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A comparative study of different DNA barcoding markers for the identification of some members of Lamiacaea

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ABSTRACT

The objective of the present work is to evaluate the efficacy of a DNA barcoding approach as a tool for the recognition of commercial kitchen spices belonging to the Lamiaceae family that are usually sold as enhancers of food flavor. A total of 64 spices samples, encompassing six different genera (i.e. Mentha, Ocimum, Origanum, Salvia, Thymus and Rosmarinus) were processed with a classical DNA barcoding approach by amplifying and sequencing four candidate barcode regions (rpoB, rbcL, matK and trnH-psbA) with universal primers. Results suggest that the non-coding trnH-psbA intergenic spacer is the most suitable marker for molecular spices identification followed by matK, with interspecific genetic distance values ranging between about 0% to 7% and 0% to 5%, respectively. Both markers were almost invariably able to distinguish spices species from closest taxa with the exclusion of samples belonging to the genus Oregano. Moreover, in a context of food traceability the two markers are useful to identify commercial processed spice species (sold as dried plant material). We also evaluated the potential benefits of a multilocus barcode approach over a single-marker and although the most suitable combination was the matK + trhH-psbA, the observed genetic distances values were very similar to the discriminatory performance of the trnH-psbA. Finally, this preliminary work provide clear evidences that the efficacy of a DNA barcoding approach to the recognition of commercial spices is biased by the occurrence of taxonomic criticisms as well as traces of hybridization events within the family Lamiaceae. For this reason, to better define a more practical and standardized DNA barcoding tool for spices traceability, the building of a dedicated aromatic plants database in which all species and cultivars are described (both morphologically and molecularly) is strongly required.

1. Introduction

The Lamiaceae is a large family of about 6990 described species belonging to 264 genera (Gurcharan, 2004) and which is characterized by square stems, opposite or whorled leaves and zygomorphic flowers usually with 2-lipped corolla. Species belonging to this family are mainly herbs or shrubs of various sizes (rarely trees) and are cosmopolites with two main centers of biodiversity corresponding to the Mediterranean basin and central Asia. Due to the production of aromatic oils and secondary metabolites, some of these plants are commonly used as spices for cooking such as basil, marjoram, mint, oregano, rosemary, sage, savory and thyme. In this context, many members of the Lamiaceae are characterized by considerable commercial importance and have been previously investigated both at the species and cultivar levels in order to characterize their variability and properties (Labra et al., 2004; Novak, Lukas, Bolzer, Grausgruber-Gröger, & Degenhardt, 2008; Trindade, 2010). Systematic and phylogenetic studies on aromatic plants were usually based on morphological characters (Gurcharan, 2004). However, in the last decades, continuous advances in molecular biology (e.g. genetics) have offered a set of new tools useful for investigating the relationships among these taxa and to characterize the peculiar chemical composition of related cultivars (Gounaris, Skoula, Fournaraki, Drakakaki, & Makris, 2002; Labra et al., 2004). Discontinuous markers like RAPDs, AFLPs and hypervariable DNA regions (such as SSRs) are among the most frequently used molecular approaches for these studies (Labra et al., 2004; Trindade, 2010). However, despite their proved efficacy, these markers are not always able to distinguish among different species or cultivars (Azizi, Wagner, Honermeier, & Wolfgang, 2009; Trindade, 2010) and they are often not universally usable on a wide panel of taxa since they have been developed on specific genera or species (Novak et al., 2008; Segarra-Moragues & Gleiser, 2009).

Recently, 'DNA barcoding', a new technique based on the analysis of short, standardized and universal DNA region/s (named 'barcode sequence/s'), has been proposed as a universal tool for species identification (Hebert, Cywinska, Ball, & DeWaard, 2003; www.barcoding.si.edu). The basic idea of the discrimination system is simple: if the sequence variation of the DNA barcode among species is higher than within species, they can be successfully discriminated from one another. In practice, a DNA sequence from such a standardized DNA region can be generated from a small tissue sample and compared to a library of reference sequences belonging to described species (e.g.

