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Molecular Profiling for Genetic Variability in *Petroselinum crispum* Based on ISSR and RAPD Markers

Heba M. M. Ibrahim[1] , Omnia F. Abou El-Leel[2] and Khalad A. Emam[3]

[1]*Genetics Department, Faculty of Agriculture, Cairo University, Egypt.*
[2]*Medicinal and Aromatic Plants Department, Hort. Res. Inst., ARC, Giza, Egypt.*
[3]*Botanical Gardens Department, Hort. Res. Inst., ARC, Giza, Egypt.*

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ABSTRACT

The genetic variability and relationships between two different subspecies of parsley (*Petroselinum crispum*) growing in Egypt, the plain leaf type (ssp. *neapolitanum,* Danert) and the curly leaf type (ssp. *crispum),* were analyzed using 5 random amplified polymorphic DNA (RAPD) and 5 inter simple sequence repeat (ISSR) markers. RAPD markers were more efficient than the ISSR assay regarding polymorphism detection in the examined parsley samples, where the highest polymorphism percentage detected by RAPD was 84.62%, while the highest recorded for the ISSR marker was 58%. The number of amplified fragments of genomic DNA of the 2 subspecies, using RAPD analysis, yielded 76 fragments, 49 of which were polymorphic. The size of specific amplicon ranged from 246 to 5685 bp. The 5 ISSR primers produced 60 bands, 26 of which were polymorphic and the size of specific amplicons ranged from 157 to 807 bp. The polymorphic amplicons generated by RAPD and ISSR markers utilized could be useful for examining the genetic diversity and phylogenies of different *Peroselinum crispum* genotypes, which may be applied to identify elite lines and utilized in parsley improvement and genetic relationship assessment.

Key words: Parsley, Petroselinum crispum, Subspecies, Polymorphism, Genetic relationship, ISSR, RAPD.

Introduction

Parsley (*Petroselinum crispum* (Mill) Nym), a member of the Apiaceae (Umbelliferae), is a biennial herb native to Europe and Western Asia. The three main types (subspecies) of parsley are the plain leaf type (ssp. *neapolitanum*, Danert), and the curly leaf type (ssp. *crispum*), which are cultivated for their foliage, and the turnip-rooted or 'Hamburg' type (ssp. *tuberosum* (Bernh.) Crov) primarily grown for its roots, In addition to their use as fresh or dried herb, parsley leaves and seeds contain essential oils that can be used in perfumes, creams and soaps (Sabry *et al.*, 2013). Moreover, parsley possesses medicinal properties, first-mentioned by the ancient Greeks (Simon, 1990). It is used as a carminative, diuretic, hypertensive, hypotensive, stomachic, nervine, immunologic, abortifacient and nutritive agent (Robbers & Tyler, 1999; Kreydiyyeh & Usta, 2002). Parsley, like many other herbs, is highly seasonal in nature. It is a very rich source of vitamins C and E, b-carotene, thiamin, riboflavin and organic minerals (Bakowski & Michalik, 1986; Wills *et a*l., 1986; Michalik & Dobrzan´ ski, 1987; Athar *et al*., 1999). Parsley essential oil of various extracts prepared from different vegetative organs has *in vitro* antioxidant effect and plays a significant role in the scavenging effects (Fejes *et al*.; 1998; Wong and Kitts, 2006). The distinctive flavor of parsley comes from p-1,3,8-menthatriene, the dominant volatile oil in the leaves (López *et al*., 1999). Monoterpene hydrocarbons, such as β-phellandrene, 1,3,8-p-menthatriene, p-α-dimethylstyrene and terpinolene, are considered to be primarily responsible for the characteristic aroma of fresh parsley (Freeman *et al*., 1975 and Kasting *et a*l., 1972). Apiole (Macleod *et al*., 1985), myristicin and myrcene have also been identified as major aroma constituents (Simon and Quinn, 1988).

