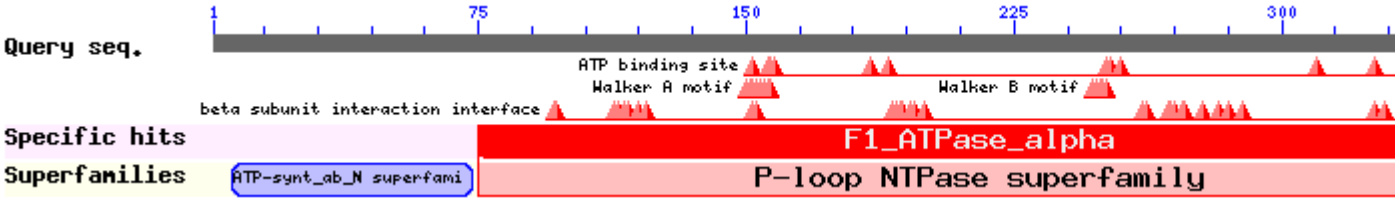
Appendix F: Blast query for Atp1 protein of A. vesiculosa

NCBI/ BLAST/ blastp suite/ **Formatting Results - RGWUGKJH015**

Graphic Summary

Show Conserved Domains

Putative conserved domains have been detected, click on the image below for detailed results.



Descriptions

Sequences producing significant alignments:	Score (Bits)	E Value	
gb|AAB87529.1| F1 ATPase a-subunit [Panax ginseng]	851	0.0	
sp|P18260.1|ATPAM_HELAN RecName: Full=ATP synthase subunit al...	850	0.0	
gb|AAK98045.1|AF301602_1 ATP1 [Daucus carota] >gb|AAK98046.1|...	850	0.0	
gb|ABD61045.1| ATPase alpha subunit [Nepenthes sp. 'Kosobe']	847	0.0	
sp|Q06735.1|ATPAM_BETVU RecName: Full=ATP synthase subunit al...	846	0.0	
ref|YP_173459.1| ATP synthase F1 subunit 1 [Nicotiana tabacum...	846	0.0	G
sp|P24459.1|ATPAM_PHAVU RecName: Full=ATP synthase subunit al...	845	0.0	
sp|Q01915.1|ATPAM_SOYBN RecName: Full=ATP synthase subunit al...	845	0.0	
ref|YP_514682.1| ATP synthase F0 subunit 1 [Oryza sativa Indi...	845	0.0	G
ref|YP_762326.1| ATPase subunit 1 [Sorghum bicolor] >gb|ABI60...	845	0.0	G
gb|ABD61047.1| ATPase alpha subunit [Simmondsia chinensis]	845	0.0	
gb|ABD61032.1| ATPase alpha subunit [Staphylea trifolia]	845	0.0	
dbj|BAE32582.1| unnamed protein product [Mus musculus]	845	0.0	
gb|AAB03873.1| F1-ATPase alpha subunit [Petunia axillaris sub...	844	0.0	
gb|ABD61028.1| ATPase alpha subunit [Heuchera sp. Qiu 95076]	844	0.0	
gb|ABD61046.1| ATPase alpha subunit [Polygonum sp. Qiu 94110]	844	0.0	
sp|P05493.2|ATPAM_PEA RecName: Full=ATP synthase subunit alph...	844	0.0	
gb|AAD41619.1|AF071550_4 ATP synthase alpha chain [Vigna radi...	843	0.0	
ref|NP_064105.2| ATPase subunit 1 [Beta vulgaris subsp. vulga...	842	0.0	G
gb|ABY55213.1| Atp1 [Bambusa oldhamii]	842	0.0	
ref|YP_398393.1| atp1 [Triticum aestivum] >emb|CAA34060.1| un...	842	0.0	G
gb|ABY83982.1| atp1 [Secale strictum]	841	0.0	
gb|ABD61048.1| ATPase alpha subunit [Hedera helix]	841	0.0	
prf||1305286A ATPase alpha,F1	840	0.0	
emb|CAA48649.1| ATPase subunit [Beta vulgaris subsp. vulgaris...	840	0.0	
ref|YP_588408.1| ATPase subunit 1 [Zea mays subsp. mays] >ref...	840	0.0	G
emb|CAQ77653.1| ATPase subunit 1 [Vitis vinifera]	840	0.0	
gb|AAA75456.1| F1 ATPase alpha subunit	840	0.0	
gb|ABO31963.1| ATPase alpha subunit [Daphniphyllum sp. Qiu 94...	840	0.0	
ref|YP_002000594.1| ATP synthase F0 subunit 1 [Oryza sativa J...	839	0.0	G
ref|YP_762487.1| ATPase subunit 1 [Tripsacum dactyloides] >gb...	839	0.0	G
gb|ABD61026.1| ATPase alpha subunit [Phytolacca americana]	839	0.0	
gb|ABD61027.1| ATPase alpha subunit [Hamamelis mollis]	838	0.0	
gb|ABF60013.1| ATPase alpha subunit [Phoenix dactylifera]	838	0.0	
gb|ABO31960.1| ATPase alpha subunit [Cercidiphyllum japonicum]	838	0.0	
sp|P05492.1|ATPAM_OENBI RecName: Full=ATP synthase subunit al...	838	0.0	

