Assessment of Taxonomic Relationship Between Some Taxa of *Clinopodium*, *Hymenocrater*. and *Melissa* of Lamiaceae in Iraqi Kurdistan Region using RAPD Marker

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Received 29 January 2015 ; Accepted 31 May 2015

Abstract

To resolve genetic relationships among traditionally recognized taxa at the molecular level, , Random Amplified Polymorphic DNA(RAPD) marker was used for Taxonomic relationship and genetic diversity among six taxa of Lamiaceae family including four taxa of *Clinopodium* (*C. vulgare* subsp. *vulgare*, *C. congstum,* and *C. umbrosum*) and two taxa of *Hymenocrater* (*H. longiflorus, H. bitiominosus*) and one of *(Melissa Melissa officinalis* subsp. *officinalis*) some were described for the first time in Kurdistan region of Iraq. DNA was extracted from the leaves of these taxa, using a modified protocol because these kinds of plants are having polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Only five primers from the ten universal primers of RAPD analysis gave reproducible polymorphic bands among the studied taxa . Out of thirty bands produced seventeen were monomorphic and the rest were polymorphic, which can be used to discriminate between the studied taxa. There were monomorphic bands within the species of the genus *Clinopodium* which shared all the amplified products (except one) using the ten primers in this study. The presence of specific band in any taxa , indicates the genetic distinctness of them so as the missing bands in one taxa , which may be used in the taxa differentiation. *S*pecies of *Hymenocrater* have the most sharing bands and discriminating from the other studied genera using RAPD marker and so was *Melissa officinalis* subsp. *officinalis,* which have unique profile and discriminated from the other studied taxa using RAPD marker.

Keywords:Lamiaceae, Random Amplified Polymorphic DNA, genetic relationships

DIYALA JOURNAL FOR PURE SCIENCES

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تقدير العلاقة الوراثية بين بعض المراتب لتصنيفية لاجنا *التابعة لعائلة ال* Melissa *وال* Hymenocrater ، Clinopodium في اقليم كردستان العراق باستخدام مؤشرات ال Lamiaceae RAPD

خلود ابراهيم حسن عادل موحان الزبيدي بسوزصادق جباري

الخلاصة

لايجاد العلاقة الوراثية بين سبعة مراتب تصنيفية تابعة لعائلة ال **Lamiaceae** على المستوى الجزيئي تم اعتماد التفاعل التضاعفي العشوائي لسلسلة ال DNA اي ال RAPD والتي شملت ثلاثة مراتب من الجنس ال *Clinopodium* (*C. vulgare* subsp. *vulgare*, *C. congstum, C. vulgare* subsp. *arundanum*) ونوعين من ال *Hymenocrater* (H. *longiflorus, H. bitiominosus*) ونويع واحد من الجنس *Melissa* (*Melissa officinalis* subsp.*fficinalis*) ، والتي بعضها تدرس لاول مرة في اقليم كوردستان العراق . تم استخلاص ال DNA من العينات باستخدام طريقة معتمدة على مادة ال CTAB وذلك لاحتواء هذا النوع من النباتات ذات الاستخدامات الطبية على كمية عالية من المواد الفينولية والتي تتداخل مع تفاعلات ال PCR اللاحقة . خمسة بادئات من البادئات العشرة المستخدمة في تطبيقات الRAPD اعطت نتائج متباينة بين المراتب المدروسة ،حيث من مجموع ثلاثون حزمة (قطعة دنا متضاعفة) سبعة عشر حزمة كانت متشابهة والبقية اي ثلاثة عشر حزمة كانت متباينة بين المراتب التصنيفية المدروسة والتي استخدمت للتمييز بينها .اذ تم الحصول على حزم مشتركة بين المراتب التصنيفية التابعة للجنس الواحد كتلك المراتب التابعة للجنس *Clinopodium* وكذلك بين الانواع التابعة للجنس*Hymenocrater* والتي اشتركت بجميع الحزم الناتجة باستخدام جميع البادئات عدا حزمة واحدة .ان وجود حزمة معينة في مرتبة تصنيفية معينة فقط يدل على تميزتلك المرتبة عن باقي المراتب المدروسة كذلك غياب حزمة معينة عنها يدل على تميزتلك المرتبة عن باقي المراتب المدروسة كذلك غياب حزمة معينة عنها يدل على تميزها وقد امكن ايجاد حزم معينة لتمييز الانواع التابعة للجنس *Hymenocrater* عن باقي المراتب التصنيفية وكذلك امكن تميز النويع التابع للجنس *Melissa* بغياب حزمة مميزة فيها باستخدام مؤشرات ال RAPD.

