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Development of molecular markers for breeding hybrid varieties and their genetic traceability in fennel (Foeniculum vulgare L.)

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Riassunto

Il finocchio (*Foeniculum vulgare* Mill.*)* è una pianta annuale appartenente alla famiglia delle Apiaceae e originaria del bacino del Mediterraneo, sebbene sia ormai diffusa in diverse parti del mondo. Quantunque ben nota ed apprezzata sia nel settore culinario che come pianta erbacea aromatica e medicinale, in alcune regioni è considerata addirittura infestante. La coltivazione del finocchio è ben affermata in tutto il bacino del Mediterraneo (anche in Italia) ma comincia a diffondersi in modo massivo anche in altri paesi come, ad esempio, USA, India e Cina. Tuttavia, nonostante la sua rilevanza agronomica e farmaceutica, i dati biologici disponibili per questa specie - in primis sequenze nucleotidiche e marcatori molecolari - sono estremamente carenti. La metodologia AFLP (*Amplified Fragment Length Polymorphism*) è utilizzata da circa 20 anni per l'analisi genetica in molte specie e, basandosi sull'individuazione di polimorfismi del DNA, consente la produzione di profili elettroforetici informativi dei genomi mediante *fingerprinting*. La continua evoluzione del protocollo (originariamente sviluppato da Vos *et al.,* nel 1995) si esplica attualmente nell'impiego di primer marcati con sonde fluorescenti in combinazione con sequenziatori automatici capillari. L'uso di elettroforesi capillare e di procedure automatizzate di analisi può, da un lato, aumentare la quantità e l'affidabilità dei loci saggiabili e quindi dei dati ottenibili, e, dall'altro, diminuire l'errore sperimentale. In questo lavoro viene presentato un metodo innovativo e robusto di analisi basato sulla tecnica AFLP e denominato per questo "M13-tailed AFLP". La validazione del metodo è quindi condotta valutando la stabilità e la diversità genetica esistente, rispettivamente, entro e tra popolazioni sperimentali di finocchio. Una differenza chiave che contraddistingue questa nuova procedura dai protocolli tradizionali, risiede nell'utilizzo di primer M13 fluoresceinati in combinazione con primer AFLP ancorati in 5' ad una sequenza complementare al primer M13. Questo consente di evitare la marcatura diretta di ogni primer AFLP,

con un notevole risparmio in termini di costi. La metodica consente, in media, l'amplificazione e la visualizzazione di circa 190 frammenti per ogni combinazione di primer (EcoRI+3/MseI+3). La riproducibilità dei *fingerprint* di campioni di DNA genomico è stata valutata tramite repliche, risultando superiore al 95%. La validazione del metodo è stata condotta utilizzando 240 piante appartenenti a diverse linee *inbred* di finocchio (maschio-sterili, mantenitori e maschio-fertili). Nel presente lavoro è inoltre descritta la scoperta e il clonaggio di regioni microsatelliti (o SSR, *Simple Sequence Repeats*) nonché lo sviluppo del primo set di marcatori molecolari utili per il *genotyping* in finocchio. Le sequenze nucleotidiche delle regioni microsatellite individuate e dei relativi primer *forward* e *reverse* sono state recentemente depositate in GenBank, submission ID 1735181. In conclusione questo progetto descrive lo sviluppo e la validazione di marcatori molecolari utili per il *genotyping* e il *fingerprinting* di linee *inbred* di finocchio. Le informazioni acquisite e i protocolli sviluppati avranno una grande utilità applicativa in programmi di miglioramento genetico assistito da marcatori finalizzati alla caratterizzazione e alla selezione di linee *inbred* parentali idonee alla costituzione di nuovi ibridi F1 commerciali.

Abstract

Fennel (*Foeniculum vulgare* Mill.) is an annual herb belonging to the family Apiaceae, native to the shores of the Mediterranean basin that has become widely naturalized in many parts of the world. It is well known as an aromatic, culinary and medicinal herb, but in some regions it is also considered as an invasive plant. Bulb fennel, which is a relevant crop in the Mediterranean basin, and particularly in Italy, is as emerging crop in other countries, such as the USA, India and China. Despite its agronomic and pharmaceutical interests, researchers face the almost complete lack of biological data for this species, being nucleotide sequences and molecular markers available very scanty. Amplified Fragment Length Polymorphism (AFLP) is a well-established molecular marker technique suitable for DNA fingerprinting, which has been successfully applied in many species during the past 20 years. The original method conceived by Vos *et al*., in 1995 has evolved constantly and it is currently based on the use of fluorescence labeled primers in combination with capillary automatic sequencers. Compared to the original procedure, it is believed that the use of capillary electrophoresis and automated procedures of analysis can, on one hand, increase data throughput and scoring reliability, and, on the other, decrease the overall experimental error. In this research we describe a new and robust AFLP-based technology, named M13-tailed AFLP, and its exploitation to assess the genetic stability and diversity existing within and between fennel inbred lines, respectively. A key difference of our procedure with respect to the most traditional protocols resides on the use of M13-labeled primers in combination with M13-anchored AFLP primers, which enables to avoid the direct labeling of AFLP primers, thus reducing the costs of the procedure. In our estimates, this method allowed the amplification and visualization of an average of 190 amplicons per primer combination (EcoRI+3/MseI+3). The reproducibility of DNA fingerprints was computed on a number replicated experiments and it appears to be higher than 95%. Validation of

the method was conducted using 240 plants that belong to inbred lines (*i.e*. male sterile mutants, maintainers and pollen donors) of fennel. Here we also describe the discovery and cloning of microsatellite regions (also known as SSR, Simple Sequence Repeats) and the development of the first set of SSR markers useful for population genetics in fennel. In particular, the SSR markers were developed and exploited in the same fennel breeding stocks. The nucleotide sequences of microsatellite regions and their specific forward and reverse primers have been recently deposited in the GenBank database, submission ID 1735181. In conclusion, this research describes the development and assessment of molecular markers suitable for fingerprinting and genotyping experimental accessions of fennel that will have great applied utility for marker-assisted breeding programs aimed at the characterization and selection of parental inbred lines and the constitution of new commercial F1 hybrids in this species.

1. INTRODUCTION

Fennel (*Foeniculum vulgare* Mill.) is a diploid species (2*n*=2*x*=22) belonging to the family Apiaceae (or Umbreilliferae, *nomen conservandum*). Although nowadays fennel is widely spread all over the world, this plant species is native to the Mediterranean basin. It is a biennal (or perennial) and glaucous green herb with hollow and erect stems able to grow to a height ranging from 70 to 200 cm. The hairless leaves are finely dissected and grow up to 40 cm long, with the ultimate segments filiform (threadlike), about 0.5 mm wide.

Fennel, as a member of the Apiaceae, produces flowers in compound umbels, with each umbel having several whorls of umbellules (Peterson, 1990). The flowering stem supports tens of showy compound umbels of flowers: each umbel section has 20-50 tiny yellow flowers on short pedicels. It is normally self-fertile and can be 100% self-pollinated if out-crossing is prevented but under normal circumstances, it is highly (80% to 90%) cross-pollinated (Ramanujam *et al*., 1964 cited in Jansen, 1981; Pillai and Nambiar, 1982). Fennel is proterandrous. That is, within each flower, pollen is shed before the stigma becomes receptive (Sundararaj *et al*., 1963). Even if there are differences between varieties, generally the five stamens of each flower emerge sequentially over a 6 to 8 h period (Sundararaj *et al*., 1963). Self-fertility and proterandry are characters that may influence the agronomic practices of this crop plant species, by affecting also breeding strategies.

The fruit is represented by a dry and grooved seed from 4 to 10 mm long. Interestingly, not only the bulb and stalk, but also leaves and seeds are all edible. *Foeniculum vulgare* is classified into two sub-species *vulgare* and *piperitum*, and the most important cultivated fennel cultivars belong to the former. For example *Foeniculum vulgare* subsp. *vulgare* var. *azoricum* (Florence fennel or bulb fennel) and *Foeniculum vulgare* subsp. *vulgare* var. *dulce* (Roman fennel or sweet fennel) are widely cultivated because of their inflated leaf bases, which form an edible bulb-

like structure (for details, see The herb society of America, http://www.herbsociety.org/factsheets/fennel.pdf). On the contrary *Foeniculum vulgare* subsp. *piperitum* (wild pepper fennel) is generally used as a flavoring, because of its essential oils that impart strong odors and flavors to foods (for additional information, see Botanical Department - University of Catania, http://www.dipbot.unict.it/alimurgiche/scheda.aspx?i=22).

1.1. Medicinal and nutritional properties

It is well known as an aromatic, culinary, medicinal herb and, in some regions, as an invasive plant (Kandil, 2002). Historical evidences related to the diffusion and usage of this plant species date back to the battle of Marathon, which took place in 490 BC, literally in a "plain of fennel", an event that gave the old Greek name "marathon" (μάραθον) to this plant. We now know that fennel was also widely used by the Romans both as a spice for the scent of the environment and essential element for the preparation of dishes (see for example "*de re coquinaria*" by Marco Gavio Apicio).

As regards its medical uses, fennel has a long history of use in traditional herbal medicine to maintain health or treat symptoms of disease. As reported by Puleo (1980) fennel contains anethole, that is has been considered an active and safe phytoestrogenic agent able to reduce pain due to menstruation (dysmenorrhea). Anethole acts also in the intestine tract as a carminative and as a mild laxative (Delaram *et a*l., 2011), as well as for the symptomatic treatment of digestive disorders, alleviating mild spasmodic gastro-intestinal pains (Van den Berg, 2014), in particular way in infants and young children (Perry *et al*., 2011).

Different studies have investigated the diuretic properties of *Foeniculum vulgare*, which probably resides on the noradrenalin contractile responses of aortic rings,

with little effect on vascular tone (Wright *et al*., 2007). Its action on the circulatory system makes it also useful for the treatment of hypertension (El Bardai *et al*., 2001). In many parts of Europe fennel based teas are traditionally used for the relief of symptoms during inflammations of mucous membranes of the upper respiratory tract and for the treatment of chronic coughs (EMA, 2008). In addition to anethole, fennel contains its own unique combination of phytonutrients, such as flavonoids rutin, quercitin, and various kaempferol glycosides, that give it strong antioxidant activity. It is mostly composed of water (>90%) and, even if it is a product extremely low in kilocalories (31 Kcal/100 gr), it represents a good source of potassium, calcium, sodium and vitamin C. Nutritional properties of fennel are summarized in Figure 1 (USDA National Nutrient Database for Standard Reference).

Nutrient	Unit	1 Value Per100 g		Unit	1 Value Per100 g
Proximates			**Vitamins**		
Water	g	90.21	Vitamin C, total ascorbic acid	mg	12.0
Energy	kcal	31	Thiamin	mg	0.010
Protein	g	1.24	Riboflavin	mg	0.032
Total lipid (fat)	g	0.20	Niacin	mg	0.640
Carbohydrate, by difference	g	7.30	Vitamin B-6	mg	0.047
Fiber, total dietary	g	3.1	Folate, DFE	µg	27
Sugars, total	g	3.93	Vitamin B-12	µg	0.00
Minerals			Vitamin A, RAE	µg	48
Calcium, Ca	mg	49	Vitamin A, IU	IU	963
Iron, Fe	mg	0.73	Vitamin E (alpha-tocopherol)	mg	0.58
Magnesium, Mg	mg	17	Vitamin D (D2 + D3)	µg	0.0
Phosphorus, P	mg	50	Vitamin D	IU	0
Potassium, K	mg	414	Vitamin K (phylloquinone)	µg	62.8
Sodium, Na	mg	52			
Zinc, Zn	mg	0.20			
Lipids					
Fatty acids, total saturated	g	0.090			
Fatty acids, total monounsaturated	g	0.068			
Fatty acids, total polyunsaturated	g	0.169			
Cholesterol	mg	0			

Figure 1. Nutritional properties of fennel.

