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Assessment of Genetic Diversity and Relationships of Five *Mentha* Species Using RAPD Marker

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ABSTRACT

Genetic relatedness was examined among 5 *Mentha* species *(M. longifolia* subsp. *typhoides, M. longifolia* subsp. *schimperi, M. spicata, M. sativa, M. peperita)* distributed in Egypt, using Randomly Amplified Polymorphic DNA (RAPD) analysis. The six decamer arbitrary primers utilized amplified 114 bands, with an average of 19 fragments per primer. The size of the amplified products ranged from 100 bp to 3000 bp. The largest size fragment was amplified by the primer N8, while primers P13 and B12 produced the smallest fragments. Similarity between the species ranged 0.28- 1.00 were observed between the five pairs of species. A dendrogram based on unweighted pair group method of arithmetic means (UPGMA) divided the 5 *mentha* species into three main clusters. These results may provide useful basic knowledge needed in mint germplasm management and expansion as well as breeding programs.

Key words: Mint, Genetic diversity, Molecular markers, Medicinal plants.

Introduction

Mint plants are classified within the genus *Mentha* L. which includes perennial vertical grown herbs. The genus *Mentha* includes twenty-five species widely distributed in moderate temperature regions of South Africa, North America, Eurasia, and Australia (Ahmed, 1986; Brickell and Zuk, 1997). Many of these species are the results of inter-specific hybrids (Aparicio *et al.*, 2000). *Mentha* species contain volatile oils and secondary metabolites in their vegetative parts, which reflects medicinal and economic importance (Simpson and Conner-Ogorzaly, 1986; Khanuja *et al*., 2000; Badr *et al*., 2002). In Egyptian flora, *Mentha* plants belong to three species , *M. spicata*, *M. pulegium*, and *M. longifolia* (Brown *et al*., 1978). *M. spicata* (spearmint) has volatile oils that are economically important for their food flavoring characteristics (Blumenthal, 2000). *M. longifolia* includes two subspecies (*M. longifolia* subsp. *typhoides* and *M. longifolia* subsp. *schemprei*) and is distributed worldwide (Brown *et al*., 1978). *Mentha* × *piperita* L., which is known as *M. piperita* (peppermint) is widely cultivated in Egypt and has economic importance and usage (Mustafa *et al*., 2005). *Mentha niliaca* is known as the Egyptian mint. It is a hybrid of *Mentha* x *niliaca* (Van *et al*., 1975). Interestingly, in their blooming habits, *M. niliaca* produces spikes of lavender flowers in summer (Uphof, 1968). Despite the medicinal importance of *M. niliaca*, and the fact that it is a domesticated species in Egypt, it has been rarely studied at the molecular level (Van *et al*., 1975).

Understanding the genetic diversity of *Mentha* plants is needed for crop improvement (Yousef *et al*., 2015). Morphological traits, cellular biochemical, and isozyme polymorphism has been used to detect the genetic variability among the *Mentha* plants (Mustafa *et al.,* 2005; Yousef *et al*., 2015). Molecular markers are efficient tools for genotype identification and estimation of relatedness through DNA fingerprinting (Williams *et al.*, 1990). Genetic variation and taxonomic relationships for three mint species and two subspecies were previously investigated using RAPD and ISSR molecular markers (Yousef et al., 2015). In that study, they used three species of genus *Mentha* (*Mentha viridis*; *Mentha piperita*; *Mentha aquatica*) and two subspecies (*Mentha spicata* var. *Morocana*; *Mentha spicata* var. *Longofolia*). However, it didn't not involve classification of the Egyptian Mint within the other cultivated species of mint in Egypt. The present study was conducted to assess genetic fingerprints of the Egyptian mint plants in order to elucidate and validate the genetic diversity and polymorphism among the cultivated species and subspecies of mint that are widely distributed in Egypt using RAPD molecular maker.