كلمات مفتاحية : عائلة ال Lamiaceae ، لتفاعل التضاعفي العشوائي لسلسلة الدنا ، العلاقة الوراثية

Introduction

The field of molecular phylogenetics has progressed tremendously in the last decades, in all kinds of organisms including plants. In the case of medicinal and aromatic plants (MAPs), especially the Lamiaceae family, deeper insight of the genetic structures of populations of these kinds of plants was achieved, which is important in managing the natural resources of them (1).

Estimation of the genetic relationship between genotypes in plant varieties and accessions is essential for effective utilization of plant genetic resources in crop improvement. Morphological characters (qualitative and quantitative) have been employed for estimating genetic diversity. Although the use of these traditional methods provided a useful tool for genetic studies but the method has been limited by the small number of phenotypic characters and to stage specific expression of characters beside they influenced by environment and it require a long time (2) .The use of molecular markers has proven its value in deferent purposes in molecular biology, especially for analysis of genetic diversity and varietal identification ,since there is no effect of stage of development, environment or management practices. There are a large number of DNA markers which enables precise classification of the cultivars and germplasm collections. It is now possible by using molecular marker, especially DNA markers to test complex hypotheses involving phylogenetic relationships, biogeographical models of dispersal, range expansion, variances,and evolutionary transitions in character states involving groups ranging from closely related species (3, 4). There are three kinds of DNA markers including :DNA hybridization (5), and DNA sequence analysis (6) besides polymerase chain reaction (PCR) based techniques, among them PCR have many advantages of sensitivity, reliability, fast and cost-effective. Of the available PCR method, Random Amplified Polymorphic DNA (RAPD) technique is the fastest and simplest (7). It involves PCR amplification of random DNA sequences from genomic DNA using short primers of arbitrary nucleotide sequence. Polymorphisms are a result of base pair substitutions or insertions/ deletions that modify the primer annealing site or insertions into the genomic sequence that separate the primer site to a distance that does not allow for amplification to occur. RAPD markers generate DNA fingerprints using a single synthetic nucleotide primer which could efficiently detect polymorphism based on comparison throughout the genome. It does not require any prior knowledge of DNA sequence (8). RAPD have been widely used in DNA fingerprinting, gene mapping and phylogenetic studies,
reconstruction of phylogenetic relationships for many organisms studying phylogeny and taxonomy within several families (9). RAPD were also applied in some genera of Lamiaceae

family (10, 11, and 1). However there is still little information available on genetic diversity of the Lamiaceae family, as it considered the sixth largest family of flowering plants and one of the most economically important. (12), so the aims of this study were, estimation of the genetic diversity between some taxa of three genera of Lamiaceae family and a systematic analysis of these collections that based on DNA profiling using RAPD markers to determine If the samples of galbanum are similar to each other in genetic point of view, and this goal require extraction of high quality of DNA from these taxa.

Materials and Methods

Sampling and DNA extraction

Six Samples of plant leaf of Lamiaceae family were collected from deferent area of Kurdistan region including three taxa of *Clinopodium* (*C. vulgare* subsp. *vulgare*, *C. congstum,* and *C. umbrosum*) and two taxa of *Hymenocrater* (*H. longiflorus, H. bitiominosus*) and one of *Melissa (Melissa officinalis* subsp. *officinalis*) . DNA from the leaves of plants was extracted using cetyl trimethyl ammonium bromide (CTAB) method as described by Liber *et.al*(13) with slight modification as follows:

Three grams of the leaves of plant of each sample , homogenate to powder with liquid nitrogen and the powder was transfer to 10 ml tube volume of CTAB extraction buffer [1.4 M NaCl, 2% CTAB (cetyl trimethylammonium bromide) which were preheated at 65^0 C for 15 minute to homogenate, mix vigorously and incubate in water bath at 65^0 C for 1 hr ,then Bring down the sample to room temperature, and add 5ml chloroform:isoamyl alcohol (24:1), mix gently by inverting for 15-20 minute spin for 10 minute .then add equal volume of chloroform: phenol: isoamyl alcohol to the aqueous phase (25:24:1), mix, keep for 5 minute and spin for 15 minute at 12000 rpm, then take the aqueous phase and add equal volume of chloroform (to remove phenol), mix and spin for 15 minute. Then Transfer the top layer in fresh tubes, measure the volume. And add 1/10 ammonium acitate and 2/3 ml of cold isopropanol. Mix gently to precipitate the nucleic acid and spin for 5-10 minute. Wash with around 3ml of 70% ethanol. Spin for 10 minute decant and dry the pellet at room temperature. After drying the pellet,

dissolve in TE(Tris ,EDTA) buffer, for detection of the DNA samples, they were electrophoresed in a 1% agarose gel and were subsequently visualized by UV illumination after ethidium bromide staining (14).