There are two different subspecies (ssp..) of parsley widely grown in Egypt (Abdel-Moemin, 2014); the plain leaf type (ssp.. *neapolitanum,* Danert) and the curly leaf type (ssp.. *crispum*) (Savage

Corresponding Author: Heba M. M. Ibrahim, Genetics Department, Faculty of Agriculture, Cairo, University, Egypt.
E-mail: heba.ibrahim@staff.cu.edu.eg

and Vanhanen, 2015). The term "subspecies" is used as a recognition of the distinctiveness distribution of organisms geographically, in contrast to the term "variety", which is a taxonomic rank below "subspecies" and is seen throughout the natural range of the geographic distribution of the species or subspecies (Clausen, 1941) and is the result of selection and breeding.

There are different molecular markers that are highly informative and provide more discriminating information and good reproducibility such as AFLP and RFLP, however, these techniques are more expensive, require more amount of DNA, and need more effort. RAPD and ISSR analysis are considered as the simplest, fastest, and has the ability to detect polymorphisms extensively, in addition to good-suited method for DNA fingerprinting (Costa *et al*., 2016)
Techniques based on analysis of DNA polymorphism such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers are widely applied to identify phylogenetic variability between plants (Williams *et al.* 1990; Zietkiewicz *et al.* 199, respectively). These techniques were applied in numerous studies, e. g. Gaber *et al*. (2007); Domblides *et al*. (2010); Khanam *et al*. (2012); Salama *et al*. (2016).

There are several comparative studies among different *Petroselinum crispum* varieties based on essential oil composition, antioxidant effect, and medical effect (Fejes *et al*., 1998; Wong and Kitts, 2006; Sabry *et al*., 2014; Soliman *et al*., 2015; Maodaa *et al*., 2016). However, despite the usefulness and importance of parsley as a medicinal plant and their medical application, molecular genetic studies on parsley to detect the genetic relationship among different genotypes are relatively few (Maxim *et al.,* 2009). To our knowledge, the only study of the genetic diversity via DNA profiling using ISSR and RAPD employed 32 random samples of the garden parsley, *Petroselinum crispum* (Mill.) Nym. ex A.W. Hill, grown in Mosco (Domblides *et al.,* 2010).

The aim of this investigation was to assess the genetic diversity and similarity of two ssp.. of parsley grown in Egypt, namely ssp.. *Neapolitanum* and ssp. *crispum*, utilizing RAPD and ISSR techniques.

Materials and Methods

1-Plant materials:

Two subspecies of parsley, the plain leaf type (ssp.. *neapolitanum*, Danert) and the curly leaf type (ssp. *crispum*) were used in the present study. Seedlings of the two subspecies of parsley were obtained from the Medicinal and Aromatic Plants Section at El-Kanater El-Khairia, Kalubia Governorate, Horticulture Research Institute, Agricultural Research Center (A.R.C), Egypt, and planted at the Experimental Farm of Faculty of Agriculture, Cairo University, Giza, Egypt in October, 2014. The seedlings were 10 – 15 cm in length, with 5 – 8 leaves and were planted in a randomized complete block design with three replicates.

2- Random Amplified Polymorphic DNA (RAPD-PCR) Analysis:

Total genomic DNA was extracted from fresh leaves using cetyltrimethyl ammonium bromide (CTAB) method by Richards (1997). Five random DNA oligonucleotide primers (Eurofins Genomics, Ebersberg, Germany) listed in Table (1) were independently used in the PCR reaction according to Williams *et al*. (1990). The PCR amplification was performed in a 25 µl reaction volume containing 2.5 µl of dNTPs (2.5 mM), 1.5 µl of Mg Cl_2 (25 mM), 2.5 µl of 10 x buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50 ng/µl), 0.3 µl of Taq polymerase (5 U/µl) and 14.7 µl of sterile ddH_2O. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Techni TC-512 PCR System (Thermo Fisher Scientific, Dublin, Ireland). The reaction mixtures were subjected to one cycle at 95 ºC for 5 minutes, followed by 35 cycles at 96 ºC for 30 seconds, 37 ºC for 30 seconds, and 72 ºC for 30 seconds, then a final cycle of 72 ºC for 5 minutes. PCR products were electrophoresed at 100 V for one hour in 1.5 % agarose gels to detect polymorphism between the two parsley varieties under study and fragments sizes were estimated with the 100bp. ladder marker. Only five primers succeeded to generate reproducible polymorphic DNA products. Table (1) lists the sequences of these DNA primers.