sp|P12862.1|ATPAM_WHEAT	RecName: Full=ATP synthase subunit al...	838	0.0
ref|YP_762500.1|	ATPase subunit 1 [Tripsacum dactyloides] >gb...	837	0.0
gb|ABD61051.1|	ATPase alpha subunit [Coffea arabica]	836	0.0
dbj|BAD38497.1|	ATP synthase F0 subunit 1 [Oryza sativa Japon...	836	0.0
emb|CAB99314.1|	F0-F1 ATPase alpha subunit [Sorghum bicolor] ...	835	0.0
gb|ABD61049.1|	ATPase alpha subunit [Eucommia ulmoides]	835	0.0
gb|ABD61043.1|	ATPase alpha subunit [Vitis sp. Qiu 94046]	833	0.0
gb|ABD61041.1|	ATPase alpha subunit [Berberidopsis beckleri]	833	0.0
gb|ABD61038.1|	ATPase alpha subunit [Iris sp. Qiu 95091]	832	0.0
gb|AAT69067.1|	F1-ATPase alpha subunit [Nicotiana tabacum]	831	0.0
gb|AAW33096.1|	F1-ATPase alpha subunit [Bougainvillea glabra]	830	0.0
gb|AAF16992.1|AF197660_1	ATPase alpha subunit [Podophyllum pe...	829	0.0
gb|ABD61040.1|	ATPase alpha subunit [Gunnera monoica]	829	0.0
gb|AAW33094.1|	F1-ATPase alpha subunit [Aesculus californica]	829	0.0
gb|ABD61025.1|	ATPase alpha subunit [Spinacia oleracea]	829	0.0
gb|AAV66476.1|	F1-ATPase alpha subunit [Gentiana procera]	829	0.0
gb|AAV66472.1|	F1-ATPase alpha subunit [Sambucus sieboldiana]	828	0.0
gb|AAT69068.1|	F1-ATPase alpha subunit [Schizanthus pinnatus]	828	0.0
gb|ABD61033.1|	ATPase alpha subunit [Chamaedorea tenella]	828	0.0
gb|AAT69069.1|	F1-ATPase alpha subunit [Montinia caryophyllacea]	827	0.0
gb|AAV66473.1|	F1-ATPase alpha subunit [Dipsacus fullonum]	827	0.0
gb|ABD61050.1|	ATPase alpha subunit [Lamium sp. Qiu 95019]	827	0.0
gb|AAV66471.1|	F1-ATPase alpha subunit [Ilex verticillata]	827	0.0
gb|ABO31968.1|	ATPase alpha subunit [Liquidambar styraciflua]	826	0.0
gb|AAV66474.1|	F1-ATPase alpha subunit [Campanula garganica]	826	0.0
gb|AAV66480.1|	F1-ATPase alpha subunit [Syringa vulgaris]	826	0.0
gb|ABO31961.1|	ATPase alpha subunit [Corylopsis glabrescens f...	826	0.0
gb|ABO86590.1|	ATPase alpha subunit [Brassica juncea]	825	0.0
gb|AAW33099.1|	F1-ATPase alpha subunit [Hibiscus rosa-sinensis]	825	0.0
gb|AAW33106.1|	F1-ATPase alpha subunit [Rubus sp. JPM-2004]	825	0.0
sp|P68541.1|ATPAM_RAPSA	RecName: Full=ATP synthase subunit al...	825	0.0
gb|ABD61029.1|	ATPase alpha subunit [Guaiacum officinale]	825	0.0
gb|AAT69043.1|	F1-ATPase alpha subunit [Odonellia hirtiflora]	825	0.0
gb|AAT69035.1|	F1-ATPase alpha subunit [Ipomoea batatas] >gb|...	825	0.0
sp|P22201.1|ATPAM_BRANA	RecName: Full=ATP synthase subunit al...	825	0.0
gb|AAT69064.1|	F1-ATPase alpha subunit [Cuscuta europaea]	824	0.0
gb|AAV66479.1|	F1-ATPase alpha subunit [Cuscuta sandwichiana]	824	0.0
gb|AAF16982.1|AF197650_1	ATPase alpha subunit [Euptelea polya...	824	0.0
gb|AAF16981.1|AF197649_1	ATPase alpha subunit [Dicentra sp. Q...	824	0.0
gb|AAX46318.1|	F1-ATPase alpha subunit [Cuscuta campestris]	824	0.0
gb|ABO86589.1|	ATPase alpha subunit [Brassica juncea]	823	0.0
ref|NP_178788.1|	ATP synthase alpha chain, mitochondrial, put...	823	0.0
gb|ABO31974.1|	ATPase alpha subunit [Ribes sp. Qiu 95022]	823	0.0
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gb|AAT69037.1|	F1-ATPase alpha subunit [Lepistemon owariensis]	822	0.0
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gb|AAF16998.1|AF197666_1	ATPase alpha subunit [Sarcandra chlo...	821	0.0
gb|AAB02743.1|	F1-ATPase alpha subunit [Phoenix reclinata]	821	0.0
ref|NP_085571.2|	ATPase subunit 1 [Arabidopsis thaliana] >emb...	821	0.0
gb|AAF17036.1|AF197704_1	ATPase alpha subunit [Tofieldia caly...	821	0.0
gb|AAV66477.1|	F1-ATPase alpha subunit [Strychnos spinosa]	820	0.0
gb|AAT69042.1|	F1-ATPase alpha subunit [Iseia luxurians]	820	0.0
gb|AAT69050.1|	F1-ATPase alpha subunit [Falkia repens]	820	0.0
gb|AAB02741.1|	F1-ATPase alpha subunit [Calamus usitatus]	820	0.0
gb|AAT69058.1|	F1-ATPase alpha subunit [Maripa repens]	820	0.0