1.2. Production of fennel

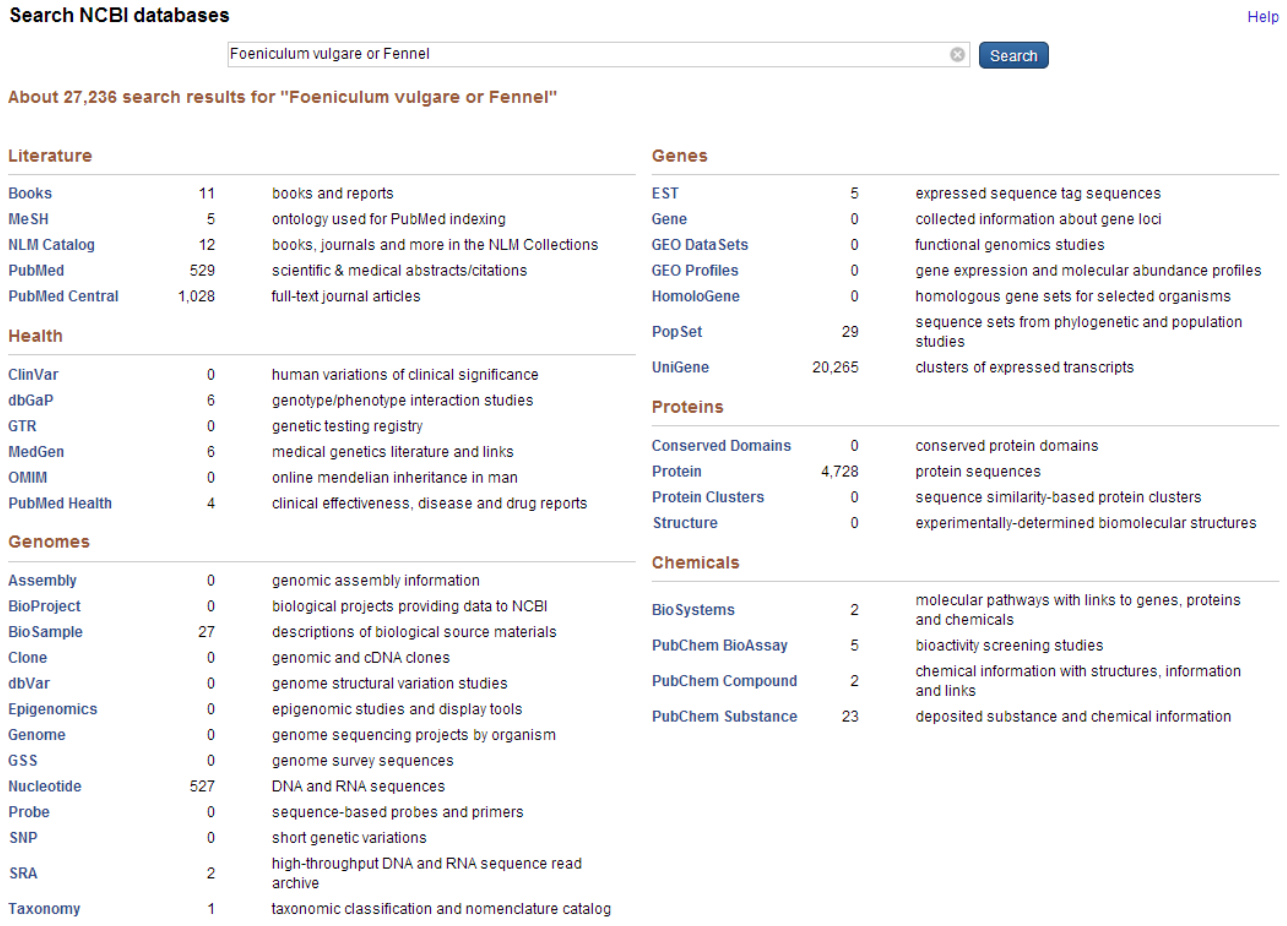
As reported by the Statistical Division of FAO in 2012, India is the world leader in production of fennel, anise, badian and coriander. The agronomic relevance of this plant species is reported on Table 1, both in terms of production (tons) and area (ha) devoted to cultivation of these products. As an example, the Indian area devoted to fennel farming (and to the other three products, 693,700 ha) covers slightly less than half of the Veneto surface (see Food and Agricultural Organization of United Nations: Economic and Social Department: The Statistical Division, http://faostat.fao.org/).

Table 1. Productivity data set of fennel by country.

	Production (tons)		**Area harvested (ha)**		**Yield (hg/ha)**
India	537330	India	693700	Palestine	78431
China	47000	Syria	69000	Netherlands	26190
Syria	45500	Bulgaria	46000	Australia	19655
Iran	44000	Iran	45500	Ukraine	17083
Bulgaria	36000	China	37000	Hungary	14231
Afghanistan	12500	Turkey	19443	Serbia	13194
Russian Fed.	12000	Afghanistan	18500	China	12703
Turkey	11820	Russian Fed.	17500	Greece	12121
Ukraine	6150	Viet Nam	6500	Spain	11764
Romania	4293	Romania	5441	Iran	9670
Viet Nam	4200	Ukraine	3600	Romania	7890
Hungary	3700	Hungary	2600	Bulgaria	7826
Spain	2941	Spain	2500	India	7746
Australia	2850	Australia	1450	Russian Fed.	6857
Palestine	2000	Serbia	720	Afghanistan	6757
Serbia	950	Greece	660	Syria	6594
Greece	800	Palestine.	255	Viet Nam	6462
Netherlands	440	Netherlands	168	Turkey	6079

Moreover, comparing these data with those of previous years, it becomes clear how the production, the area devoted to cultivation and the yield are growing year after year.

Despite its agronomic and pharmaceutical interests, researchers face the almost complete lack of biological and genomic data for this species, being nucleotide sequences and molecular markers available very scanty. In fact as resulting from a consultation of the Entrez database residing at NCBI (http://www.ncbi.nlm.nih.gov/gquery/?term=Foeniculum+vulgare) some fields are totally lacking (Figure 2).



Search NCBI databases Help

Foeniculum vulgare or Fennel Search

About 27,236 search results for "Foeniculum vulgare or Fennel"

Literature

Books	11	books and reports
MeSH	5	ontology used for PubMed indexing
NLM Catalog	12	books, journals and more in the NLM Collections
PubMed	529	scientific & medical abstracts/citations
PubMed Central	1,028	full-text journal articles

Health

ClinVar	0	human variations of clinical significance
dbGaP	6	genotype/phenotype interaction studies
GTR	0	genetic testing registry
MedGen	6	medical genetics literature and links
OMIM	0	online mendelian inheritance in man
PubMed Health	4	clinical effectiveness, disease and drug reports

Genomes

Assembly	0	genomic assembly information
BioProject	0	biological projects providing data to NCBI
BioSample	27	descriptions of biological source materials
Clone	0	genomic and cDNA clones
dbVar	0	genome structural variation studies
Epigenomics	0	epigenomic studies and display tools
Genome	0	genome sequencing projects by organism
GSS	0	genome survey sequences
Nucleotide	527	DNA and RNA sequences
Probe	0	sequence-based probes and primers
SNP	0	short genetic variations
SRA	2	high-throughput DNA and RNA sequence read archive
Taxonomy	1	taxonomic classification and nomenclature catalog

Genes

EST	5	expressed sequence tag sequences
Gene	0	collected information about gene loci
GEO DataSets	0	functional genomics studies
GEO Profiles	0	gene expression and molecular abundance profiles
HomoloGene	0	homologous gene sets for selected organisms
PopSet	29	sequence sets from phylogenetic and population studies
UniGene	20,265	clusters of expressed transcripts

Proteins

Conserved Domains	0	conserved protein domains
Protein	4,728	protein sequences
Protein Clusters	0	sequence similarity-based protein clusters
Structure	0	experimentally-determined biomolecular structures

Chemicals

BioSystems	2	molecular pathways with links to genes, proteins and chemicals
PubChem BioAssay	5	bioactivity screening studies
PubChem Compound	2	chemical information with structures, information and links
PubChem Substance	23	deposited substance and chemical information

Figure 2. Consultation of fennel available data on the Entrez database residing at NCBI.

Breeding strategies based on the production of hybrid lines has been applied in fennel, like other crop species, as hybrid breeding as it normally permits to maximize the genetic gain of a variety from the effects of heterosis. Heterosis, or hybrid vigor, refers to the phenomenon in which an F1 progeny obtained by crossing genetically divergent inbred lines or pure lines exhibits greater biomass, resistance to biotic agents and abiotic stresses, faster development and higher fertility than the two

lines used as parents (Barcaccia *et al*., 2006; Figure 3). Critical steps are the production of parental inbreds, particularly for the selection of self-compatible genotypes to be used as pollen donors, and the identification of male-sterile genotypes, to be used as seed parents.

The constitution of hybrids, a process that could be very expensive, is made economically possible in fennel by the use of male sterility. This strategy -that has already been exploited in a number of crop plants such as sunflower, sugar beet, leaf chicory and sorghum- provides the key factor that a male-sterile line could be used as seed parent as it avoids any possibility of self-pollination by maximizing cross-pollination with the designed pollinators.

Male sterility, that is the inability of the plant to produce viable pollen, is a trait maternally inherited, under cytoplasmic control (CMS) in fennel. The genetic control of male sterility in fennel resides upon the expression of a mitochondrial sterility-associated locus and a dominant “restore fertility gene” residing on the nuclear genome. Starting from this premise, the phenotype of the progeny resulting from the cross between a male-sterile mutant (S genotype at the mitochondrial locus) and a maintainer (N genotype at the mitochondrial locus) depends on the genotype of the nuclear “restore fertility gene” carried by the maintainer (genotypes: RFrf , RFRF or rfrf). As a consequence, the percentage of male fertile plants in the progeny can vary from complete presence of fertile plants (maintainer genotype: RFRF) to complete presence of sterile plants (maintainer genotype: rfrf), depending on the composition of the RF locus carried by the maintainer. Accordingly, maintainers characterized by the presence of the “restore fertility gene” in heterozygosis (RFrf) will produce 50% of fertile individuals, carrying the dominant allele at the RF locus. One relevant agronomic implication of this type of inheritance resides in the fact that the maintenance of a male-sterile parental line (ms, seed parent) requires its controlled crossing with a male-fertile maintainer.

The constitution of the F1 hybrid varieties involves two main aspects:

1) Choice of the best parental (both female and male) inbred lines by evaluating the degree of homozygosity of each line, being this needed very high to ensure genetic stability and phenotypic uniformity;

2) Choice of the best combination in all possible pair-wise parental inbred lines by evaluating the extent of genetic diversity among them as well as their specific combining ability at the morphological level. The specific combining ability (SCA) effect of two populations expresses the differences of gene frequencies between them and their genome divergence, as compared to the diallel parents (Viana and de Pina Matta, 2003).

Figure 3. Comparison between parental inbred lines (on the left and right sides) and their F1 hybrids (in the center) in fennel.

Then, the identification of parental inbred lines leading to superior hybrid combinations is a crucial factor. Such activities using conventional breeding methods are expensive and time consuming, and, furthermore, the large number of possible hybrid combinations to be produced from a relatively small number of inbred lines, render the evaluation of all possible combinations unfeasible (Legesse *et al.*, 2008).

The efficiency of breeding programs aimed at the constitution of F1 hybrids could be improved if the inbred lines could be selected for their within-line genetic stability and also screened for their between-line genetic diversity using molecular markers. In fact, they are not influenced by environmental factors and are also fast, efficient and more sensitive than phenotype-based field trials to detect large numbers of distinct differences between genotypes at the DNA level (Reif *et al.*, 2012).

1.3. SSR markers as a molecular tool for DNA genotyping

Microsatellites (also referred as Simple Sequence Repeats, shorten SSR) is a class of molecular polymorphism commonly used for linkage analysis, gene mapping and to study inheritance patterns. Microsatellites, or SSR markers, are short sequences, one to four nucleotides in length, repeated in tandem. Mutations affecting the number of repeats in a given microsatellite motif happen with high frequency in a genome and this influences the variability proper of an SSR locus, a characteristic that makes them useful as genetic markers. Microsatellites are considered co-dominant markers as they allow the complete definition of the molecular phenotype of the considered locus, therefore distinguishing between homozygous (presence of a single marker allele in double dose) and heterozygous (presence of two marker alleles) genotypes. The practice is based on a selective PCR amplification using a couple of primers for each target microsatellite region to be amplified. Compared to other, dominant, molecular marker types, the SSR analysis requires a higher degree of preliminary genomic information for its exploitation in a given species. In the case of fennel, no SSR sequences are available neither in literature nor in database.

1.4. AFLP markers as a molecular tool for DNA fingerprinting

The Amplified Fragment Length Polymorphism (acronym AFLP) is a PCR-based technique capable of producing multi-locus and reliable fingerprints of genomes, which has been successfully applied in many species during the past 20 years.

The AFLP marker systems falls into the dominant type of markers, which means that individuals showing the presence of an amplified fragment at a given AFLP locus could be either homozygous or heterozygous at that genomic locus (showing so a double dose or a single dose of the marker allele corresponding to the amplified fragment) and that the only certain genotype at the same AFLP locus is that determined by the complete lack of the amplified fragment (assumed to be homozygous for the absence of the marker allele) (Bensch and Åkesson, 2005). AFLP markers have significant advantage over other procedures because of the nucleotide sequence variability that can be assessed simultaneously at a number of independent loci is much bigger than that obtainable using other dominant marker systems, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (SSR) markers (Pejic *et al*., 1998; Torabi *et al*., 2012). Moreover, the technique requires no prior sequence knowledge, has a relatively low cost per amplified marker, it has a high level of reproducibility and it is very easily transferable across species and laboratories (Garecia *et al*., 2004).