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Materials and Methods

Plant material:

Leaf tissues of *Mentha spicata L., Mentha piperita L., Mentha sativa L., Mentha longifolia (L.) L. subsp. schimperi (Briq.) Briq., and Mentha longifolia (L.) L. subsp. typhoides (Briq.) Harley,* were collected from the experimental Plants Station at the Faculty of Pharmacy, Cairo University. The leaf materials were transported on ice and stored at -80 °C until subjected to grinding in liquid nitrogen for DNA extraction.

Genomic DNA Extraction and DNA amplification:

Total genomic DNA was extracted from field-grown mentha frozen leaves using cetyltrimethyl ammonium bromide (CTAB) method described by Porebski *et al.* (1997). DNA quality and quantity were examined using a NanoDrop UV/Vis spectrophotometer (Thermo Fisher Scientific, CA, USA) and 1 % (w/v) agarose gel, looking for a single absorbance peak at 260 nm, a 260/280 absorbance ratio of 1.8-2.0, and no evidence of substantial band shearing or contamination (either RNA or polysaccharide). Eleven decamer primers were used for DNA amplification, five of them were from the OP series (Eurofins Genomics, Ebersberg, Germany), according to (Barcaccia *et al.,* 2006). The amplification mixture for each DNA sample contained 50 ng of each template, 10.5 µl nuclease-free water, 12.5 µl Master Mix (MBI Fermentas, NY, USA), and 1µl (25 pM) primer in a 25µl of total reaction volume. The polymerase chain reactions (PCR) were performed in a gradient thermal cycler, (Applied Biosystem thermal cycler (Thermo Fisher Scientific, CA, USA), with an initial denaturation at 94 ºC for 1 min. followed by 44 cycles of denaturation at 94 ºC for 30 seconds, annealing at 40 ºC for 1 min. and extension at 72 ºC for 2 min., and a final cycle with the same steps except extension which was for 7 minute at 72 ºC. PCR products were used immediately or stored at 4 ºC till use. Amplifications were checked by examining the PCR products on 1.7 % agarose gel prepared in 0.5 X TAE (Tris Acetate EDTA) buffer at constant voltage of 70 volts for 3 hours. The gel was stained for 30 minute with ethidium bromide, visualized, and photographed with a SynGene gel documentation unit (SynGene, Cambridge, UK). The molecular weight of amplified fragments was estimated and scored using a 100 bp molecular marker DNA ladder (Thermo Fisher Scientific, CA, USA).

Table 1: The sequences of the six random primers used in the fingerprint analysis.

Primers		Sequences
1	C1	TTCGAGCCAG
2	P13	GGAGTGCCTC
3	N8	ACCTCAGCTC
4	B12	CCTTGACGCA
5	H5	AGTCGTCCCC
6	P8	GGAGCCCAG
7	OP-A01	5´ CAGGCCCTTC 3´
8	OP-A07	5´ GAAACGGGTG 3´
9	OP-B07	5´ GGTGACGCAG 3´
10	OP-B11	5` GTAGACCCGT 3`
11	OP-C12	5` TGTCATCCCC 3`

Data Analysis:

The RAPD data were manually scored and established by organizing the binary data only, scoring the polymorphic fragment as (1) for present band and (0) for the absent band. Absent and present binary data of 5 individuals and 132 polymorphic fragments was used for the analysis. the band-based approach was used for the analysis based on Bonin *et al*., 2007). Dice coefficient (Dice, 1945; Nei and Li, 1979) was used to calculate the similarity among the 5 species and subspecies. The UPGMA method was used for phylogenetic tree construction and cluster analysis (Rohlf, 2005). To estimate the reliability of the clustering pattern, Bootstrap values based on 1000 re-sampling was used in Free Tree software (Hampl *et al*., 2001). The NTSYSpc version 2.20 (Rohlf, 2005) was also used

to measure the similarity coefficient and to prepare Principal Co-ordinates Analysis (PCoA) of the correlations matrix to test the relationship among the mentha samples.