Screening of PCR

Ten RAPD primers were used in this study (Table1) obtained from Cinagene Company (with 60% - 70% GC content) were screened from a single individual. PCR reactions were performed with 1×PCR buffer without MgCl2; 2.5 mM MgCl2;50ng DNA, 0.3 µM each primer; 0.25 mM (each) dNTP; 25 U/ml Taq DNA polymerase (sigma). PCR reactions were carried out in PCR thermal cycler((Biotech,U.K) .Amplification consisted of 40 cycles: 1 min at 94°C, 1 min at 36 of primers extension at 72 ºC and final extension of 2 minutes at 72 ºC . The amplified products were electrophoresed in a 1.2% agarose gel and were subsequently Visualized by UV illumination after staining by ethidium bromide

Results and discussion

Extraction of DNA

The Genomic DNA was successfully extracted from the fresh leaves using cetyl trimethyl ammonium bromide (CTAB) based method, the purity of the DNA was ranged between 1.4-1.6, this method was also used by (13,15). DNA can also be extracted from fresh, preserved or dried samples, however fresh material is recommended (15). It s worth to mention that the Extraction of DNA from plants is not an easy issue ,because most of the plants have a very hard cell wall which need vigorous method to breaking the cell wall to get the intact DNA, however the protocol used in this study was efficient since suitable amount of amplifiable DNA is obtained ,this is due to the materials used in this protocol that include CTAB ; which was a detergent that helps lyses the cell membrane by removal of membranes lipids and make a complex with the DNA in the sample to protect it from the next steps of extraction. To protect the DNA from endogenous nucleases EDTA were included in the extraction buffer which acts as chelating agents for magnesium ions that is a necessary cofactor for most nucleases (15).

DNA extracts often contain a large amount of proteins, for proteins removal, Phenol-Chloroform were used to denaturation of protein which was precipitated and removed by

centrifuge. Finally the DNA precipitated in salt solution with Ammonium acetate, and isopropanol, the presence of Ammonium acetate play an important role for the yield of DNA, since the salt provides positive ions which formed ionic bond with the negatively charged phosphate of DNA, thus neutralizing the effect of negative repulsion of DNA and helped the DNA molecules to come closer and compact to perecipate in the presence of alcohol (isopropanol). It is also worth mentioning, that obtaining high-quality/-quantity intact genomic DNA from medicinal plants (have heigh content of polyphenols) is problematic , and encountered many problems include degradation of DNA due to endonucleases, co isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions, These results were in agreement with (16,17) who mentioned that the polyphenolics and polysaccharides interfere with the enzymatic applications of PCR. Therefore modifications were made to minimize polysaccharide co isolation and to simplify the procedure for processing large number of samples by repeating the step of DNA purification twice and adding polyvinyl pyrrolidone; 6% (PVP). These results were in agreement with (18).

Application of RAPD PCR

To resolve relationships among traditionally recognized taxa , RAPD marker was used which depend on Polymerase Chain Reaction that their main require was the primers. The amplification showed differences between banding pattern, this resulted because every primer has different contents of G + C and difference base sequences. Among the ten primers used only five primers were selected for further analyses. The selected primers generated polymorphic bands, the number of bands generated by each primer was varied , ranged between four bands in primer No.7 (CCTTCAGGCA) to eight bands in Primer No.6 (GTCCACTGTG) with molecular size ranged between 100-3000base pair (bp) in the studied taxa. Primer No.1produced eight fragments (Figure 1) with molecular size ranged between (100-3000) bp, it produced the highest numbers of bands, some of these bands were monomorphic between all studied taxa due to their binding with the conserved sequences of these taxa, other bands were specific for some of the taxa also which may be used in the

discrimination of these taxa. The presence of specific band indicated the genetic distinctness of the studied taxa and the missing bands also in one taxon may be of use in the taxa differentiation. For example the band with 550 bp were disappeared just in the species of *Hymenocrate* (*H.longiflorus*, *H. bitiominosus*) which can be considered as marker for these species. The results of RAPD marker using primer No.4 (GAACGGACTC) represented in Figure 2, six bands were produced with molecular size ranged between (150-1000) bp ,the band with 450 bp was missing in the two studied species of *Hymenocrater* which can be used in discriminating of these two species .All the studied species of *Clinopodium* have the same bands due to the genetic similarity between them. *Melissa officinalis* subsp. *officinalis* was discriminated using the primer No.7 (CCTTCAGGCA) in Figure 3 which produced the less numbers of bands (4). The size of the amplified products ranged from 200 bp to750 bp.