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individuals stored in institutional collections) providing a rapid and reproducible taxonomic recognition (Hebert et al., 2003; Wong & Hanner, 2008; Bruni et al., 2010). This method opens new perspectives for the identification of aromatic herbs which is useful not only to clarify taxonomic uncertainties within the family Lamiaceae (DNA taxonomy, Lefébure, Douady, Gouy, & Gibert, 2006), but also to investigate commercial aspects related to spices traceability from field to market.

Within the food market, internationally approved specifications provided by the American Spice Trade Association (ASTA — http://www.astaspice.org) and the European Spice Association (ESA — http://www.esa-spices.org/) define minimum quality thresholds for herbs and spices only by considering their phytochemical profile and the amount of their essential oil. However, herbs and spices tend to be grown by smallholders and it is difficult to monitor and control growers who might be inclined to use different herbs to increase the agricultural yield. For example, Mediterranean oregano has recently been adulterated with plants having leaves similar in color (i.e. silvery gray) and size of such as *Rhus coriaria* L., *Cistus* spp. (Marieschi, Torelli, Poli, Sacchetti, & Bruni, 2009). In this context, the definition of a universal identification system based on a standardized molecular approach would allow not only the characterization of the spices species starting from a small plant fragment, but also the traceability of commercial spices products sold in powder form. Indeed, these kinds of products, usually sold as enhancers for food flavor, could be accidentally or intentionally contaminated by other less-valuable plants species (Sasikumar, Syamkumar, Remya, & Zachariah, 2004; Dhanya & Sasikumar, 2010). In this context, the search for a suitable genomic region to perform a universal DNA barcoding approach on Lamiaceae family is an element of primary importance. The selected regions should be universally applicable to a large number of plant species, like the mitochondrial *coxI* (cytochrome c oxidase subunit 1) in metazoans (Hebert et al., 2003). Due to the inadequacy of this marker for plants, several alternative regions have been proposed, such as the plastidial rpoB, rpoC1, rbcL and matK genes. Moreover, intergenic plastidial spacers (trnH-psbA, atpF-atpH and psbK-psbI) and the nuclear internal transcribed spacers have also been proposed as efficient DNA barcode regions (Chase et al., 2005; Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005; Fazekas et al., 2008).

Recently, the CBOL Plant Working Group (www.barcoding.si.edu/plant_working_group.html) proposed the combinations of matK with rbcL to increase the efficacy of the method in plant species identification, especially for critical groups (Hollingsworth, Forresta, et al., 2009). In spite of this useful proposal, both the identification and the combination of the most appropriate regions for plant DNA barcoding remain contentious (Le Clerc-Blain, Starr, Bull, & Saarela, 2009; Bruni et al., 2010; Chen et al., 2010).

The objective of the current study is to evaluate the universal applicability of a DNA barcoding approach to reach a univocal identification of aromatic plant species starting from different plant portions and processed kitchen spices subjected to industrial modifications (e.g.: drying, shredding, and storage) that make them morphologically unrecognizable. In this study, we provide an evaluation of the performances of four candidate barcoding loci and different combinations of the same in the recognition of various Lamiaceae species and cultivars, analysing DNA extracted from different plant materials. In addition, closely related taxa were analysed to better define the discrimination power of the tested markers and combinations.

2. Materials and methods

2.1. Plant sampling

A panel of 43 samples representing 16 different species was selected to test for the performance of four different candidate genomic regions for DNA barcoding analyses (Table 1). In most cases, different commercial cultivars were considered for each species. Fresh samples were collected from greenhouse of Milan Botanical Garden (www.biodip.unimi.it/it/dipartimento/cascina.htm), Certify Seed and Garden Center Ingegnoli (www.ingegnoli.it/), Garden Center Biovivaio Gran Burrone (www.biovivaiogranburrone.com) and Garden Center Viridea (www.viridea.it/it/home.asp). Sampling details can be retrieved from Table 1. A dichotomous key (Pignatti, 1982) was used to verify the correctness of the taxonomic assignment for each collected sample.

Samples were stored at −20 °C and following the protocol specified by the Biorepositories initiative (http://www.biorepositories.org) they were vouchered with the id name 'MIB:zpl:' followed by a progressive numeric code. This string identifies the Institutional collection locality where the samples are currently stored (University of Milano-Bicocca).