gb|AAF16978.1|AF197646_1	ATPase alpha subunit [Cocculus trilo...	820	0.0
gb|AAF16984.1|AF197652_1	ATPase alpha subunit [Persoonia kate...	820	0.0
gb|AAT69052.1|	F1-ATPase alpha subunit [Bonamia media]	820	0.0
gb|AAT69036.1|	F1-ATPase alpha subunit [Astripomoea malvacea]	820	0.0
gb|AAM95202.1|	ATPase F1 alpha subunit [Schoenocephalium cucu...	820	0.0
gb|AAQ74508.1|	F1-ATPase alpha subunit [Cercidiphyllum japoni...	820	0.0

>gb|AAB87529.1| F1 ATPase a-subunit [Panax ginseng]
Length=507

Score = 851 bits (2198), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 423/432 (97%), Positives = 426/432 (98%), Gaps = 0/432 (0%)

```
Query  1    YTNFQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASGVKGIALNLENENVGIVVFGSD  60
            YT+FQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASGVKGIALNLENENVGIVVFGSD
Sbjct  22   YTHFQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASGVKGIALNLENENVGIVVFGSD  81

Query  61   TAIKEGDLVKRNTGSIVDVPAGKAMLGRVVDALGVPIDGRGATGWSERRRVEVKAPGIIER  120
            TAIKEGDLVKRNTGSIVDVPAGKAMLGRVVDALGVPIDGRGA    ERRRVEVKAPGIIER
Sbjct  82   TAIKEGDLVKRNTGSIVDVPAGKAMLGRVVDALGVPIDGRGALSDHERRRVEVKAPGIIER  141

Query  121  KSVHEPMQTGLKAVDSLVPIGRGQRELIIGDRQTGKTAIAIDTILNQKEMNSRATSESET  180
            KSVHEPMQTGLKAVDSLVPIGRGQRELIIGDRQTGKTAIAIDTILNQK+MNSR+TSESET
Sbjct  142  KSVHEPMQTGLKAVDSLVPIGRGQRELIIGDRQTGKTAIAIDTILNQKQMNSRSTSESET  201

Query  181  LYCVYVAIGQKRNSTVAQLVQILSEANALEYSILVAATASDPAPLQFLAPYSGCAMGEYFR  240
            LYCVYVAIGQKRNSTVAQLVQILSEANALEYSILVAATASDPAPLQFLAPYSGCAMGEYFR
Sbjct  202  LYCVYVAIGQKRNSTVAQLVQILSEANALEYSILVAATASDPAPLQFLAPYSGCAMGEYFR  261

Query  241  DNGMHALIIYDDLSKQAVAYRQMSLLLRRPPGREAFPGDVFYLHSRLLERAAKRNSDQTGA  300
            DNGMHALIIYDDLSKQAVAYRQMSLLLRRPPGREAFPGDVFYLHSRLLERAAKRNSDQTGA
Sbjct  262  DNGMHALIIYDDLSKQAVAYRQMSLLLRRPPGREAFPGDVFYLHSRLLERAAKRNSDQTGA  321

Query  301  GSLTALPVIETQAGDVSAYIPTNVISITDGQICSETELFYRGIRPAINVGLSVSRVGSAA  360
            GSLTALPVIETQAGDVSAYIPTNVI ITDGQICSETELFYRGIRPAINVGLSVSRVGSAA
Sbjct  322  GSLTALPVIETQAGDVSAYIPTNVIPITDGQICSETELFYRGIRPAINVGLSVSRVGSAA  381

Query  361  QLKAMKQVRGSSKLELAQYREVAALAQFGSDLDAATQALLNRGARLTEVPKQPQYAPLPI  420
            QLKAMKQV GSSKLELAQYREVAALAQFGSDLDAATQALLNRGARLTEVPKQPQYAPLPI
Sbjct  382  QLKAMKQVCGSSKLELAQYREVAALAQFGSDLDAATQALLNRGARLTEVPKQPQYAPLPI  441

Query  421  EKQILVIYAAVN  432
            EKQILVIYAAVN
Sbjct  442  EKQILVIYAAVN  453
```