Results and Discussion

Eleven arbitrary RAPD accessions were used in this study to screen the genetic diversity among five different Mentha plants grown in Egypt (3 spices and 2 subspecies; Table 1). None of the OP series primers generated scorable amplified fragments with the five different *Mentha* specimens under study. However, different sets of the OP series were successfully used in assigning the genetic relationship of other different medicinal plants including Mentha and *Petroselinum (Kabir et al., 2014;* Salama *et al*., 2016; Ibrahim *et al*., 2017a*; Ibrahim et al., 2017b).* The profile and densitograms for the other six RAPD markers used here were mentioned in Fig. (1) and Tables (2). These primers produced clear and scorable bands with a total of 114 amplified fragments applied to all genotypes under study. The amplified DNA fragments ranged from 3000 to 10 bp (Fig. 1). P8 and N8 primers produced the maximum number of fragments, 31 and 25, respectively, (Fig. 1 and Table 2), and also generated the highest number of polymorphic amplicons (30 and 25, respectively). The other four primers (P13, C1, B12, and H5) amplified 58 DNA fragments, with an average of 14 amplicons per primer across the 5 mint samples. Maximum polymorphism was observed with primers N8 and H5 (25 and 23 polymorphic bands, respectively). The overall DNA patterns generated by primers C1 and B12 were 9 and 8 polymorphic bands, respectively. There were 3 polymorphic bands out of 11 scorable genomic DNA bands of the five mint leaf samples amplified by primer p13 (Table 2).

Table 2: Total number of amplicons, monomorphic and polymorphic amplicons as revealed by RAPD primers among the five Mentha plants.

Primer	Total Number of Amplicons	Polymorphic amplicons	Monomorphic amplicons	% of Polymorphism
C1	13.0	9.0	4.0	69%
P13	11.0	3.0	8.0	27%
N8	25.0	25.0	0.0	100%
B12	11.0	08.0	3.0	72%
H5	23.0	23.0	0.0	100%
P8	31.0	30.0	1.0	96%
Total	114	98	16	77.3%

The similarity matrix generated by using the numerical analysis technique "NTSYS-PC" version1.5 program (Rohlf, 2005) is presented in Table (3). Interestingly, the similarity between *M. longifolia* subsp. *typhoides and M. Spicata* showed the highest resemblance (44%), which was higher than the resemblance (39%) between the two subspecies that fall within the same species origin (*M. longifolia* subsp. *typhoides and M. longifolia* subsp. schimperi. However, in another study using isozyme analysis, to illustrate the genetic distance between them, these two different plants exhibited high level of variation (Mustafa *et al.,* 2005). Also, the genetic similarity between the subsp. *typhoides and M. Sativa* was similar to that o between the subsp. schimperi and *M. sativa* (38%). These results suggest that the four Mentha plants (*M. longifolia* subsp. *typhoides and M. longifolia* subsp. *schimperi*, *M. sativa*, and *M. spicata*) may share similar background, while subsp. *typhoides and M. Spicata* may be considered to have the least diversity and thus relatedness compared to the rest of the studied accessions.

Table 3: Similarity Matrix for Jaccard's Coefficient of 5 Mentha Accessions

	M. sativa	*M. subsp. schimperi*	*M. piperita*	*M. Subsp. typhoides*	*M. spicata*
M. sativa	1.00				
M. subsp. schimperi	0.38	1.00			
M. piperita	0.32	0.28	1.00		
M. Subsp. typhoides	0.38	0.39	0.31	1.00	
M. spicata	0.31	0.35	0.32	0.44	1.00