3- Inter Simple Sequence Repeat (ISSR-PCR) Analysis:

ISSR-PCR reactions utilized five randomly selected primers (Eurofins Genomics, Ebersberg, Germany) listed in Table (1). Amplifications were conducted in 25 µl reaction volume containing the following reagents: 2.5 µl of dNTPs (2.5 mM), 2.5 µl MgCl2 (2.5 mM), and 2.5 µl of 10 x buffer, 3.0 µl of Primer (10 pmol), 3.0 µl of template DNA (25 ng/µl), 1 µl of Taq polymerase (1U/ µl) and 12.5 µl of sterile dd H2O. The PCRs were one cycle at 94 ºC for 4 min. followed by 45 cycles of 1 min. at 94 ºC, 1 min. at 57 ºC, and 2 min at 72 ºC. The reaction was finally stored at 72 ºC for 10 min. The PCR products were separated in a 1.5 % agarose gels and fragments sizes estimated with the 100bp. ladder marker (Thermo Fisher Scientific, CA, USA).

Table 1: RAPD and ISSR primers sequences used in the DNA fingerprint analysis.

ISSR Primer		RAPD Primer	
Primer Name	Sequence	Primer Name	Sequence
HB-8	5`GAG AGA GAG AGA GG 3`	OP-A01	5´ CAGGCCCTTC 3´
HB-9	5` GTG TGT GTG TGT GC 3`	OP-A07	5´ GAAACGGGTG 3´
HB-10	5` GAG AGA GAG AGA CC 3`	OP-B07	5´ GGTGACGCAG 3´
HB-11	5` GTG TGT GTG TGT TGT CC 3`	OP-B11	5` GTAGACCCGT 3`
HB-12	5` CAC CAC CAC GC 3`	OP-C12	5` TGTCATCCCC 3`

4- Statistical analysis:

The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA molecular weight markers. The bands scored from DNA profiles generated by each primer were tabulated and the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively). Calculation was achieved using Dice similarity coefficients (Dice, 1945) as implemented in the computer program SPSS-10.

Results and Discussion

Randomly amplified polymorphic DNA (RAPD) markers:

The five decamer arbitrary RAPD primers amplified DNA fragments for the two parsley subspecies (Table 2 and Fig. 1). Polymorphism levels differed from one primer to another. OP-A07 and OP-B11 primers exhibited low level of polymorphism (50% and 58.82%, respectively). However, OP-A01 and OP-B07 primers exhibited moderate levels of polymorphism (64.29% and 66.67%, respectively). OP-C12 primer exhibited high level of polymorphism (84.62%). The number of total amplified fragments (TAF), polymorphic fragments (PF), monomorphic fragments (MF) and specific markers (SM) for each sample using the five primers are shown in Table (2). OP-A01 primer produced fourteen fragments with molecular size ranging from 246 to 2708 bp.. (Fig.1; Table 2) with 64.29% polymorphism, nine of which were species-specific markers with 246, 1121, 1635, 2100, 2708 bp.. for ssp.. *crispum* and 269, 604, 1500, 2071 bp. for ssp.. *neapolitanum*, Danert), where other five fragments were common in the two subspecies. OP-A07 primer resulted in fourteen DNA fragments with molecular size ranging from 279 to 3464 bp.. with polymorphic percentage (50.00%) in which seven of them were species-specific with 807 bp. for ssp.. *crispum* and 279, 401, 1082, 1490, 1646, 3080 bp. for ssp.. *neapolitanum* (Danert and the other seven fragments were common t in both genotypes. OP-B07 primer produced eighteen DNA fragments with molecular size ranging from 314