The AFLP methodology is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: (i) restriction of the genomic DNA with specific combinations of endonucleases and ligation of oligonucleotide adapters to the restriction site ends, (ii) selective amplification of subsets of restriction fragments, and (iii) gel electrophoresis analysis of the amplified fragments or amplicons (Vos *et al*., 1995). In the first step the genomic DNA is digested with two different restriction enzymes, a six-base cutter (*e.g*. *Eco*RI; 5′-G↓AATTC-3′ or *Pst*I; 5′-CTGCA↓G-3′) and a four-base cutter (*e.g*. *Mse*I; 5′-T↓TAA-3′) to generate hundreds of thousands of anonymous DNA fragments with sticky ends to which specific adapters are ligated. The second step is based on a PCR-based amplification (pre-amplification) of restricted/ligated DNA

fragments and is carried out using sets of primers complementary to the sequences of adaptors/enzymes combinations used in the previous enzymatic reaction. The amplified DNA population resulting from this pre-amplification procedure is then used for the final amplification, in a reaction mixture employing primer combinations different from the previous set by the presence of (one to three) additional bases toward the 3-prime ends of the primers.

The EcoRI-specific primer (or PstI-specific primer) used in the final amplification step is traditionally labeled on its 5-prime end with radioactive isotopes (*e.g*. ^{33}P) or fluorophores (*e.g*. fluorescein, FAM) dependent on the available detection system. The final products can be separated by length either using PAGE systems or DNA sequencers. Therefore the original method conceived by Vos *et al*. in 1995 has evolved constantly and it is currently based on the use of fluorescence labeled primers in combination with capillary automatic sequencers. If compared to the first protocol, it is believed that the use of capillary electrophoresis and automated procedures of analysis can, on one hand, increase data throughput and scoring reliability, and, on the other, decrease the overall experimental error. However, a major gap remains the use of labeled primers, which is one of the key aspects affecting the cost of the procedure.

Very few works concerning the evaluation of genetic diversity in fennel through the use of molecular markers have been produced and the only paper available on the use of AFLP markers is that by Torabi *et al*. (2012).

2. MATERIALS AND METHODS

2.1. Genomic DNA purification

A total of 240 genomic DNA samples were extracted and purified from leaves of different varieties of fennel (*Foeniculum vulgare* Mill.) using DNeasy 96 plant kit (Qiagen), by following the procedure provided by the suppliers. Both quality and concentration of DNA samples were estimated by 1% Agarose (Life Technologies) 1X Sybr Safe (Life Technologies) gel electrophoresis and by spectrophotometric analysis (NanoDrop 2000c UV-Vis, Thermo Scientific).

2.1. Protocols for SSR markers detection

For amplification and sequencing of the microsatellite-containing regions in fennel we took advantage of heterologous sequences available for the two taxonomically related species *Daucus carota* and *Apium graveolens*. Sequences and primers were retrieved from Acquadro *et al*. (2006) and from http://www.vcru.wisc.edu/sdata (Table 2). PCR amplifications of the microsatellite-containing regions were done with the reaction mix described in Table 3.

Table 2. Basic information on the heterologous primers used for SSR marker analysis in fennel.

Primer name		Sequences (5'-3')	Original species	Motif	Fragment size (bp)
ECMS-1	For	CTACATTTCTTCCTCTCCCAC	*Apium graveolens*	(TCC)8	180-310
	Rev	TTCTCTCCATTCTCTCAAACA			
ECMS-6	For	TTGACTGGTATTCTTGTTCATC		(GAT)16	315-330
	Rev	ATCCATTTCTCTCTTGTTTCA			
ECMS-9	For	GGCAATGAGTGGTGCTCT		(ATA)12	320-330
	Rev	CGCAAGTCGTGAAGATAAGT			
ECMS-13	For	GCTGTAATGTGGAATGAAGAA		(AT)16	278-280
	Rev	AAACCAAGTGTAGCAAGTAGAA			
ECMS-16	For	AGGTTTCAGTTTCTGGTAGTGT		(CAA)8	278-290
	Rev	TGTTGCTGTGTAGGCATCT			
ECMS-19	For	CCCAAGTCATCAATCCCAAT		(TTTTG)3-(AG)7	146-162
	Rev	GCGGGGACACTCCACTAC			
ECMS-39	For	GCTACAACACCAACAGCA		(GCA)5...(GCA)7*...(GCA)7*...(GCA)5*...(GCA)10*	360-375
	Rev	GCTACAACACCAACAGCA			
GSSR-5	For	ATAATAAACCCAACCAGACCCC		(AC)9	120
	Rev	ATCAGGCAAATCCCATACTGAC		(AC)6	
GSSR-24	For	GCCAACCATCAAAATCACTTCT		(TC)12	183
	Rev	GAATAACTGCCTGCAATACCG			
GSSR-35	For	AATTCACAATCACCGACTCTCC	*Daucus carota*	(GA)13	173
	Rev	ACGTCAAAGCTCCTGTTCATTT			
GSSR-37	For	CGAGGGAGAATGACGAAAATTA		(TATG)7	197
	For	TCTGTGACGAGTAGGATCAGGA		(TTGC)3	
GSSR-154	For	CTTATATGTGATGGCGTCGAAA		(TC)11	328
	Rev	GACTGCACCGCTCCTAACTC			

Table 3. SSR amplification mix.

Reagent	Working dilution	Amount (µl)
Buffer	10X	2
$MgCl_2$	50 mM	0,8
dNTPs	10 mM	0,8
Primer F	50 ng/µl (≈6pmol/µl)	0,5
Primer R	50 ng/µl (≈6pmol/µl)	0,5
Taq Polymerase	5 U/µl	0,1
Sterile H_20		14,3
DNA	10 ng/µl	1,0
Total		20,0

Presence and quality of amplification products were checked by DNA gel electrophoresis on 1% Agarose (Life Technologies) 1X Sybr Safe (Life Technologies) gels. The use of heterologous primers led to the production of amplification profiles that were evaluated for their complexity and banding patterns.

Amplification reactions yielding a single amplification product were directly sub-cloned and sequenced, while for all PCR reactions providing complex profiles of multiple bands, individual bands were extracted from the agarose gel and processed singularly.

Criteria followed to choose the bands to be extracted and further processed were:

1. Size of the band, and its correlation to the expected size of the same microsatellite region in *Daucus carota* and *Apium graveolens*
2. Amplification of the product in replicated experiments performed on different fennel plant accessions.

PCR products extracted from gel were purified using GenElute™ Gel Extraction Kit (Sigma Aldrich) and individually sub-cloned with the kit StrataClone PCR Cloning, by following the indication provided by the supplier (Agilent Technologies). Following transformation and cell culture, a minimum of 8 positive colonies per transformation reaction was tested by colony PCR (for additional details, please see Table 4). PCR products having the desired size and intensity were then enzymatically

prepared for sequencing by applying the purification procedure ExoI-FAP, as described by the supplier of the enzymes (Fermentas). The sequence of cloned fragments was determined at BMR Genomics (Padova, Italy), using the 3730xl DNA Analyzer original BigDye® Terminator v3.1 kit (Applied Biosystems) and M13 for/ T7 primers.

Sequences were then screened for the presence of SSR motifs with the software GENEIOUS v. 3.6 (www.geneious.com). In case of successful amplification and DNA sequencing of SSR containing regions, homologous M13-anchored primers were designed to specifically amplify the repetitive region with the software GENEIOUS v. 3.6. Primer sets were tested for their composition and ability to produce primer-dimers with the software PerlPrimer v. 1.1.21. (http://perlprimer.sourceforge.net/). Finally, primer sets were also tested on three fennel samples (a male sterile individual, MS1; a maintainer individual, Mant1; a male fertile individual MF23) in order to conduct preliminary analyses on their functionality and effectiveness.

Table 4. colony PCR mix.

Reagent	**Working dilution**	**Amount (µl)**
Buffer	10X	2
$MgCl_2$	50 mM	1,2
dNTPs	10 mM	0,8
T7 primer	50ng/µl (≈6pmol/µl)	0,5
M13 rev primer	50ng/µl (≈6pmol/µl)	0,5
Taq Polymerase	5U/µl	0,1
Sterile H_20		14,9
E. coli		1 colony
Total		20,0

2.3. Protocols for AFLP markers detection

Preliminary investigations were made in order determine the most suitable protocol for AFLP marker detection.

Table 5. Primers and adapters (all purchased from Invitrogen) applied in the AFLP procedure. For each primer or adapter, the restriction enzyme, the primer sequence and the usage concentration are reported.

Not tailed Primers	**Sequence (5'-3')**	**Conc. (µM)**
EcoRI+C	GACTGCGTACCAATTCC	6
PstI+A	GACTGCGTACATGCAGA	6
MseI+A	GATGAGTCCTGAGTAAA	6
MseI+C	GATGAGTCCTGAGTAAC	6
MseI+CAA	GATGAGTCCTGAGTAACAA	6
MseI+ACC	GATGAGTCCTGAGTAAACC	6
5'-Tailed primer		
*EcoRI+C	TTGTAAAACGACGGCCAGTGACTGCGTACCAATTCC	6
*EcoRI+CA	TTGTAAAACGACGGCCAGTGACTGCGTACCAATTCCA	6
*EcoRI+CAA	TTGTAAAACGACGGCCAGTGACTGCGTACCAATTCCAA	6
*PstI+A	TTGTAAAACGACGGCCAGTGACTGCGTACATGCAGA	6
*PstI+AG	TTGTAAAACGACGGCCAGTGACTGCGTACATGCAGAG	6
*PstI+AGC	TTGTAAAACGACGGCCAGTGACTGCGTACATGCAGAGC	6
Adapters		
EcoRI-Adaptor I	CTCGTAGACTGCGTACC	5
EcoRI-Adaptor II	AATTGGTACGCAGTC	5
PstI-Adaptor I	CTCGTAGACTGCGTACATGCA	5
PstI- Adaptor II	TGTACGCAGTCTAC	5
MseI-Adaptor I	GACGATGAGTCCTGAG	50
MseI-Adaptor II	TACTCAGGACTCAT	50

2.2.1. Protocol 1

Restriction and ligation reactions were conducted using restriction endonuclease working at different temperature: EcoRI and PstI worked at 37°C, MseI at 65°C. All the enzymes and their related buffer were purchased from Thermo scientific. A first

40 µl restriction reaction containing water, Buffer TANGO 10X (8 µl), EcoRI (or PstI) 10 U/µl (0,5 µl), DNA 500 ng/30µl (30µl) was incubated at 37°C for 3h. After that, MseI 10 U/µl (0,5 µl) was added to the reaction mixture and the whole incubated for 3h at 65°C. Finally, a master mix composed by water, T4 buffer 10X (5 µl), T4 DNA ligase 5U/µl (1 µl), EcoRI (or PstI,) and MseI adapters (1 µl each one, for usage concentrations see Table 5) were added to reach a final volume of 50 µl and for a final incubation of 4 h at 37°C. The higher concentration of MseI adapters relative to EcoRI adapters (see Table 5) reflects the higher number of restriction sites available for the four-base cutter MseI. Quality of the enzymatic reaction and of the subsequent ligation was assessed by 1% Agarose (Life Technologies) 1X Sybr Safe (Life Technologies) gel electrophoresis. The restricted/ligated products were then diluted 1:10 and used as template for the following pre-selective amplifications.

Pre-amplifications and selective amplifications were performed with two different strategies.

In one strategy, the pre-amplification reaction was carried using canonical primer: EcoRI+1 (or PstI+1) and MseI+1 (1^{st} variant, Table 6), while amplification was accomplished with a three primer system: 5' tailed EcoRI+2/3 (or 5' tailed PstI+2/3), MseI+3 and fluorescent-labeled M13 primer (1^{st} variant, Table 7). In a second strategy, pre-amplification was carried out using 5'tailed EcoRI+1/2/3 (or 5' tailed PstI + 1/2/3) and MseI+1 (2^{nd} variant, Table 6), while amplification was conducted using fluorescent-labeled M13 primer and MseI+3 (2^{nd} variant, Table 7). In both cases, the product of the pre-amplification procedure was checked by DNA gel electrophoresis on 1% Agarose (Life Technologies) 1X Sybr Safe (Life Technologies), then diluted 1:10 and used as template for the following selective amplification.

The amplification products were separated by capillary electrophoresis for precise visualization of amplified-labeled fragments.

Table 6. Composition of the pre-amplification master mix used for AFLP markers detection.

Reagent	Working dilution	1st variant µl	2nd variant µl
Buffer	10X	5,0	5,0
$MgCl_2$	50mM	3,0	3,0
dNTPs	10 mM	1,0	1,0
EcoRI+1 (or PstI+1)	50ng/µl (≈6pmol/µl)	1,5	-
MseI+1	50ng/µl (≈6pmol/µl)	1,5	1,5
5'tailed EcoRI+1/2/3 (or 5' tailed PstI+ 1/2/3)	50ng/µl (≈6pmol/µl)	-	1,5
Taq Polimerase	5U/µl	0,2	0,2
Sterile H_20		32,8	32,8
Restricted, ligated and diluted 1:10 DNA		5,0	5,0
Total		50,0	50,0

Table 7. Composition of the amplification master mix used for AFLP markers detection.