In order to evaluate the efficacy of a multi-marker DNA barcoding approach in delimiting species boundaries (and even cultivars variability) among the most commercialized Lamiaceae, samples were divided into six taxonomical groups, according to their genus (see Table 1).

Group I — This group consists of seven mint samples belonging to morphologically identified plants of congeneric species: *Mentha spicata* L., *Mentha aquatica* L. and their supposed hybrid *Mentha piperita* L. (see Table 1 for more details).

Group II — This group includes three different morphologically identified basil species (*Ocimum basilicum* L., *Ocimum gratissimum* L. and *Ocimum tenuiflorum* L.) as well as several cultivars of *O. basilicum* L. The selected cultivars show small differences in their morphology and chemical composition (Labra et al., 2004) and are mostly used as culinary herbs or ornamental plants.

Group III — This group encompasses two different origanum species: *O. majorana* L. and *O. vulgare* L. These are the most commonly commercialized origanum species. In addition, *O. pseudodictamnius* Sieber and *O. heracleoticum* L. were also considered (see Table 1 for more details).

Group *IV* — This group is constituted by salvia species only, including some ornamental taxa such as *Salvia rutilans* L. and *Salvia uliginosa* L.; these species are not edible and for this reason it is very important to set up a molecular approach able to distinguish them from *Salvia officinalis* L., commonly used as fresh or dried spice for cooking (see Table 1 for more details).

Group V — Consists of samples of thyme as cultivated plant (*Thymus vulgaris* L.) (see Table 1 for more details).

Group VI — This group consists of three samples of *Rosmarinus officinalis* (i.e.: the most common cultivar "Arp") collected from different plant collections (see Table 1 for more details).

Moreover, a total of 21 additional samples of commercial processed spices (e.g. dry and/or crumbled leaves) were collected in order to verify the usefulness of DNA barcoding as a tool for spices traceability. In particular, at least three commercial spice samples were analysed for each one of the six groups. These samples were purchased from supermarkets and drugstores and only products clearly labelled with a commercial spice name (e.g. mint, basil, and oregano) were sampled while flavor enhancers with an unclear label or made of mixed spices were excluded. Further details can be retrieved from Table 1.

2.2. DNA isolation and amplification

Fresh young leaves (100 mg) belonging to the selected plants species of Lamiaceae included in the six groups (Table 1) were used for DNA analysis. In the case of commercial spices, a total of 20 mg of powder or shredded material was used for DNA extraction.

Table 1
List of analysed spices samples divided in different groups (Gr) according to their taxonomy and provenance. For each sample the voucher number (V.N.) the Reference Species name, the cultivar name or common name, for the commercial samples, are provided. The Accession Numbers corresponding of DNA sequences of the four analysed markers are also included. Fresh samples were collected (C) from greenhouse of Milan Botanical Garden (MBG), certify seed and garden center Ingegnoli (ING), garden center Biovivaio Grand Burrone (BGB) and garden center Viridea (VIR). Commercial samples were collected in four different European Commercial Companies and to ensure their anonymity each sample was marked with the following codes CP1, CP2, CP3 and CP4.