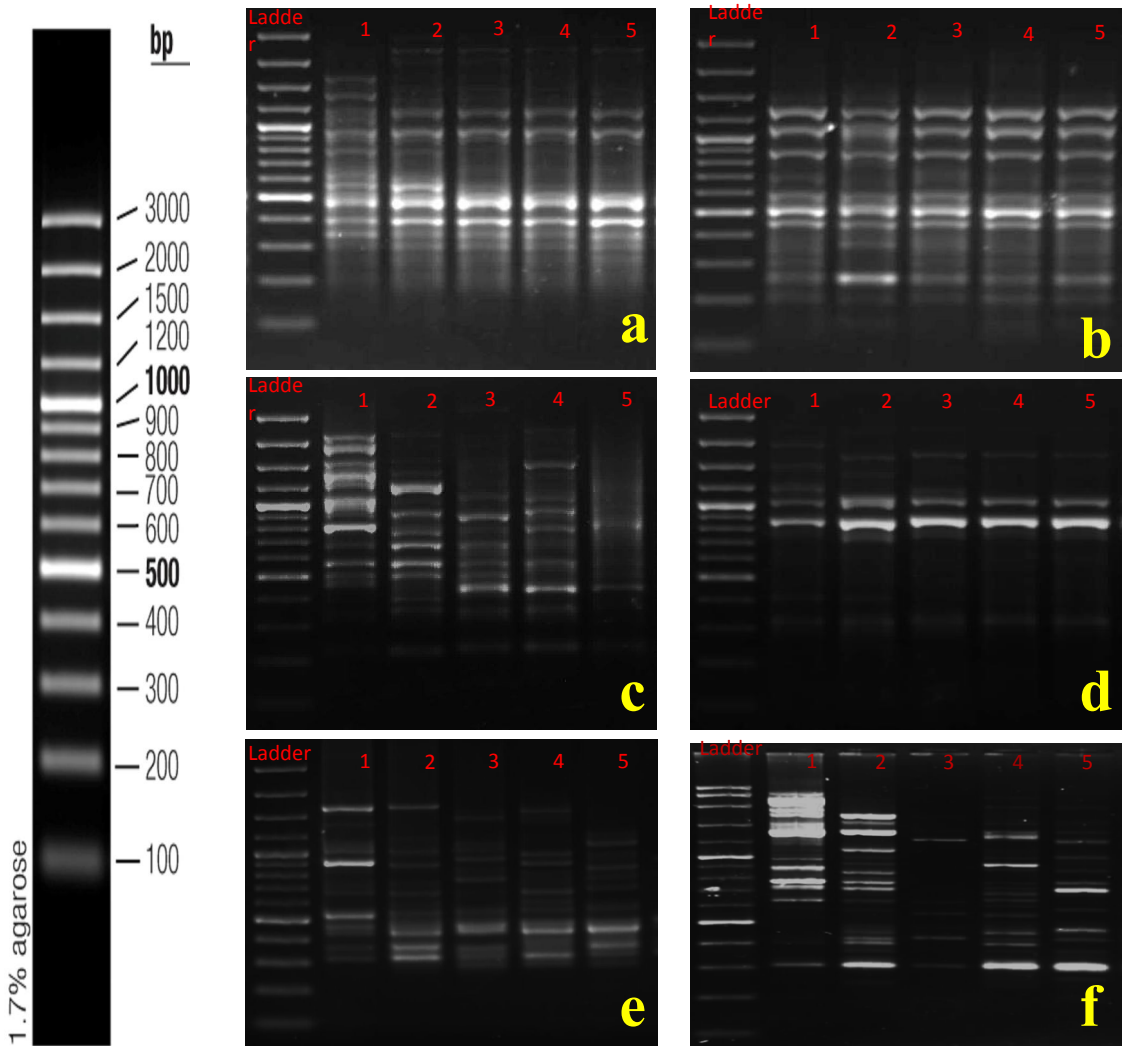

Fig. 1: RAPD profile for the five-mentha species as detected with primers C1 (a), P13(b), N8 (c), B12 (d), H5 (e) and P8 (f). 1- *M. spicata,* 2- *M. piperita,* 3- *M. sativa, 4- M. Longolisa subsp. Schimperi, 5- M. Longifolia subsp. typhoides*. M: 1 kb DNA Ladder

Dendrogram using UPGMA were generated based on the similarity matrix to classify the five Mentha plants into three main clusters (Fig. 2a and b). The dendrogram in Fig. (5a) revealed that *Mentha* species and subsp. were divided into three distinct groups, *M. longifolia* subsp. *typhoides and M. Spicata* in a group, and *M. longifolia* subsp. schimperi, *M. sativa* in a separate group, while, *M. piperita* clustered individually in a third group. This data were supported when a similar UPGMA dendrogram was generated using a statistically different multivariate software to analyze the data based on Nei's (1978; Fig. 5b) by generating bootstrap values. A two-dimensional principal coordinate analysis was generated using NTSYSpc, 2.20 software to further support these clustering patterns based on the Jaccard's similarity coefficients (Fig. 3; Rohlf, 2005).