Reagent	Working dilution	1st variant µl	2nd variant µl
Buffer	10X	2	2
$MgCl_2$	50mM	1,2	1,2
dNTPs	10 mM	0,4	0,4
fluorescently-labelled M13 primer	50ng/µl (≈6pmol/µl)	0,48	0,6
EcoRI+3	50ng/µl (≈6pmol/µl)	0,6	0,6
5'tailed EcoRI+2/3 (or 5' tailed PstI+2/3)	50ng/µl (≈6pmol/µl)	0,2	-
Taq Polimerase	5U/µl	0,1	0,1
Sterile H_20		10,02	10,1
Preamplified and diluted (1:10) DNA		5	5
Totale		20	20

2.2.2. Protocol 2

For the restriction and ligation procedure, a mix containing water, 10 µl of RL buffer 1X (One Phor All 100 mM, DTT 25mM, BSA 0,25 ng/µl), EcoRI or PstI 20 U/µl (0,25µl New England Biolabs), MseI 4 U/µl (1,25µl, New England Biolabs), EcoRI (or PstI) and MseI adapters (1 µl each one, for usage concentration see Table 5), ATP 10 mM (1 µl) and T4 DNA ligase 7.5 U/µl (0,133 µl, New England Biolabs) were added to 30 µl of DNA (500 ng/30µl), for a final volume of 50. This reaction was then incubated for 4 hours at 37°C. The subsequent steps (pre-amplification, amplification and capillary electrophoresis) are identical to those described in the *protocol 1,* so also in this case two different strategies were used and is possible referring to table 6 and 7.

2.2.3. Validation of the AFLP procedures

Experimental conditions tested in the preliminary investigations were validated using a collection of 240 genomic DNA samples (Table 8). After having defined critical steps, such as protocol for anchor usage, enzyme combinations and primer combinations to be used in the selective amplification step, the above mentioned samples were all processed.

All fragment analysis data derived from capillary electrophoresis were evaluated with the software Peak Scanner^{Tm} v. 1.0 (Life Technologies).

Table 8. Biological materials studied in this research (set of analysis).

Population	No. of individuals	Population	No. of individuals
MS1	8	MF162	8
MS2	8	MF164	8
MS3	8	MF165	6
MS5	8	MF166	5
MS6	8	MF742	5
MS7	8	MF743	4
MS9	8	MF13	4
MS10	8	MF TR	4
Mant1	8	MF MN	4
Mant2	8	MF FALSO MN	3
Mant3	8	MF SARNO	5
Mant5	8	MF WAN	4
Mant6	8	MF ROMA86	4
Mant7	8	MF ROMA 92	4
Mant9	8	MF ROMAGNA	4
Mant10	8	MF MA 98	4
MF23A	8	MF MA 99	4
MF23B	8	MF CH	4
MF15	8	MF 163	4

MS: male sterile line; Mant: maintainer line; MF: male fertile line.

Statistical analysis of the peaks exported from Peak Scanner^{Tm} v. 1.0 (Life Technologies) was performed with RawGeno v. 2.0 (Arrigo *et al*., 2012) that allows to transform raw data into binary data. Descriptive genetic diversity and differentiation statistics were calculated using the PopGene software package v. 1.32 (Yeh *et al*., 1997). A cluster analysis was performed according to the

unweighted pair-group arithmetic average method (UPGMA), and the dendrogram was constructed from the symmetrical mean genetic distances matrix. These calculations were conducted using PopGene software package v. 1.32 (Yeh *et al*., 1997). The proportion of genetic similarity (GS) in all pair-wise comparisons of individuals was calculated using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) v. 2.21q (Rohlf, 1993) by applying the coefficient of Dice (Dice, 1945).

3. RESULTS

3.1. Genotyping by SSR markers

Two fennel genomic DNA samples were amplified using the 12 couples of heterologous primers shown in Table 2. Most PCR amplifications were characterized by the presence of multiple bands of similar intensity (Figure 4, ECSM1). In rare cases the electrophoresis gel showed one or two bands displaying higher intensity than the other faint bands (Figure 4, ECSM16).

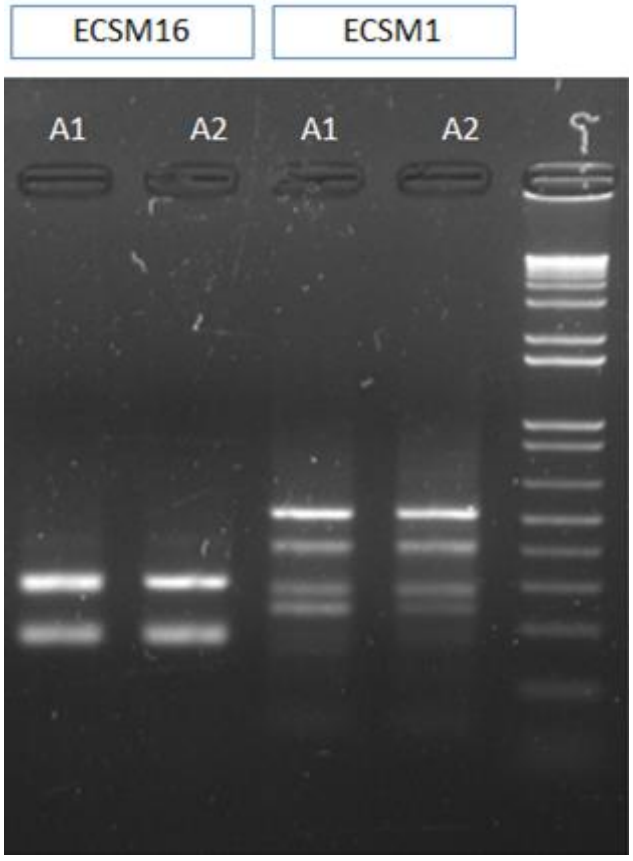


Figure 4. Two different heterologous primer pairs tested on two fennel samples.

We considered that direct sub-cloning of multiple PCR products with different intensities would not have been efficient. Therefore in all cases in which more than two bands were visualized (Figure 4, ECSM1), before sub-cloning them, we

proceeded by excision and purification of single PCR products from the gel (Figure 5).

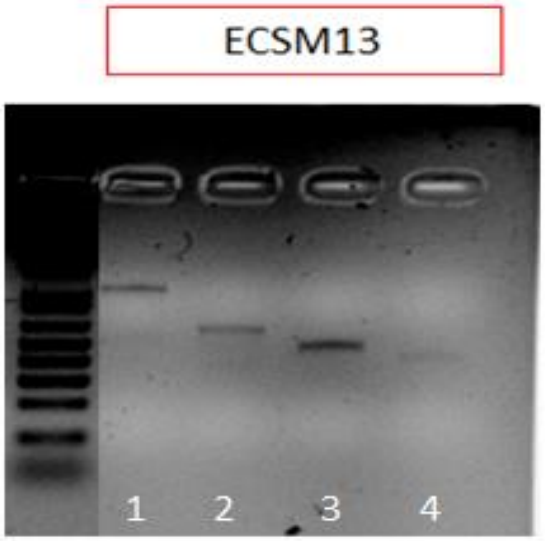


Figure 5. Example of four bands (obtained by PCR amplifying with ECSM13 heterologous primer pair) correctly extracted and purified.

Starting from 12 primer combinations, we were able to amplify and obtain high quality sequences for four products. This led to the identification of four distinct sequences carrying an SSR motif. Two of them are characterized by the presence of perfect, tri-nucleate repeats [$(AGC)_n$ and $(AGA)_n$], whereas the remaining sequences contain imperfect microsatellites with complex repeats [$(ACA)_n(ATA)_n$ and $(GT)_n(AG)_n$].

Homologous primers designed to specifically amplify the newly identified fennel SSR loci were named FvSSR1, FvSSR2, FvSSR3 and FvSSR4. All amplification experiments performed with these primer pairs confirmed the efficacy of the primer combinations over the four genomic loci.

The nucleotide sequences of microsatellite regions and specific primer pairs required for their amplification were recently submitted to the GenBank database (ID 1735181). As an example, the results of the amplification developed on three

fennel samples (MS1, Mant1 and MF23) and with the four pairs of homologous primers, are shown in Figure 6. Primer combinations FvSSR2, FvSSR3 and FvSSR4 could generate single PCR products with length ranging from 100 bp to 450 bp (Figure 6). Primer combination FvSSR1 generated two products of different intensity in all replicated reactions conduced in our tests. The profile amplified by this primer combination is such that, of the two amplified fragments, a smaller band is intense and always detectable while a longer product is frequently less intense and in some samples not visible (Figure 6, see fvSSR1 1° primer pair). The situation remained unchanged also adopting more stringent PCR conditions, acting on annealing temperatures and decreasing $MgCl_2$ concentration. For this locus, a new primer pair was also designed but it revealed similar results (Figure 6, see fvSSR1 2° primer pair).

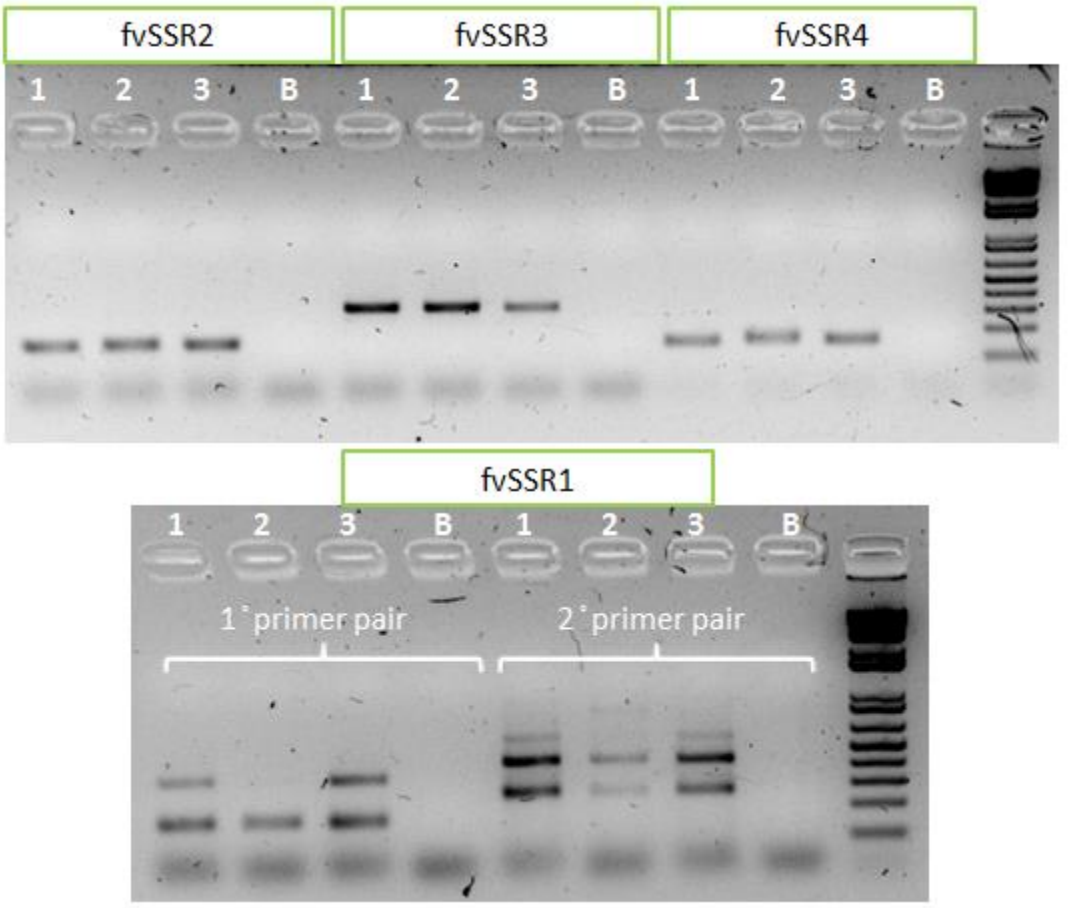


Figure 6. Agarose gel of three fennel samples (1: MS1, 2: Mant1 and 3: MF23 B: negative control) amplified using 4 primer combinations drawn on SSR region.

Additional studies are currently in progress to determine the nature of the amplification pattern displayed by the primer pair of the locus FvSSR1.

3.2. Fingerprinting by AFLP markers

3.2.1. Definition and optimization of the M13-tailed AFLP method

Experimental procedures needed for setting up the M13-tailed AFLP method were exploited using DNA samples that scored an estimated concentration higher than 50 ng/µl, and 260/230 and 260/280 nm absorbance reading ratios varying between 1,8 and 2. Genomic DNA samples not reaching the minimal qualitative or quantitative requirements for this molecular procedure were re-extracted to ensure optimal qualitative standards to the AFLP analysis.