>sp|P18260.1|ATPAM_HELAN RecName: Full=ATP synthase subunit alpha, mitochondrial
emb|CAA39428.1| F1 ATPase; adenosinetriphosphatase [Helianthus annuus]
emb|CAA37022.1| unnamed protein product [Helianthus annuus]
emb|CAA37613.1| F1 ATPase; adenosinetriphosphatase [Helianthus annuus]
Length=510

Score = 850 bits (2197), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 422/432 (97%), Positives = 425/432 (98%), Gaps = 0/432 (0%)

```
Query  1    YTNFQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASGVKGIALNLENENVGIVVFGSD  60
            YTNFQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASGVKGIALNLENENVGIVVFGSD
Sbjct  22   YTNFQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASGVKGIALNLENENVGIVVFGSD  81

Query  61   TAIKEGDLVKRNTGSIVDVPAGKAMLGRVVDALGVPIDGRGATGWSERRRVEVKAPGIIER  120
            TAIKEGDLVKRNTGSIVDVPAGKAMLGRVVDALGVPIDGRGA    ERRRVEVKAPGIIER
Sbjct  82   TAIKEGDLVKRNTGSIVDVPAGKAMLGRVVDALGVPIDGRGALSDHERRRVEVKAPGIIER  141

Query  121  KSVHEPMQTGLKAVDSLVPIGRGQRELIIGDRQTGKTAIAIDTILNQKEMNSRATSESET  180
            KSVHEPMQTGLKAVDSLVPIGRGQRELIIGDRQTGKTAIAIDTILNQK+MNSR+TSESET
Sbjct  142  KSVHEPMQTGLKAVDSLVPIGRGQRELIIGDRQTGKTAIAIDTILNQKQMNSRSTSESET  201

Query  181  LYCVYVAIGQKRNSTVAQLVQILSEANALEYSILVAATASDPAPLQFLAPYSGCAMGEYFR  240
            LYCVYVAIGQKRNSTVAQLVQILSEANA+EYSILVAATASDPAPLQFLAPYSGCAMGEYFR
Sbjct  202  LYCVYVAIGQKRNSTVAQLVQILSEANAMEYSILVAATASDPAPLQFLAPYSGCAMGEYFR  261

Query  241  DNGMHALIIYDDLSKQAVAYRQMSLLLRRPPGREAFPGDVFYLHSRLLERAAKRNSDQTGA  300
            DNGMHALIIYDDLSKQAVAYRQMSLLLRRPPGREAFPGDVFYLHSRLLERAAKRNSDQTGA
Sbjct  262  DNGMHALIIYDDLSKQAVAYRQMSLLLRRPPGREAFPGDVFYLHSRLLERAAKRNSDQTGA  321

Query  301  GSLTALPVIETQAGDVSAYIPTNVISITDGQICSETELFYRGIRPAINVGLSVSRVGSAA  360
            GSLTALPVIETQAGDVSAYIPTNVI ITDGQICSETELFYRGIRPAINVGLSVSRVGSAA
Sbjct  322  GSLTALPVIETQAGDVSAYIPTNVIPITDGQICSETELFYRGIRPAINVGLSVSRVGSAA  381

Query  361  QLKAMKQVRGSSKLELAQYREVAALAQFGSDLDAATQALLNRGARLTEVPKQPQYAPLPI  420
            QLK MKQV GSSKLELAQYREVAALAQFGSDLDAATQALLNRGARLTEVPKQPQYAPLPI
Sbjct  382  QLKTMKQVCGSSKLELAQYREVAALAQFGSDLDAATQALLNRGARLTEVPKQPQYAPLPI  441

Query  421  EKQILVIYAAVN  432
            EKQILVIYAAVN
Sbjct  442  EKQILVIYAAVN  453
```