to 3397 bp. with polymorphic percentage (66.67 %), and twelve of them were species-specific with 694, 925, 1123, 1261, 1409, 1518, 2070, 2903 bp. for ssp.. *crispum* and 405, 555, 858, 1048 bp. for ssp.. *neapolitanum* (Danert), while the other six fragments were common in both genotypes. OP-B11 primer amplified seventeen DNA fragments with molecular size ranging from 416 to 5685 bp. with polymorphic (58.82%) fragments and ten of them were species-specific markers at 437, 1969, 2374 bp. for ssp.. *crispum* and 416, 471, 1073, 1897, 2231, 4093, 5685 for ssp.. *Neapolitanum* (Danert) and the other seven fragments were common in both genotypes. OP-C12 primer amplified thirteen DNA fragments with molecular size ranging from 367 to 3506 bp. with polymorphic percentage (84.62 %) in which eleven of them were species-specific markers with 690, 776, 2238, 2896, 3506 bp. for ssp.. *crispum* and 367, 481, 646,729, 871, 1156 bp. for ssp.. *neapolitanum* (Danert), while the other two fragments were common in both genotypes.

Table 2: Species-specific RAPD and ISSR markers for two parsley varieties genotypes. TAF = Total Amplified Fragments, MF= Monomorphic Fragments, PF= Polymorphic Fragments, SM= Specific Markers, bp.= Base Pair.

Primers code	Range of M.S.	TAF	MF	PF	SM (bp.)		Polymorphism (%)
RAPD primers					ssp.. *crispum*	ssp.. *neapolitanum*	
OP-A01	246-2708	14	5	9	246, 1121, 1635, 2100, 2708	269, 604, 1500, 2071	**64.29**
OP-A07	279-3464	14	7	7	807	279, 401, 1082, 1490, 1646, 3080	**50.00**
OP-B07	314-3397	18	6	12	694, 925, 1123, 1261, 1409, 1518, 2070, 2903	405, 555, 858, 1048	**66.67**
OP-B11	416-5685	17	7	10	437, 1969, 2374	416, 471, 1073, 1897, 2231, 4093, 5685	**58.82**
OP-C12	367-3506	13	2	11	690, 776, 2238, 2896, 3506	367, 481, 646, 729, 871, 1156	**84.62**
Total RAPD		**76**	**27**	**49**			
ISSR primers							
HB-08	157-682	12	6	6	157, 345, 682	276, 391, 5282	**50.00**
HB-09	127-807	15	8	7	237, 308, 418, 635, 807	127, 222	**46.67**
HB-10	128-581	10	7	3	317, 472, 581	-------	**30.00**
HB-11	252-585	11	8	3	814	302, 825	**27.27**
HB-12	174-804	12	5	7	316, 396, 438	311, 451, 582, 804	**58.33**
Total ISSR		**60**	**34**	**26**			
Total		**136**	**61**	**75**			