Preliminary analyses indicated that both with protocol I -based on the use of two endonucleases working at different temperature (EcoRI or PstI working at 37°C, and MseI working at 65°C, see protocol I for additional details)- and protocol II (all enzymes working at 37°C) is possible to achieve optimal results. Moreover, the procedure composed by a pre-amplification reaction carried using canonical primers (EcoRI+1 or PstI+1 and MseI+1, 1st variant, see Table 6), followed by an amplification step accomplished with a three primers system (5'-tailed EcoRI+2/3 or 5' tailed PstI+2/3, MseI+3 and fluorescently-labeled M13 primer, 1st variant, see Table 7) proved to be more robust than the other strategy tested (pre-amplification carried out using 5'tailed EcoRI+1/2/3 or 5' tailed PstI + 1/2/3 and MseI+1 and amplification conducted using fluorescently-labeled M13 primer and MseI+3).

On the basis of these findings, we decided to adopt the 1st variant of the protocol I for the molecular analysis of the 240 fennel samples.

As far as the combination of enzymes and primers concerned, two different endonuclease combinations (EcoRI/MseI and PstI/MseI) were initially tested on

some randomly chosen fennel samples. The restriction and ligation steps proved to be efficient in both cases as shown by 1% gel electrophoresis of restricted/ligated DNA fragments (Figure 7). Indeed, as shown in Figure 7, both combinations provided pools of fragments ranging in size between 100 bp and 2,000 bp.

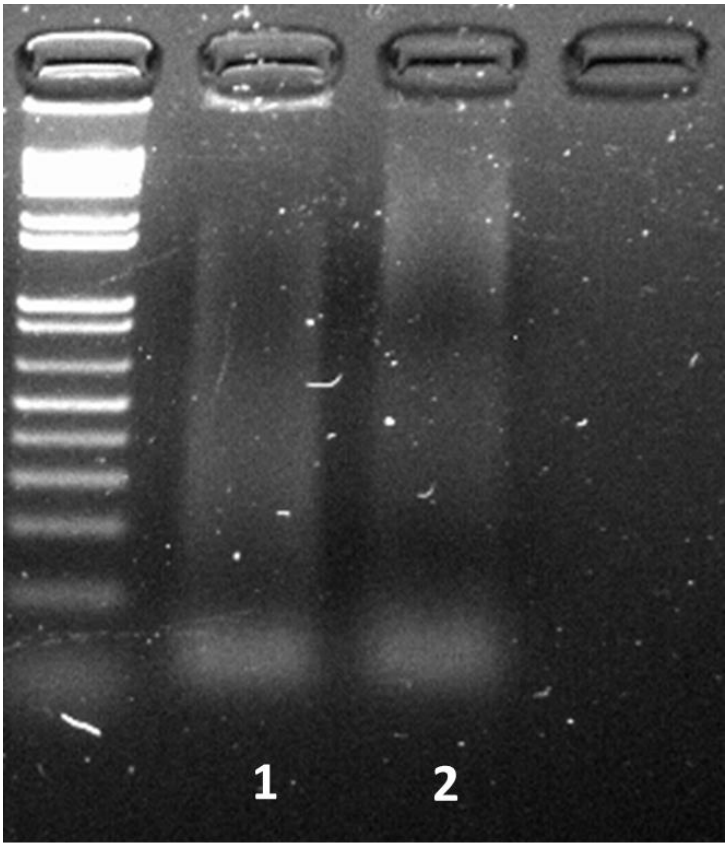


Figure 7. Agarose gel electrophoresis of restricted/ligated products obtained with two different enzyme combinations (1: EcoRI/MseI; 2: PstI/MseI).

Then, four different pre-amplifications were set up using four different primer combinations (EcoRI+C/MseI+A; EcoRI+C/MseI+C; PstI+A/MseI+A; PstI+A/MseI+C). Pre-amplification of ligated DNA fragments with two selective bases (one base for each primer), resulted in a significant reduction on the number of fragments visible on 1% agarose gel electrophoreses, by compressing the population of detectable fragments in the size range varying between 100 bp and 1,000 bp (Figure 8).

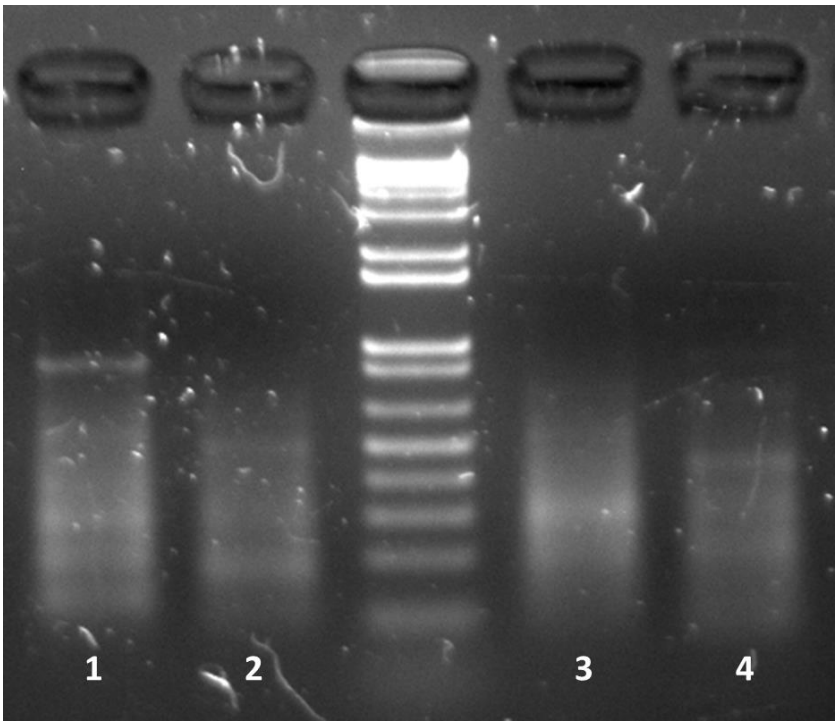


Figure 8. Agarose gel electrophoresis of pre-amplified products using 4 primer combinations (1: EcoRI+C/MseI+A; 2: EcoRI+C/MseI+C; 3: PstI+A/MseI+A; 4: PstI+A/MseI+C).

Selective amplification reactions were performed with 8 different primer combinations (EcoRI+CA/MseI+ACC, EcoRI+CAA/MseI+ACC, EcoRI+CA/MseI+CAA, EcoRI+CAA/MseI+CAA, PstI+AG/MseI+ACC, PstI+AGC/MseI+ACC, PstI+AG/MseI+CAA, PstI+AGC/MseI+CAA) by using the labeled-M13 primer as third primer (see the materials and methods section for additional details on primer sequence used in these experiments, Table 5).

The amplification efficiency was tested on 4% agarose electrophoresis gels (Figure 9). As shown in Figure 9, the size of the amplified fragments ranged between 100 bp and 650 bp. Some of the analyzed samples showed a homogenous profile, characterized by a continuous smear of multi-size amplified fragments (*e.g.* EcoRI+CAA/MseI+CAA). On the contrary, one primer combination (EcoRI+CAA/MseI+ACC) revealed the presence of few major bands with higher intensity than the other amplified fragments of the same reaction.

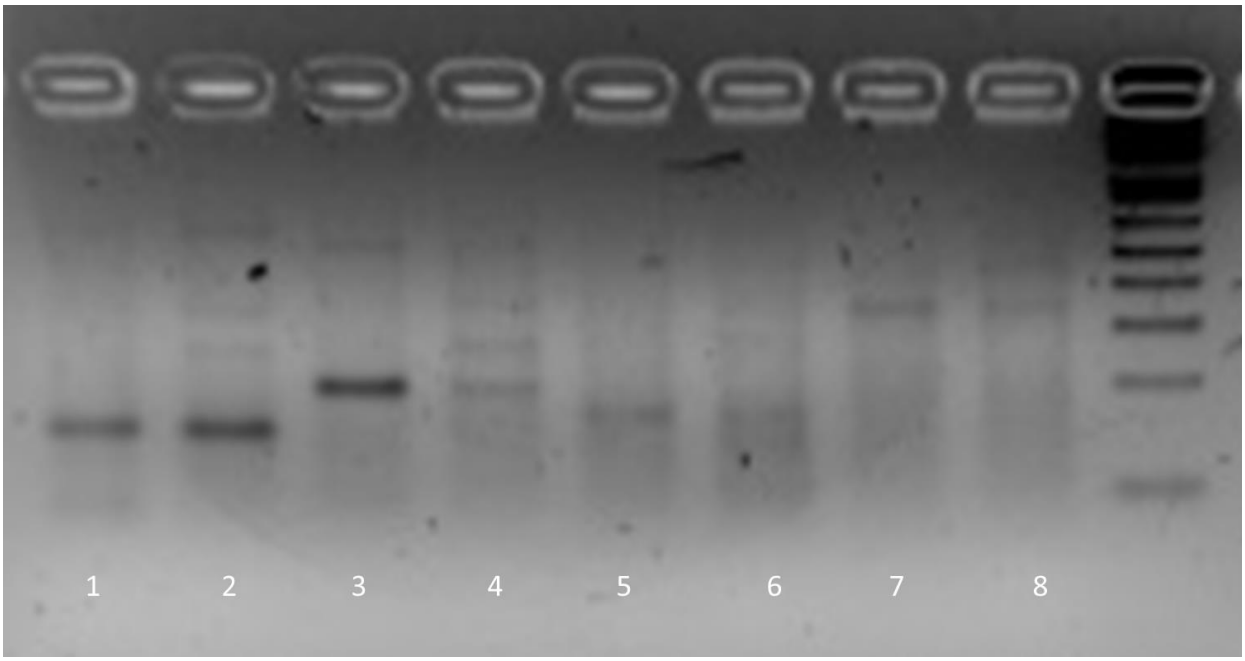


Figure 9. Agarose gel electrophoresis of final amplified products with 8 primer combinations (1: EcoRI+CA/MseI+ACC, 2: EcoRI+CAA/MseI+ACC, 3: EcoRI+CA/MseI+CAA, 4: EcoRI+CAA/MseI+CAA, 5: PstI+AG/MseI+ACC, 6: PstI+AGC/MseI+ACC, 7: PstI+AG/MseI+CAA, and 8: PstI+AGC/MseI+CAA).

More accurate investigations on number and quality of amplified fragments were done through capillary electrophoresis of PCR reactions. Graphical outputs of capillary electrophoresis runs are shown in Figures 10 (panels 1 and 2).

In general, from the comparison of results summarized in Figure 9 and Figure 10 we noticed a selective loss of amplified fragments longer than 400 bp. Likewise the previous preliminary experiments, capillary electrophoresis of amplified PCR products showed amplification profiles in which one or few bands had significant greater intensity than the average of the population of amplified fragments (Figures 9 and 10). The number of amplified fragments for each primer combination ranged from 50 to 400. The highest number of amplified fragments visible in our range of sizes was scored by the two primer combinations EcoRI+CAA/MseI+CAA (130 detectable peaks, Figure 10 panel 1) and PstI+AG/MseI+CAA (113 detectable peaks, Figure 10 panel 2).