The results of primer No. 9 (GGTGCGGGAA) represented in (Figure 4) which produced six bands with molecular size ranged between 100-750 bp. The species *H. bituminosus*, was discriminate using this primer by missing the band of 750bp in this species and it's presence in all the studied taxa. The species *C. congestum*, was only discriminated from the other species of *Clinopodium* by the presence of the band of 250 bp. The result of the primer No.10 (GTAGACCCGT) was represented in Figure 5 where *Melissa officinalis* subsp. *officinalis* can be detected by missing the band of 500 bp in this species only and the two studied species of the genus *Hymenocrater* were also discriminated by the missing band of 180 bp. The presence of the band of 550 bp using this primer can be used to discriminate between the species of *Clinopodium* as this band present only in *C. vulgare* subsp. v*ulgare* and absence in the other two taxa. However the studied taxa of all the species of *Clinopodium* had shared the other entire bands produced using the ten primers in this study. So the more shared bands obtained from RAPD analyses between objects, the larger is the similarity between them.

These results were in agreement with Matos *et al*.(19). The case of the similarity between these species due to the common features between them and this may confirmed that *C. vulgare* subsp. *vulgare* belong to the genus *Clinopodium* as it was approved using the morphological characters such as the calyx tube, bracteoles (20) and the cytological study that conducted by

Matos *et al*.(19) beside that recorded in Flora Iranica and flora of turkey, which confirmed that this subspecies is belong to the *Clinopodium* genus. This meant that molecular data for the studied taxa of Lamiaceae support their taxonomic treatments, and as RAPD provided more precise information concerning genetic relationships, this is in agreement with Abdel Khalik *et. al*(21), Recently RAPD also was used in taxonomic study for many taxa within Lamiaceae family (22,23).

Conclusion

The present study showed the usefulness of RAPD analysis in distinguishing between the three genera, and their results were in broad agreements with traditional classification. So despite the reproducibility problem, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time and labor.

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Table 1. The primers, used in PCR processes

No.	Primer names(sequences)	No.	Primer names(sequences)
1-	AATCGGGCTG	2-	TTCCGAACCC
3-	CCGCATCTAC	4-	GAACGGACTC
5-	AATGCGGGAG	6-	GTCCACTGTG
7-	CCTTCAGGCA	8-	GTCTACGGCA
9-	GGTGCGGGAA	10-	GTAGACCCGT