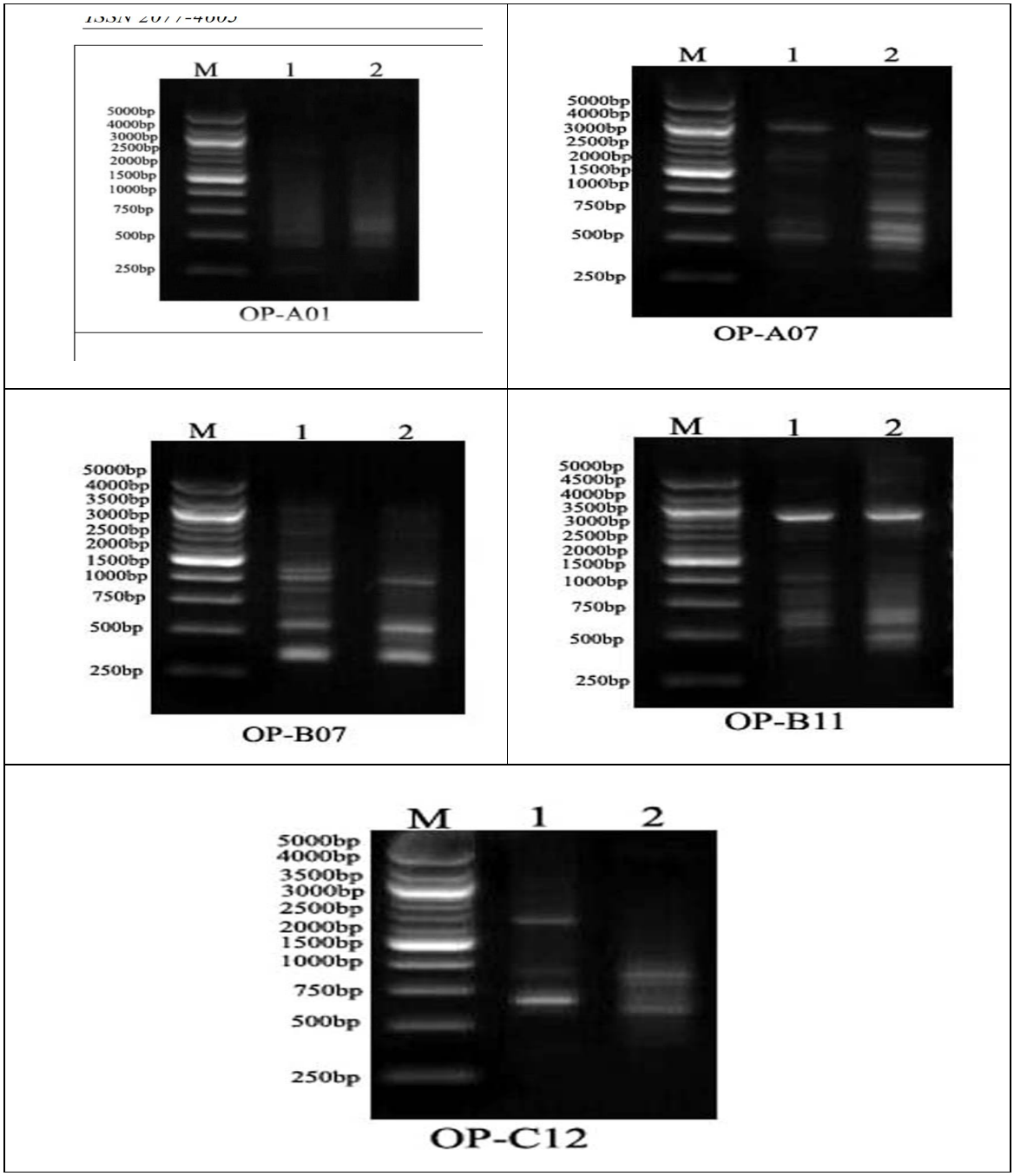

Fig. 1: RAPD-PCR analysis of two parsley varieties genotypes (second season) 1- The curly leaf type (ssp.. *crispum*), 2- The plain leaf type (ssp. *neapolitanum*, Danert). M- Molecular weight marker (100 bp.).

Inter Simple Sequence Repeats (ISSRs) markers:

The five ISSR primers succeeded in amplifying DNA fragments for the two parsley subspecies (Fig. 2; Table 2). Polymorphism levels differed from one primer to another, i.e. HB-12 primer recorded the highest level of polymorphism (58.33%), while, HB-08, HB-09 and HB-10 primer exhibited moderate level of polymorphism (50.00, 46.67 and 30.00 %) respectively and the lowest level of polymorphism (27.27%) represented by HB-11 as shown in Table 2. HB-08 Primer showed 12 DNA fragments with molecular size ranging from 157 to 682 bp. with 50% polymorphism,