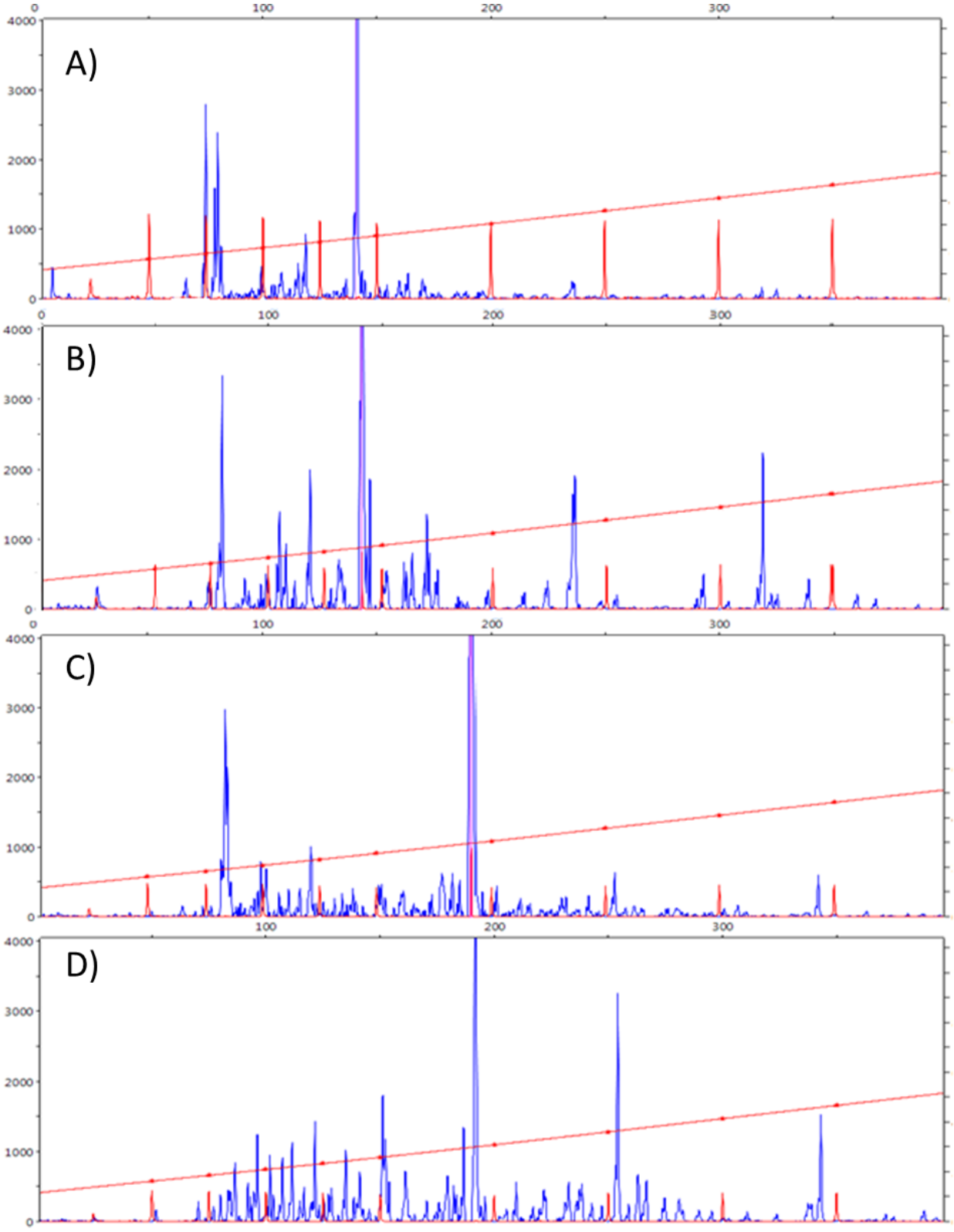


Figure 10, panel 1. Capillary electrophoresis of PCR products amplified with EcoRI/MseI primer combinations: A) EcoRI+CA/MseI+ACC, B) EcoRI+CAA/MseI+ACC, C) EcoRI+CA/MseI+CAA, D) EcoRI+CAA/MseI+CAA.

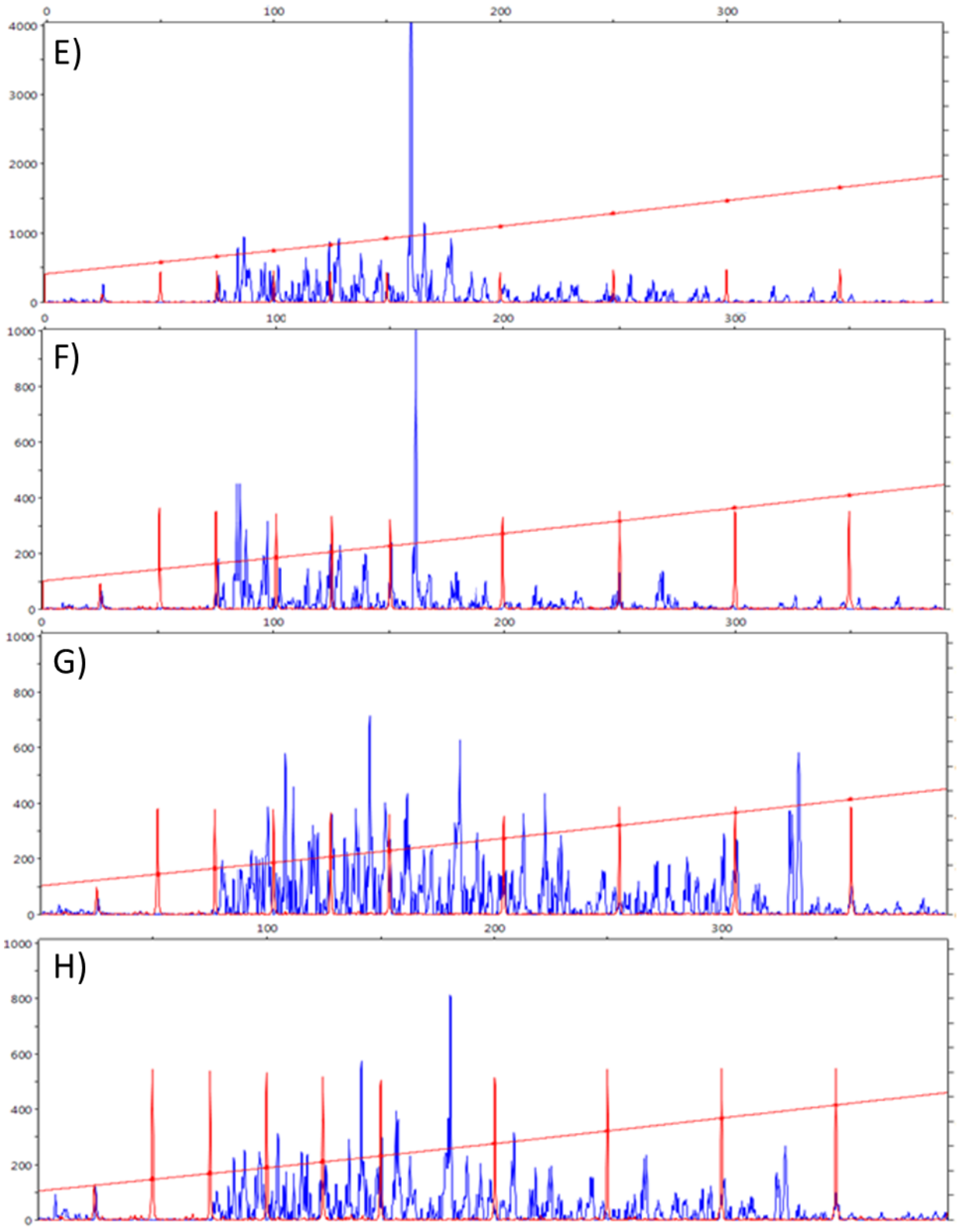


Figure 10, panel 2. Capillary electrophoresis of PCR products amplified with PstI/MseI: E) PstI+AG/MseI+ACC, F) PstI+AGC/MseI+ACC, G) PstI+AG/MseI+CAA, H) PstI+AGC/MseI+CAA.

Moreover, setting up AFLP experiments using biological e technical replicates it was possible to assess the reliability and reproducibility of the protocol, of the primer pairs and of the enzyme combinations chosen. The repeatability resulted to be higher than 97%.

3.2.2. Validation of the AFLP procedures on fennel commercial lines

Experimental conditions tested in the preliminary investigations were validated on a fennel collection of 240 genomic DNA samples. Particular attention was given to critical steps such as: i) protocol for anchor usage (protocol I, 1st variant); ii) restriction enzyme combinations (EcoRI/MseI); and iii) primer combinations to be used in the selective amplification step (EcoRI+CAA/MseI+CAA).

Capillary electrophoresis of amplification products was initially evaluated for their peak composition and size distribution. Only fragments with a size ranging from 60 bp and 300 bp were selected for subsequent analysis as this allowed a higher level of standardization the sample dataset. Finally, as many as 203 amplicons were scored from a single amplification procedure. A graphical representation of marker abundance and distribution among all analyzed samples is shown on Figure 11.

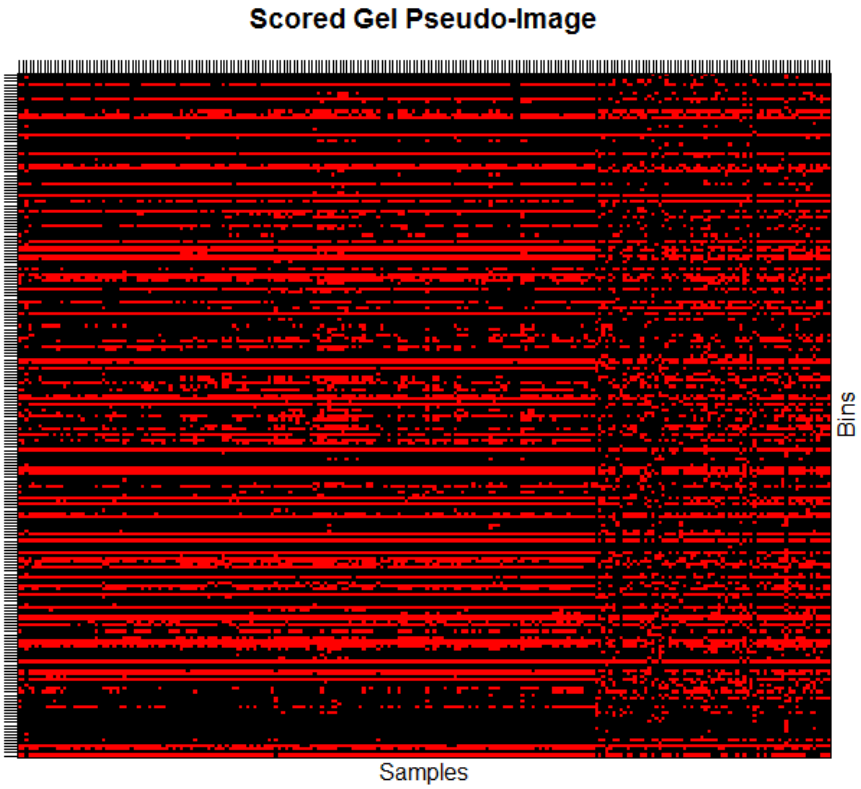


Figure 11. Virtual gel correlating AFLP markers (Bins) and DNA samples (Samples). Red bins indicate the presence of an amplified AFLP markers.

Transformation of raw data into binary data was done upon the application of specific threshold of fluorescence studied to minimize the possible influence of the background and maximize the efficiency of peak calling, which allowed the construction of binary data matrix like that reported as an example in Figure 12.

	FB5	FB50	FB51	FB52	FB53	FB54	FB55	FB56	FB57	...
299.49	0	0	1	0	0	0	1	0	0	
297.32	0	0	0	0	1	0	0	0	0	
295.76	0	0	0	0	0	0	0	0	0	
293.02	0	0	0	1	0	0	0	0	1	
290.46	1	0	1	0	0	0	1	1	0	
289.38	0	0	0	0	1	0	0	0	0	
285.75	0	0	0	0	0	0	0	0	0	
283.44	1	0	0	0	0	0	0	0	1	
281.6	0	0	0	0	0	0	0	0	0	
279.15	0	0	0	0	1	0	1	0	0	
277.94	0	1	0	0	0	0	0	0	0	
275.84	1	1	1	1	1	1	1	1	1	
271.56	0	0	0	0	0	0	1	0	0	
269.11	0	0	0	0	0	0	0	0	0	
267.73	1	0	1	1	0	0	1	1	1	
265.65	0	0	0	0	0	0	0	0	0	
263.84	1	0	0	1	1	1	0	1	0	
260.67	0	0	0	0	0	0	0	0	0	
...										

Figure 12. Example of output matrix by Rawgeno. Presence of a determined peak is defined as 1, whereas the absence of the corresponding peak is indicated as 0.

Descriptive statistics over all loci along with information on the amount of genetic diversity found across fennel accessions are reported in Table 9. The average number of polymorphic loci within each inbred line was as high as 53 (26%) and ranged from a minimum number of 17 (8%) to a maximum number of 152 (72%).

Table 9. Number of polymorphic loci (and %) within each inbred line; H = Nei's (1973) genetic diversity; I = Shannon's information index of phenotypic diversity (Lewontin, 1972).