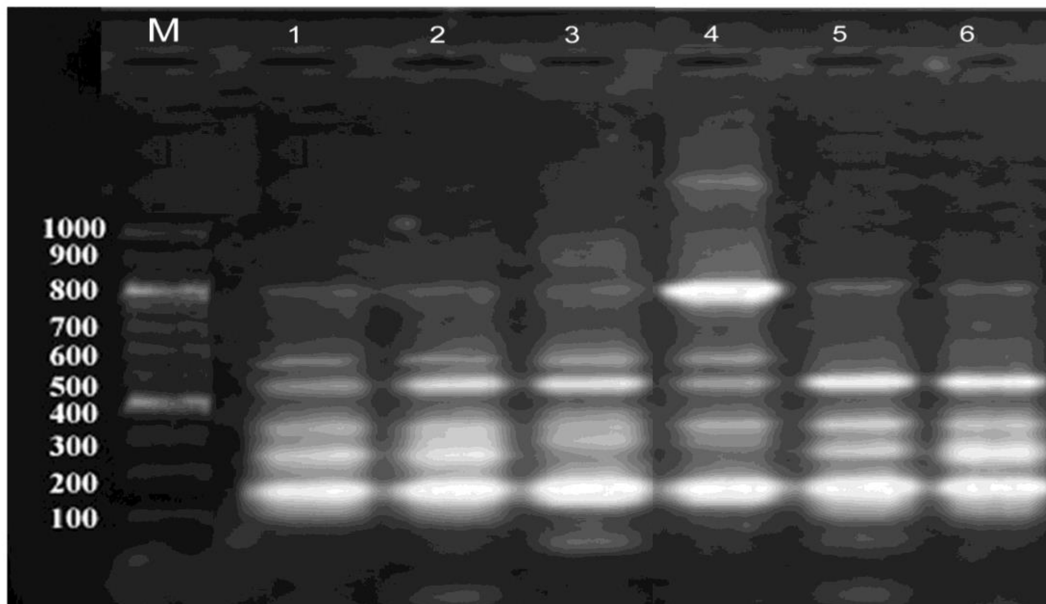

Figure 1. RAPD profiles of six taxa of Lamiaceae using the Primer No.6 (GTCCACTGTG). M: represent molecular weight size marker 1 kb ladder). 1: *Clinopodium vulgare* subsp. *vulgare*, 2: *Clinopodium umbrosum*, 3: *Clinopodium congstum*, 4: *Melissa officinalis* subsp. *officinalis*, 5: *Hymenocrater longiflorus*, 6: *Hymenocrater bitiominosus*.

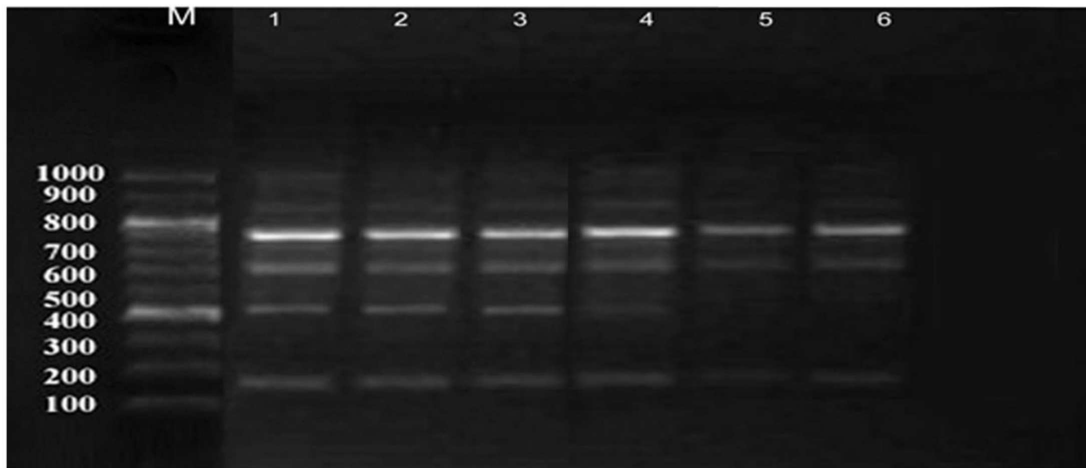

Fig 2.RAPD profiles of six taxa of Lamiaceae using the Primer No.4 (GAACGGACTC). M: represent molecular weight size marker 1 kb ladder). 1: *Clinopodium vulgare* subsp. *vulgar*e, 2: *Clinopodium umbrosum*, 3: *Clinopodium congstum*, 4: *Melissa officinalis* subsp. *officinalis*, *5: Hymenocrater longiflorus*, 6: *Hymenocrater bitiominosus*