In the present study, The genetic relatedness and finger printing explored among 5 *Mentha* (*M. longifolia* subsp. *typhoides, M. longifolia* subsp. *schimperi, M. spicata, M. sativa, M. peperita*) through randomly amplified polymorphic DNA (RAPD) markers can be applied to identify and collect genetically elite germplasm, which may help plant breeder in generating a distinct exclusive Mentha germplasm. However, finger printing using different markers such as ISSR, RAPD with enough number of primers per study to successfully achieve more accurate determination of the genetic similarity among diverse collection of Mentha plants grown in Egypt.

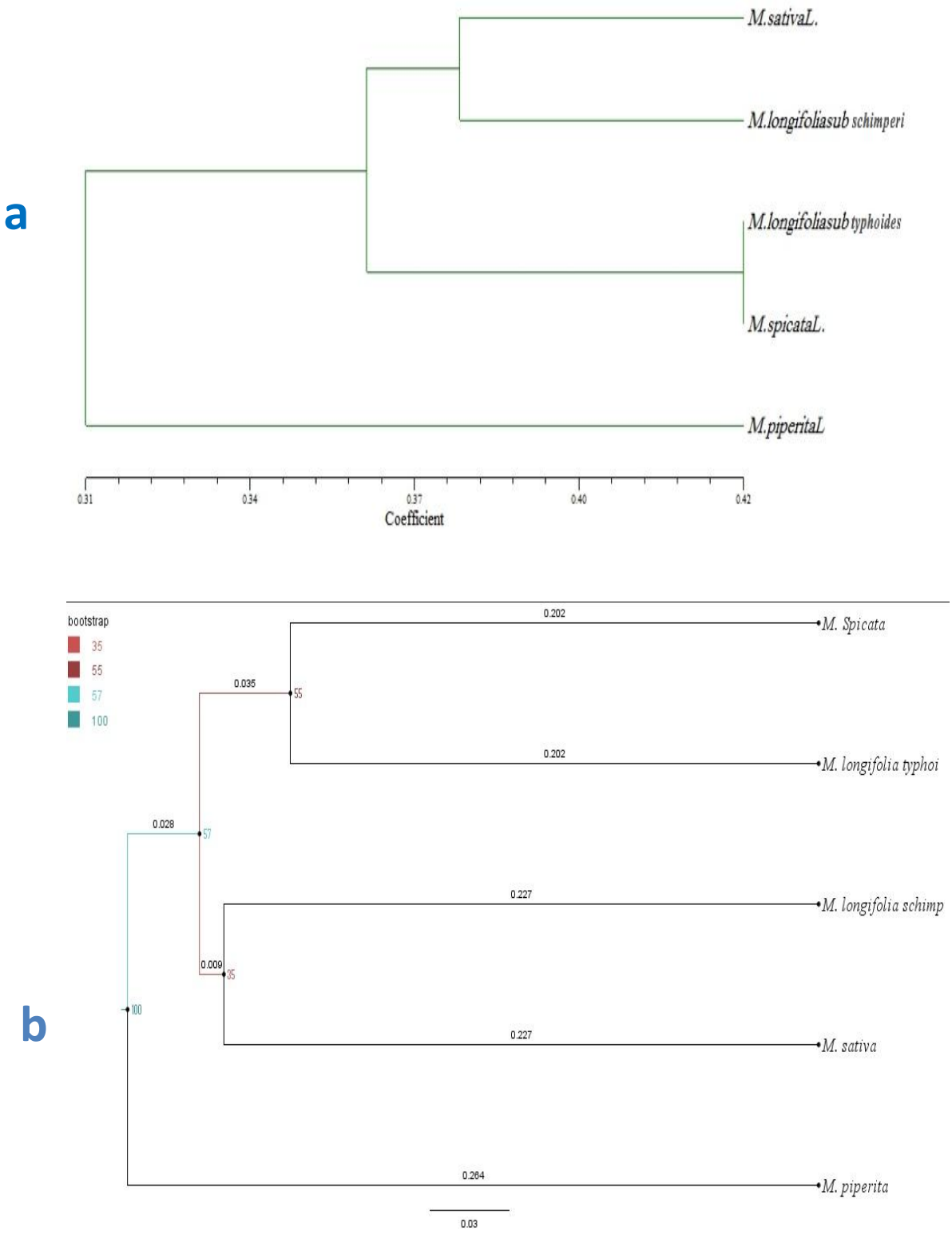