and six of them were positive species- specific markers at 157, 345, and 682 bp. for *ssp.. crispum* and 276, 391, and 5282 bp. for *ssp. neapolitanum.* The other six DNA fragments were common and existed in all samples. HB-09 primer showed fifteen DNA fragments with molecular weight sizes ranged from 127 to 807 bp., with polymorphism of 46.67%; seven of them were positive species-specific markers (237, 308, 418, 635, and 807 bp.) for *ssp. crispum*, and two fragments (127, 222 bp.) for ssp. *neapolitanum*. The other eight DNA fragments were presented in all samples. While, HB-10 primer showed ten DNA fragments with molecular size ranging from 128 to 581bp. with polymorphism of 30.00 %. Three of these bands were positive species- specific markers (317, 472, 581bp.) for ssp. *crispum* and another seven DNA fragments were presented in all genotypes which are considered as common fragments. Also, HB-11 primer resulted in generating eleven DNA bands with molecular weight size ranging from 252 to 585p with 27.27% polymorphism. Out of these generated fragment, only three of them showed positive species- specific markers at 814 bp. for *ssp. crispum* and 302, 825 bp. for *ssp. neapolitanum*.
HB-12 primer showed twelve DNA fragments with molecular size ranging from 174 to 804 bp. with 58.33% polymorphism and seven of them were positive species- specific markers at 316, 396, and 438 bp. for ssp. *crispum*, and for ssp. *neapolitanum* there were four bands (311, 451, 582, and 804 bp.). The other five fragments were present in all sample, which are considered as common fragments.

This investigation compared the ISSR and RAPD profiles as genetic markers to characterize the two parsley subspecies. Our results indicated that the two subspecies can be distinguished from one another using two different types of genetic markers. The RAPD markers produced a larger number of the random DNA fragments scored, and therefore were more efficient to detect the polymorphism in this study, as the highest polymorphism detected was 84.62% as compared to the results obtained by ISSR markers (58.33%), in contrast to what is generally reported literature about the superior efficiency of ISSR in assessing the genetic relationship among deferent genotypes (Nagaoka and Ogihara, 1997; Ajibade *et al*., 2000; Singh *et al*., 2014). However, Rayar *et al*. (2015) reported that the efficiency of the ISSR markers didn't meaningfully differ than the RAPD when determining the genetic variability and polymorphism of *F. pseudomonas* strains. Moreover, Guasmi *et al*. (2012) and Izzatullayeva *et al*. (2014) reported that RAPD markers are either more efficient in exhibiting variation or equally effective when studying the genetic diversity of barley and sugar beet, respectively.

The number of total monomorphic fragments is higher for ISSR than RAPDs. ISSRs have a high capacity to reveal polymorphism and offer great potential to determine intra and inter genomic diversity as compared to other arbitrary primers like RAPDs (Zietkiewicz *et al*., 1994). A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target regions of different genetic nature (function and structure) of the genome (Rayar *et al.*, 2015). However, ISSR markers are expected to reveal higher polymorphism reproducibility than RAPD, as the level of polymorphism observed, specifically, among subspecies are expected to be less when using ISSR primers because the ISSR primers hardly amplify the non-coding regions of the genome while RAPD amplifies both coding and non-coding regions of the genome, thus generating more amplicons (Costa *et al.*, 2016), The ability to resolve genetic variation among different genotypes may be more directly related to the level of polymorphism detected with each marker technique rather than a function of which technique is employed. Gupta *et al*. (2008) and Mahdy (2012) reported the same conclusion with *Jatropha curcas* and *Corchorus olitorius* L. and *Lactuca sativa* L, respectively.

The different banding patterns produced from RAPD and ISSR markers show genetic variation between the two Parsley subspecies, and reported significant differences between the two parsley subspecies. Unfortunately, the only previous genetic variation study of *Petroselinum crispum* were among random parsley genotypes within one species cultivated in Mosco using five RAPD and six ISSR primers (Domblides *et al.,* 2010). It should be noted that the number of primers utilized for each of RAPD and ISSR in that study as well as the current investigation may not be enough to reveal all the existing polymorphism (Izzatullayeva *et al*., 2014). However, the results reported here illustrate the potential of similar studies for speedy detection of genetic diversity and DNA fingerprinting of closely related genotypes with relatively minor genetic variability(ies) such as varieties and sub species. Therefore, further studies are needed to accurately examine the population and evolutionary origins of *Petroselinum crispum* (parsley) genotypes in Egypt as this investigation illustrated the importance of accurate genetic diversity and variability studies for plant improvement.