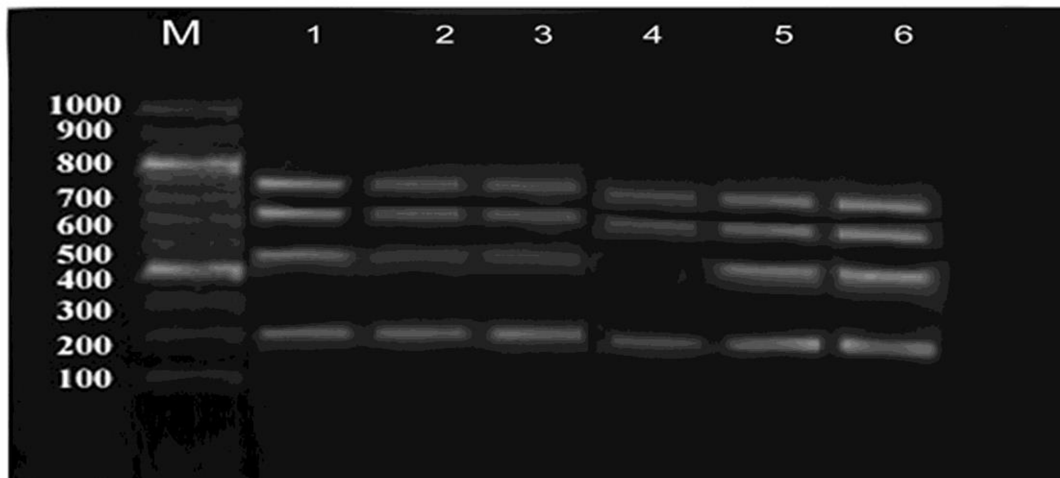

Fig 3. RAPD profiles of six taxa of Lamiaceae using the Primer No.7 (CCTTCAGGCA). M: represent molecular weight size marker 1 kb ladder). 1: *Clinopodium vulgare* subsp. *vulgar*e, 2: *Clinopodium umbrosum*, 3: *Clinopodium congstum*, 4: *Melissa officinalis* subsp. *officinalis*, 5: *Hymenocrater longiflorus*, 6: *Hymenocrater bitiominosus*.

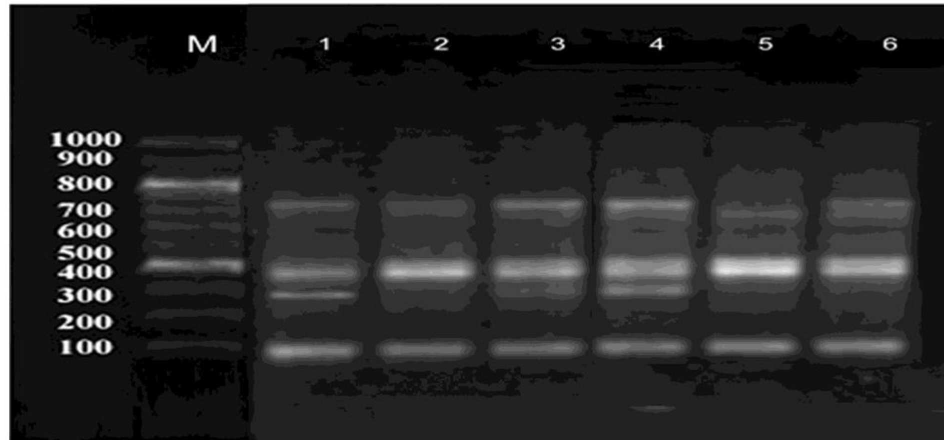

Fig 4. RAPD profiles of six taxa of Lamiaceae using the Primer No.9 (GGTGCGGGAA). M: represent molecular weight size marker 1 kb ladder). 1: *Clinopodium vulgare* subsp. *vulgar*e, 2: *Clinopodium umbrosum*, 3: *Clinopodium congstum*, 4: *Melissa officinalis* subsp. *officinalis*, 5: *Hymenocrater longiflorus*, 6: *Hymenocrater bitiominosus*.

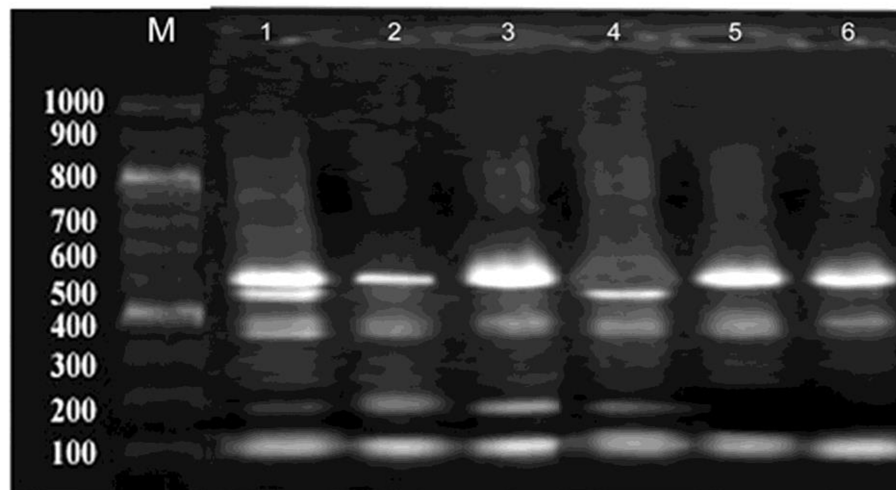

Fig 5. RAPD profiles of six taxa of Lamiaceae using the Primer No.10 (GTAGACCCGT). M: represent molecular weight size marker 1 kb ladder). 1: *Clinopodium vulgare* subsp. *vulgar*e, 2: *Clinopodium umbrosum*, 3: *Clinopodium congstum,* 4: *Melissa officinalis* subsp. *officinalis*, 5: *Hymenocrater longiflorus*, 6: *Hymenocrater bitiominosus*.