Inbred lines	No. (%) polymorphic loci	H	I
MS1	33 (16%)	0,0577	0,0862
MS2	36 (18%)	0,0609	0,0915
MS3	32 (16%)	0,0550	0,0820
MS5	32 (16%)	0,0557	0,0825
MS6	134 (66%)	0,2042	0,3129
MS7	116 (57%)	0,1805	0,2758
MS9	152 (75%)	0,2079	0,3286
MS10	107 (53%)	0,1744	0,2633
Mant1	129 (64%)	0,1936	0,2983
Mant2	121 (60%)	0,2019	0,3054
Mant3	122 (60%)	0,1946	0,2944
Mant5	123 (61%)	0,2106	0,3137
Mant6	87 (43%)	0,1331	0,2032
Mant7	24 (12%)	0,0413	0,0613
Mant9	23 (11%)	0,0423	0,0624
Mant10	35 (17%)	0,0593	0,0892
MF23A	33 (16%)	0,0608	0,0902
MF23B	28 (14%)	0,0481	0,0724
MF15	28 (14%)	0,0514	0,076
MF162	52 (26%)	0,0861	0,1296
MF164	33 (16%)	0,0519	0,0788
MF165	49 (24%)	0,0865	0,1284
MF166	29 (14%)	0,0554	0,0814
MF742	59 (29%)	0,1022	0,1532
MF743	36 (18%)	0,0696	0,1022
MF13	37 (18%)	0,0737	0,1071
MF TR	33 (16%)	0,0621	0,0915
MF MN	36 (18%)	0,0598	0,091
MF FALSO MN	26 (13%)	0,0495	0,0731
MF SARNO	22 (11%)	0,0382	0,0572
MF WAN	32 (16%)	0,0574	0,0857
MF ROMA86	30 (15%)	0,0533	0,0796
MF ROMA 92	33 (16%)	0,0568	0,0856
MF ROMAGNA	17 (8%)	0,0331	0,0483
MF MA 98	34 (17%)	0,0646	0,0951
MF MA 99	24 (12%)	0,0439	0,0654
MF CH	28 (14%)	0,0524	0,0775
MF 163	23 (11%)	0,0419	0,0624

Nei's (1973) genetic diversity calculated within population varied from 0,033 to 0,210. A higher degree of genetic diversity was observed within maintainer lines (average H-index= 0,135) and male-sterile, seed parent lines (average H-index = 0,125). Conversely, genetic diversity calculated within male-fertile, pollen donor lines was on average as low as 0,060 (Table 9).

Genetic diversity estimates among populations in all possible pair-wise combinations, as calculated according to Nei's (1978), ranged from a minimum value of 0,020 (*e.g*. MS3 vs. MF163) and a maximum value of 0,280 (MS9 vs. MF CH, see Table 10).

Genetic diversity calculated among 22 male-fertile lines accessions was lower than 10%, varying from 0,010 (MF 23A vs. MF 23B) and 0,140 (MF MA 99 vs. MF 743) with an average estimate equal to 0,070. Among all possible male-sterile lines, genetic diversity values ranged from 0,020 (*e.g*. MS1 vs. MS2) and 0,230 (*e.g*. MS9 vs. MS5), with an average estimate of 0,100. When we considered the maintainer lines, genetic diversity ranged from 0,030 (Mant7 vs. Mant9) to 0,200 (Mant2 vs. Mant7), with an average estimate of 0,090. Genetic diversity among maintainers and male-sterile lines allowed a precise definition of diversity/similarity relationships existing between lines belonging to these two main groups (Table 10 and Figure 14). Higher values of genetic similarity (on average, S = 0,022) was observed among the male-sterile lines: MS6, MS7, MS9, MS10 and the maintainer lines: Mant1, Mant2, Mant3, Mant5 e Mant6. Furthermore, MS1, MS2 and MS3 male-sterile lines showed to be related to Mant9, as they scored an average value of genetic diversity as low as 0,024. Similarly, a very low level of genetic diversity (0,042) was found between the male-sterile MS5 and the maintainer line Mant7 (see Table 10 for additional details).

In general, the estimation of the genetic similarity coefficients (Dice, 1945) for single inbred lines underlined a high level of genetic uniformity within lines, as supported

by an average genetic similarity equal to 0,870. By contrast, four different male-sterile lines, namely MS6, MS7, MS9 and MS10, exhibited a degreed of genetic uniformity within population that was lower than 0,75 (see Figure 13 and Table 11). These data were also in agreement with the estimates of genetic diversity computed among individuals of each inbred line. In all mentioned cases, genetic diversity scored values higher than 0,17, with a maximum value of 0,21 (Table 10 and Table 11). Similarly, the extent of within population genetic uniformity, calculated as coefficient of genetic similarity, appeared to be lower than 0,80 (Figure 13) for maintainer lines Mant1, Mant2; Mant3, Mant5 and Mant6, whereas genetic diversity values were on average higher than 0,19 (Table 11).

Table 10. Genetic diversity statistics in accordance with Nei's (1978). For male-sterile inbred lines, genetic distances (below diagonal), genetic diversity estimates (on diagonal) e genetic identity estimates (above diagonal) are reported. For both maintainer lines and pollinator lines, genetic distance estimates with respect of male-sterile inbred lines are reported.

		Male-sterile lines							
	LINE	**MS1**	**MS2**	**MS3**	**MS5**	**MS6**	**MS7**	**MS9**	**MS10**
Male-sterile lines	**MS1**	0,0577	0,9849	0,9786	0,9763	0,8869	0,8521	0,8165	0,865
	MS2	0,0152	0,0609	0,9792	0,975	0,8898	0,8563	0,8213	0,8631
	MS3	0,0217	0,0211	0,0550	0,9681	0,9014	0,8709	0,8395	0,8742
	MS5	0,024	0,0253	0,0325	0,0557	0,8684	0,8309	0,7984	0,8435
	MS6	0,120	0,1167	0,1039	0,1411	0,2042	0,9745	0,9664	0,9437
	MS7	0,1601	0,1551	0,1382	0,1852	0,0259	0,1805	0,9801	0,9572
	MS9	0,2027	0,1969	0,175	0,2251	0,0342	0,0201	0,2079	0,9449
	MS10	0,1451	0,1473	0,1344	0,1702	0,058	0,0437	0,0567	0,1744
Maintainer lines	**Mant1**	0,1597	0,1619	0,1434	0,1796	0,0509	0,0328	0,037	0,0333
	Mant2	0,1863	0,1853	0,1689	0,2048	0,0544	0,0401	0,0356	0,0376
	Mant3	0,0871	0,0861	0,0764	0,0998	0,0453	0,0466	0,0687	0,0407
	Mant5	0,1324	0,1279	0,1145	0,1400	0,0567	0,0518	0,0645	0,0370
	Mant6	0,0643	0,0570	0,0526	0,0842	0,0579	0,0675	0,1002	0,0518
	Mant7	0,0451	0,0339	0,0342	0,0457	0,1412	0,1640	0,2151	0,1688
	Mant9	0,0354	0,0326	0,0162	0,0466	0,1114	0,1467	0,1824	0,1472
	Mant10	0,0499	0,0503	0,0498	0,0555	0,1244	0,1515	0,1883	0,1313
Pollinator lines	**MF23A**	0,0345	0,0295	0,0184	0,0499	0,1042	0,1435	0,1757	0,1453
	MF23B	0,0313	0,0293	0,0152	0,0454	0,1145	0,1531	0,1895	0,1515
	MF15	0,0657	0,0514	0,0472	0,0594	0,1400	0,1663	0,2044	0,1414
	MF162	0,0493	0,0522	0,0449	0,0675	0,1107	0,1372	0,1695	0,1068
	MF164	0,0380	0,0326	0,0342	0,0335	0,1344	0,1810	0,2199	0,1677
	MF165	0,0796	0,0636	0,0665	0,0636	0,1598	0,1908	0,2251	0,1621
	MF166	0,0652	0,0542	0,0543	0,0511	0,1538	0,1937	0,2319	0,1895
	MF742	0,0872	0,0740	0,0825	0,0940	0,1642	0,1830	0,2263	0,1408
	MF743	0,1163	0,0976	0,1105	0,1092	0,1979	0,2296	0,2665	0,1681
	MF13	0,0906	0,0738	0,0800	0,0796	0,1731	0,1976	0,2349	0,1627
	MF TR	0,0401	0,0395	0,0395	0,0400	0,1468	0,1723	0,214	0,1441
	MF MN	0,0582	0,0500	0,0437	0,0816	0,0891	0,1183	0,1497	0,1107
	MF FALSO MN	0,0773	0,0772	0,0818	0,0690	0,1774	0,2176	0,2459	0,1828
	MF SARNO	0,0425	0,0319	0,0295	0,0457	0,1291	0,1624	0,2055	0,1531
	MF WAN	0,0598	0,0575	0,0585	0,0625	0,1651	0,2042	0,237	0,1853
	MF ROMA86	0,0304	0,028	0,0246	0,0353	0,1182	0,1647	0,2033	0,1605
	MF ROMA 92	0,0333	0,0342	0,0432	0,0340	0,1540	0,1942	0,2336	0,1772
	MF ROMAGNA	0,0340	0,0197	0,0248	0,0336	0,1294	0,1596	0,202	0,1560
	MF MA 98	0,0622	0,0572	0,0597	0,0519	0,1603	0,1927	0,2265	0,1727
	MF MA 99	0,0536	0,0589	0,0377	0,0713	0,1096	0,1312	0,1665	0,1363
	MF CH	0,0794	0,0746	0,0838	0,0612	0,1998	0,236	0,2764	0,2164
	MF 163	0,0296	0,0253	0,0225	0,042	0,1196	0,1648	0,2037	0,1545

Table 11. Within line estimates of genetic similarity (Dice, 1945) and genetic diversity (Nei, 1978), as measures of genetic uniformity and genetic stability.

Llines	MS1	MS2	MS3	MS5	MS6	MS7	MS9	MS10	Mant1	Mant2
Genetic stability (Dice, 1945)	0,9165	0,9180	0,9221	0,9386	0,6032	0,6104	0,5359	0,7441	0,6758	0,6749
Genetic diversity (Nei, 1978)	0.0577	0.0609	0.0550	0.0557	0.2042	0.1805	0.2079	0.1744	0.1936	0.2019
St.dev.	0,0508	0,0501	0,0494	0,0387	0,2726	0,2896	0,2764	0,1876	0,1951	0,2684
Lines	**Mant3**	**Mant5**	**Mant6**	**Mant7**	**Mant9**	**Mant10**	**MF23 A**	**MF23 B**	**MF15**	**MF162**
Genetic similarity (Dice, 1945)	0,7332	0,7233	0,8027	0,9469	0,9432	0,9346	0,9073	0,9266	0,9405	0,8886
Genetic diversity (Nei, 1978)	0.1946	0.2106	0.1331	0.0413	0.0423	0.0593	0.0608	0.0481	0.0514	0.0861
St.dev.	0,2151	0,2117	0,1474	0,0391	0,0393	0,0596	0,0609	0,0494	0,0385	0,0739
Lines	**MF164**	**MF165**	**MF166**	**MF742**	**MF743**	**MF13**	**MF TR**	**MF MN**	**MF FALSO MN**	**MF SARNO**
Genetic similarity (Dice, 1945)	0,9311	0,9100	0,9330	0,8912	0,9272	0,9265	0,9282	0,8996	0,9458	0,9481
Genetic diversity (Nei, 1978)	0.0519	0.0865	0.0554	0.1022	0.0696	0.0737	0.0621	0.0598	0.0495	0.0382
St.dev.	0,0408	0,0682	0,0611	0,0895	0,0714	0,0682	0,0708	0,1023	0,0665	0,0404
Lines	**MF WAN**	**MF ROMA86**	**MF ROMA 92**	**MF ROMAGNA**	**MF MA 98**	**MF MA 99**	**MF CH**	**MF 163**		
Genetic similarity (Dice, 1945)	0,9322	0,9271	0,9298	0,9579	0,9257	0,9253	0,9414	0,9389		
Genetic diversity (Nei, 1978)	0.0574	0.0533	0.0568	0.0331	0.0646	0.0439	0.0524	0.0419		
St.dev.	0,0618	0,0694	0,0645	0,0412	0,0678	0,0716	0,0523	0,0546		

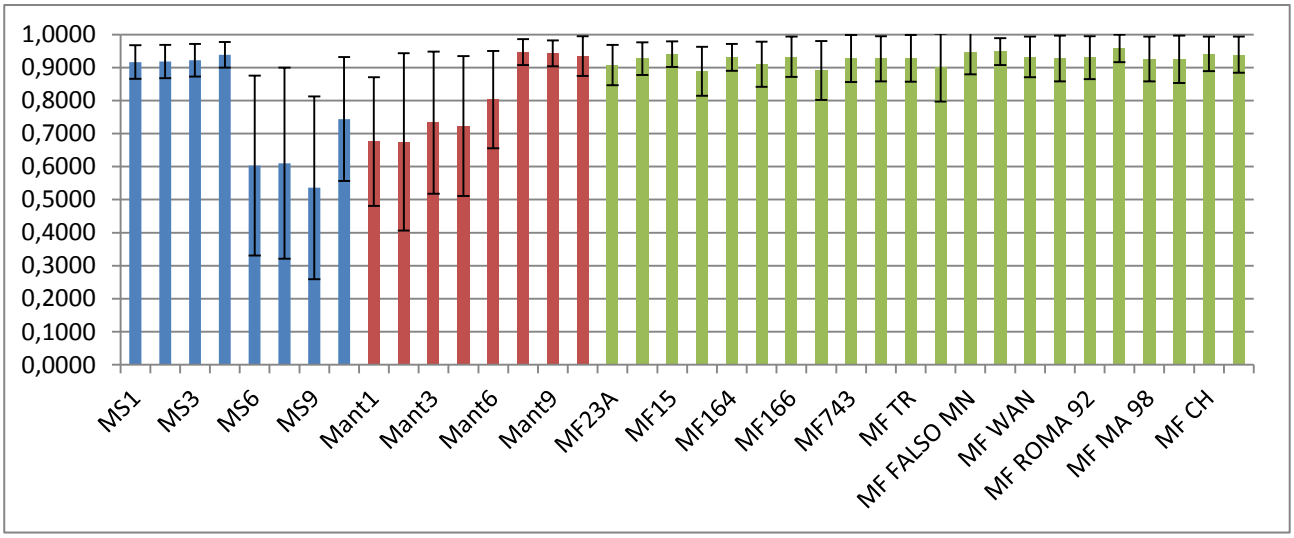


Figure 13. Genetic uniformity within inbred line (● Male sterile lines; ● Maintainer lines; ● Male fertile lines).