Fig. 2: UPGMA-based dendrogram of *mentha* and closely related genera Lavender generated from 106 RAPD markers. Confidence limits for the dendrogram are based on 1000 bootstrap replications. Bootstrap values were cut off at 50% and presented at the nodes. Dendrogram was assessed by FreeTree (Hampl *et al*., 2001).

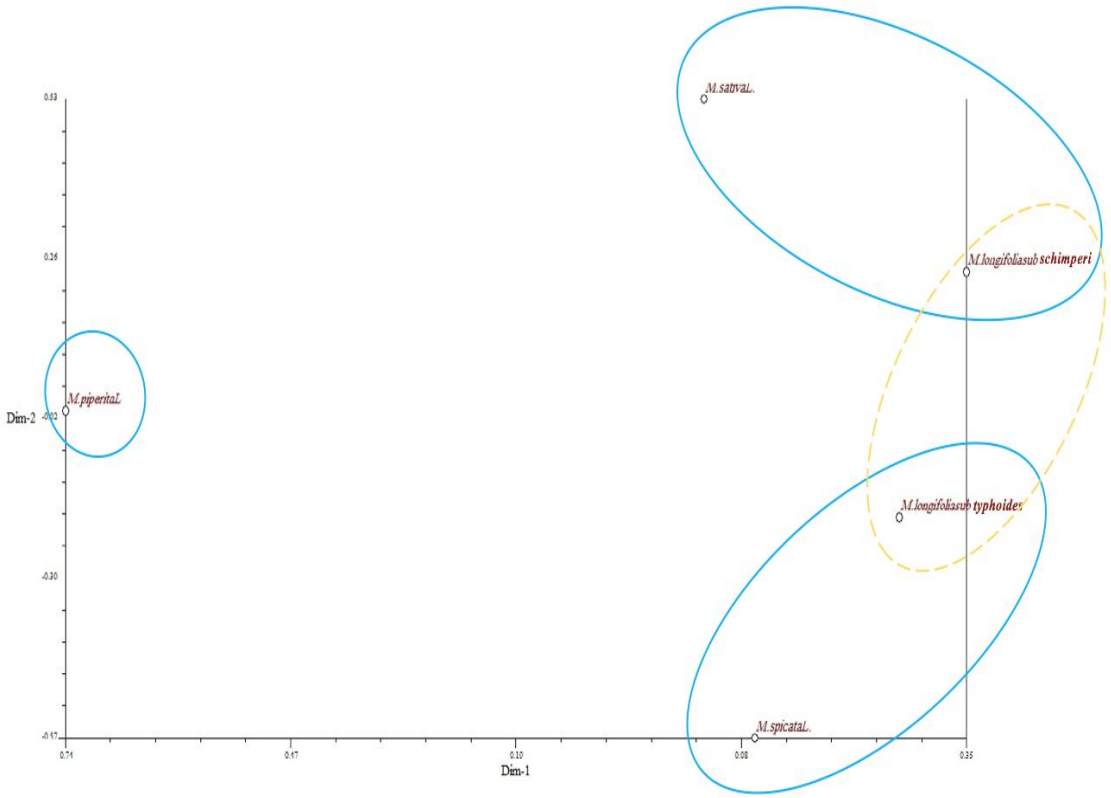

Fig. 3: PCoA based on 114 RAPD markers. The matrix plot is processed by NTSYSpc, 2.20 (Rohlf, 2005). There are three groups including *l. Officials group*, *L. lawande, M. French, M. Pepper, and M. Habak group,* and *L. Mowafe, M. baladi and M. Saudi*.

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