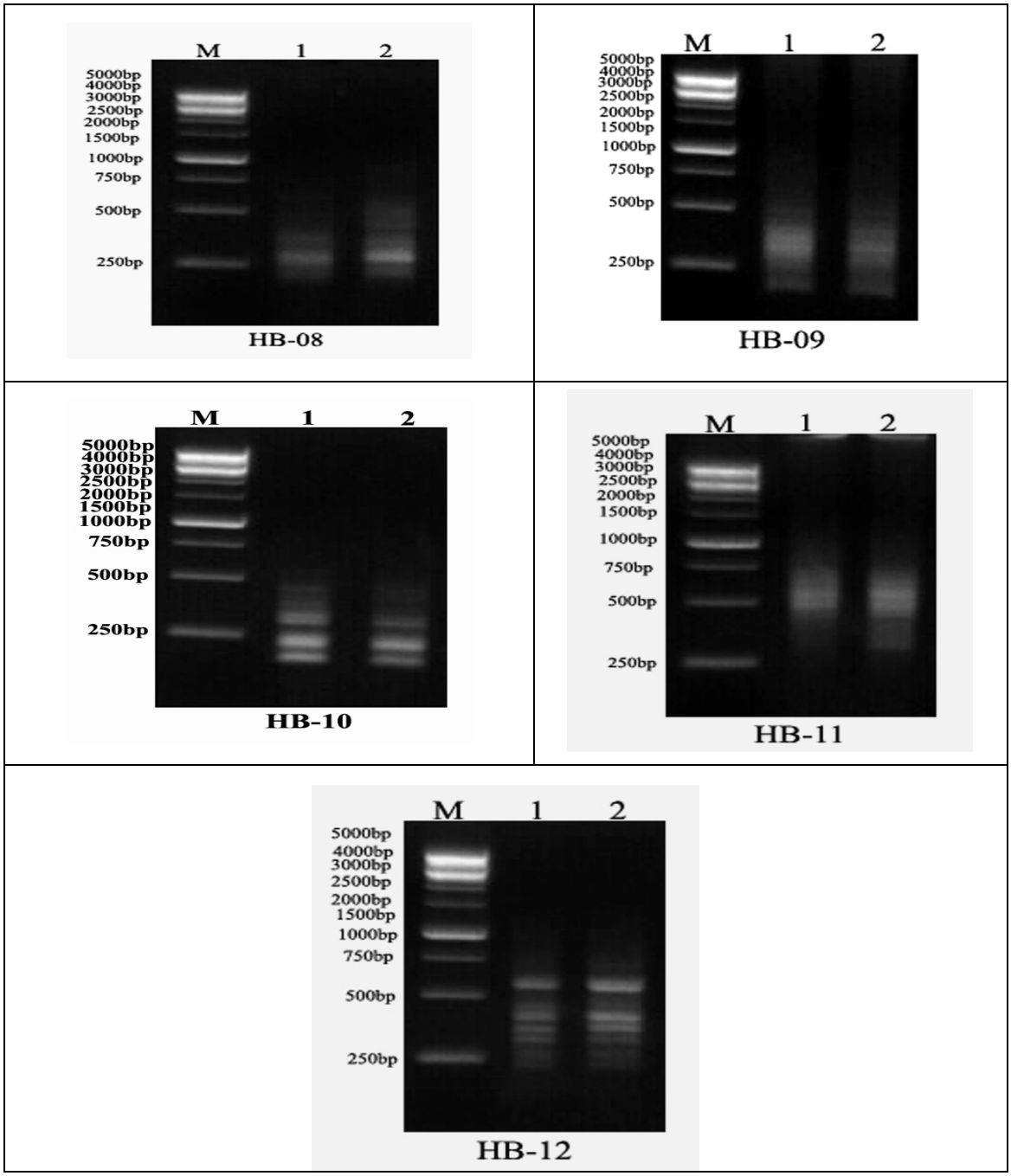

Fig. 2: ISSR-PCR analysis of two parsley varieties genotypes (second season). 1- The curly leaf type (ssp.. *crispum*), 2- The plain leaf type (ssp. *neapolitanum*, Danert). M- Molecular weight marker (100 bp.).

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