The genetic diversity existing among fennel lines was displayed by constructing an UPGMA dendrogram. As shown in Figure 14, all analyzed lines were grouped in two main clusters. A first cluster included male-sterile lines MS6, MS7, MS9 and MS10 and maintainer lines Mant1, Mant2, Mant3, Mant5 and Mant6, whereas the remaining accessions, that is male-sterile lines MS1, MS2, MS3 and MS5, and maintainer lines Mant7, Mant9 and Mant10, were grouped in a second distinct cluster (Figure 14).

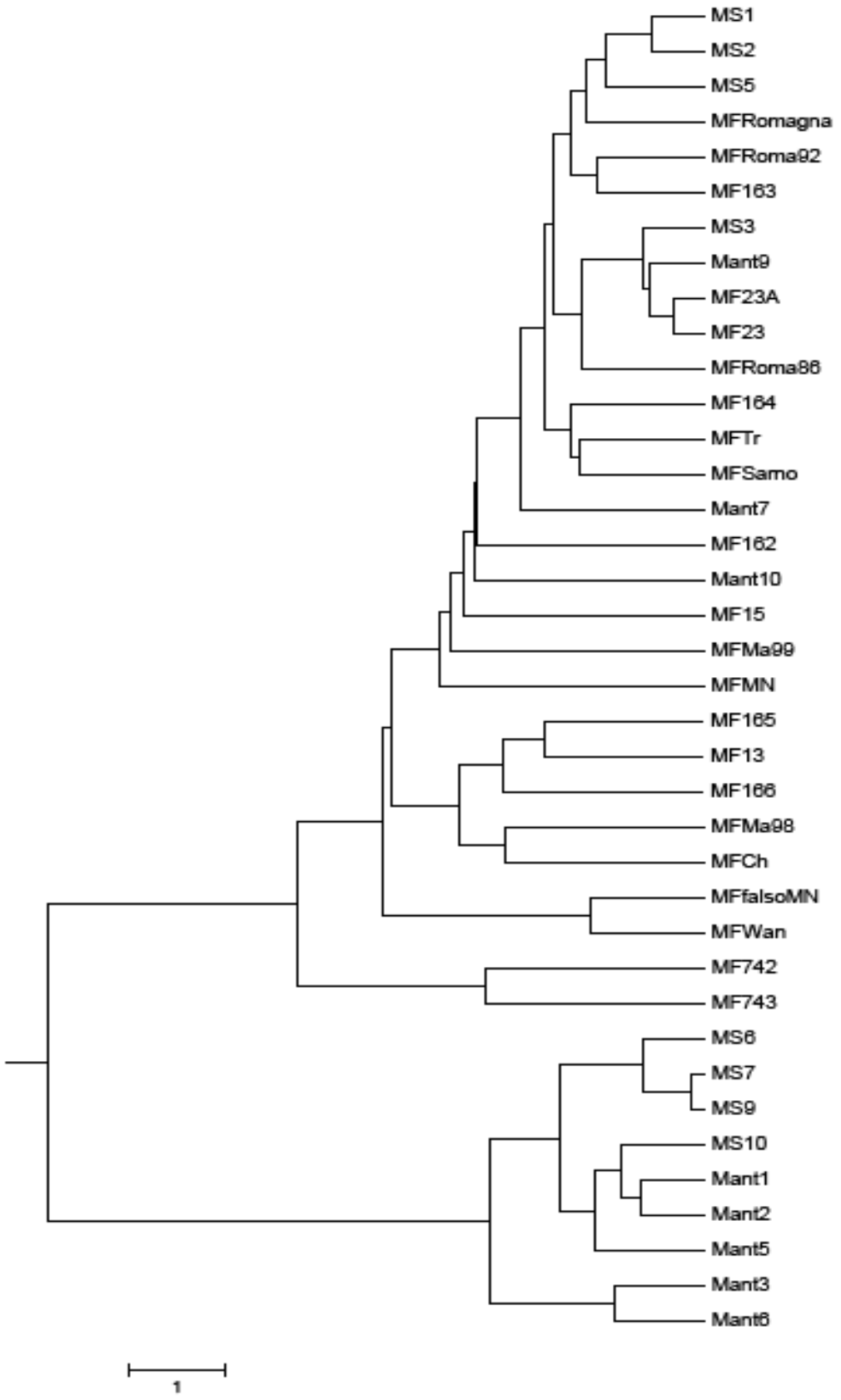


Figure 14. UPGMA dendrogram including all fennel lines based on genetic distance estimates.

4. DISCUSSION

4.1. Development of SSR markers

The use of heterologous primers for amplifying and cloning microsatellites in fennel proved to be applicable at the methodological level and successful in practice, allowing to explore genome information of not-yet explored species starting from well-known phylogenetically related species. In this study, some molecular data regarding SSR motifs already available for *Daucus carota* and *Apium graveolens* were used to identify informative, polymorphic and discriminant microsatellite regions in *Foeniculum vulgare.* In fact, as reported by Plunkett and Downie (1999), these three species belong to the Apiaceae family and are strictly related. Twelve pairs of heterologous primers (seven from *Apium graveolens* and five from *Daucus carota)* were tested on fennel genomic DNA samples and the sub-cloning of the amplification product, followed by a Sanger sequencing, enabled to identify four SSR motifs. Then it was possible to design specific primers (homologous primer pairs) to selectively amplify the four target genomic loci in *Foeniculum vulgare*. Preliminary tests performed using a core collection of fennel genomic DNA samples, in order to verify the specificity of the new-designed primers, provided excellent results, especially for the FvSSR2, FvSSR3 and FvSSR4. In fact, as already shown in triplicated experiments, they were able to amplify univocally the specific genomic region containing the target microsatellite motif. Otherwise the primer pair for FvSSR1 proved to amplify one or two genomic regions depending on the DNA sample used as template. This result remained unchanged also adopting more stringent PCR conditions, in terms of annealing temperatures, and decreasing $MgCl_2$ concentration. For this locus, a new primer pair was designed but it revealed similar results. A possible explanation is that fennel genome may be characterized by duplicated regions and that one of these two target microsatellites has undergone an insertion/deletion of about 200 bp in length between the two primer binding

sites. In fact, in both cases the PCR amplification, performed in replicated experiments using the two different primer pairs designed on the same SSR region (fvSSR1), yielded two distinct bands of different length, in which the strong and the faint PCR products always differed each other of about 200 bp. Further investigations are therefore necessary for understanding the genetic origin of this finding. Probably, the sub-cloning and sequencing of all amplicons obtained by using each of the two primer pairs designed for fvSSR1 will help us to comprehend which kind of insertion/deletion eventually occurred. Moreover, the analysis of segregating populations is likely crucial to understand if these marker alleles belong to duplicated genomic loci.

Currently we are using the fluorescent-labeled primers combined with the subsequent capillary electrophoresis to get essential data about the polymorphism information content of each of the four SSR markers. Finally the four SSR regions discovered will be validated by genotyping the 240 fennel genomic DNA samples already characterized by using the M13-tailed AFLP fingerprinting analysis.

4.2. Development of AFLP markers

4.2.1. Optimization of the M13-tailed AFLP methodology

Amplified Fragment Length Polymorphism (AFLP) is a well-established molecular marker technique suitable for DNA fingerprinting and exploitable also for studies of population genetics. This methodology has significant advantages over other procedures because genetic variability can be assessed at a number of independent loci that is much bigger than that usually obtained using co-dominant SSR or SNP markers. However, a major gap remains the use of labeled primers, which is one of the aspects that most affect the cost of the procedure. In this research we describe a new and robust AFLP-based technology, named M13-tailed AFLP, derived from the

use of different non-labeled primers with a 5'-tail complementary to a unique labeled M13 primer. The tuning of this new technology suitable for multilocus DNA fingerprinting has started by testing two different protocols, each one executed using two variants. As a first step, it was necessary to understand the reproducibility of each experiment using restriction enzymes purchased from different companies and working at different conditions. The endonucleases working at different temperatures (*i.e*. *Eco*RI and *Pst*I working at 37°C, and *Mse*I working at 65°C, purchased from Thermo Scientific) and those working at the same temperature (they were all working at 37°C, purchased from New England Biolabs) showed both high reproducibility and similar restriction efficiency. Furthermore, comparing the two PCR protocols, we documented the production of genomic profiles having the same number of peaks, in the same positions and with the same intensities. These findings suggested that the brand and the working conditions of the restriction enzymes used in our experiments were not affecting the genomic fingerprinting results. As a consequence, the choice of the enzymes to be used remains purely arbitrary and mostly depends on economical requirements.

The three primers system adopted in amplification reactions (5'-tailed EcoRI+2/3 or 5'-tailed PstI+2/3, MseI+3 and fluorescently-labeled M13 primer, as 1st variant) showed a higher efficiency, greater repeatability and less variability than the second variant. Moreover, the number of amplified fragments was higher in the first variant as well as the number of false positives was smaller compare to the second variant. On the whole, all these observations and evaluations were considered sufficient to justify the choice of the first variant of the AFLP protocol to be adopted for subsequent genomic analyses.

Finally for the choice of the best primer and enzyme combinations, two main aspects were evaluated: the highest, discriminable and more intense number of peaks obtained and the highest number of polymorphic markers scored across

genomic DNA samples of fennel. As reported in the results section, the combination EcoRI/MseI answered to the specific requirements mentioned above, even if we would have expected to observe a highest number of peaks using PstI, owing to its sensitivity to methylation, as happens in other plant species. All the primer combinations with two selective bases produced profiles with too many peaks that makes difficult to discriminate among each other. As a consequence, this finding made obligatory the choice of both primers with three selective bases, such as EcoRI+CAA/MseI+CAA.

After the choice of the protocol variant for AFLP analysis, the enzyme and primer combinations were tested in order to assess the reliability and reproducibility of AFLP fingerprints setting up experiments by using biological e technical replicates. The repeatability resulted to be higher than 97% confirming the robustness of the method.

4.2.2. Validation of the AFLP procedures

DNA fingerprinting analysis through the “M13-tailed AFLP” molecular markers was executed in order to generate genetic-molecular profiles of the inbred lines of fennel. This characterization enabled to define the genetic similarity and stability within inbred lines and the genetic diversity among inbred lines, and then to determine the best parental lines and cross combinations. From the collected data it was clear that the genetic uniformity and stability of each population is very high, being plant individuals that belong to the same line genetically similar. Some of the fennel accessions pertaining to the pollinator group and to the maintainer group scored genetic similarity estimates lower than the average value. In particular, MS6, MS7, MS9 and MS10 as well as Mant1, Mant2, Mant3, Mant5 and Mant6 revealed a within line genetic similarity coefficients lower than 80%, thus suggesting the need to proceed with additional inbreeding steps (by means of full-sibling and selfing for male-sterile lines and maintainers, respectively).

Observing the genetic relationships of the experimental population as a whole, inbreds MS1, MS2 MS3 and Mant7, Mant9 and Mant10 proved to be genetically very related to some male-fertile lines, with genetic similarity values greater than 97%. This finding was not expected because each male-sterile line (*e.g*. MS1) would have to be genetically similar to its maintainer line (*e.g*. Mant1). On the contrary each couple of male-sterile and male-fertile lines would have to be genetically divergent each other in order to maximize heterozygosity in their F1 progeny. Some maintainers and pollinators resulted genetically related. It is possible that the reason why it happens is that the first ones derive from the second ones or vice versa. At the same time it is not clear why some male-sterile lines and their maintainers are not as closely related as wanted (*e.g*. MS1 and Mant1). These results are of particular interest and require careful considerations and additional investigations in order to select the appropriate inbred lines to be used in the breeding programs. It is worth mentioning that some of the male-sterile lines (*i.e*. MS6, MS7, MS9 and MS10) revealed very high similarity levels with some maintainer lines (*i.e*. Mant1, Mant2, Mant3, Mant5 and Mant6), showing estimates higher than 97% between two groups, and also a quite high genetic distance with the other male-fertile lines or pollinators, that is higher than 0,1.

In conclusion, this research describes the development and assessment of molecular markers suitable for fingerprinting and genotyping accessions of fennel that will have great applied utility for marker-assisted breeding programs aimed at the characterization and selection of parental inbred lines, and the constitution of new commercial F1 hybrids in this species.

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