Gr	V. N.	Reference species name	Cultivar name	Common name	C	Code	Accession numbers			
							matK	psbA-trnH	rpoB	rbcL
Group I	MIB:Zpl:03291	*Mentha piperita* L.	Piperita		MBG	MP1	FR719055	FR726096	FR720471	FR720529
	MIB:Zpl:03292	*Mentha piperita* L.	Piperita		VIR	MP2	FR719056	FR726097	FR720472	FR720530
	MIB:Zpl:03780	*Mentha piperita* L.	Piperita		ING	MP3	FR719057	FR726098	–	FR720531
	MIB:Zpl:03781	*Mentha aquatica* L.	Gigante		ING	MA1	FR719058	FR726099	–	FR720532
	MIB:Zpl:03782	*Mentha aquatica* L.	–		BGB	MA2	FR719059	FR726100	–	FR720533
	MIB:Zpl:03783	*Mentha spicata* L.	Crispa		BGB	MS1	FR719060	FR726101	FR720473	FR720534
	MIB:Zpl:03784	*Mentha spicata* L.	Maroccan		BGB	MS2	FR719061	FR726102	FR720474	FR720535
	MIB:Zpl:03301	–	–	Mint	CP2	MEC1	FR719062	FR726103	FR720475	FR720536
	MIB:Zpl:03306	–	–	Mint	CP3	MEC2	FR719063	FR726104	FR720476	FR720537
	MIB:Zpl:03785	–	–	Mint	CP4	MEC3	–	FR726105	FR720477	FR720538
Group II	MIB:Zpl:03288	*Ocimum basilicum* L.	Italian classic		MBG	OB1	FR719064	FR726106	FR720478	FR720539
	MIB:Zpl:03289	*Ocimum basilicum* L.	Italian classic		ING	OB2	FR719065	FR726107	FR720479	FR720540
	MIB:Zpl:03786	*Ocimum basilicum* L.	Italian classic		BGB	OB3	FR719066	FR726108	FR720480	FR720541
	MIB:Zpl:02997	*Ocimum gratissimum* L.	Vana tulsi		MBG	OG	FR719067	FR726109	FR720481	FR720542
	MIB:Zpl:02998	*Ocimum tenuiflorum* L.	Krisha Tulsi		MBG	OT	FR719068	FR726110	FR720482	FR720543
	MIB:Zpl:03299	–	–	Basil	CP2	BC1	FR719069	FR726111	FR720483	FR720544
	MIB:Zpl:03787	–	–	Basil	CP3	BC2	FR719070	FR726112	FR720484	FR720545
	MIB:Zpl:03788	–	–	Basil	CP4	BC3	FR719071	FR726113	FR720485	FR720546
	MIB:Zpl:02884	*Ocimum basilicum* L.	Mostruoso mammouth		ING	OBcv1	FR719072	FR726114	FR720486	FR720547
	MIB:Zpl:02885	*Ocimum basilicum* L.	Green leaves		ING	OBcv1	FR719073	FR726115	FR720487	FR720548
	MIB:Zpl:02886	*Ocimum basilicum* L.	Gecom		ING	OBcv2	FR719074	FR726116	FR720488	FR720549
	MIB:Zpl:02887	*Ocimum basilicum* L.	Red leaves		ING	OBcv3	FR719075	FR726117	FR720489	FR720550
	MIB:Zpl:02888	*Ocimum basilicum* L.	Verde a palla		ING	OBcv4	FR719076	FR726118	FR720490	FR720551
	MIB:Zpl:02889	*Ocimum basilicum* L.	Italiano		ING	OBcv5	FR719077	FR726119	FR720491	FR720552
	MIB:Zpl:02890	*Ocimum basilicum* L.	Napoletano		ING	OBcv6	FR719078	FR726120	FR720492	FR720553
	MIB:Zpl:02996	*Ocimum basilicum* L.	Scernese		ING	OBcv7	FR719079	FR726121	FR720493	FR720554
Group III	MIB:Zpl:03290	*Origanum majorana* L.	Sweet		BGB	OM1	FR719080	FR726122	FR720494	FR720555
	MIB:Zpl:03789	*Origanum majorana* L.	Sweet		VIR	OM2	FR719081	FR726123	FR720495	FR720556
	MIB:Zpl:03790	*Origanum majorana* L.	Sweet		MBG	OM3	FR719082	FR726124	FR720496	FR720557
	MIB:Zpl:03791	*Origanum majorana* L.	Sweet		ING	OM4	FR719083	FR726125	–	FR720558
	MIB:Zpl:03293	*Origanum vulgare* L.	Aureum		MBG	OV1	FR719084	FR726129	FR720497	FR720559
	MIB:Zpl:03294	*Origanum vulgare* L.	Aureum		VIR	OV2	FR719085	FR726130	FR720498	FR720560
	MIB:Zpl:03792	*Origanum vulgare* L.	Aureum		BGB	OV3	FR719086	FR726131	FR720499	FR720561
	MIB:Zpl:03793	*Origanum vulgare* L.	Gigante		ING	OV4	FR719087	FR726132	–	FR720562
	MIB:Zpl:03794	*Origanum vulgare* L.	Vulgaris		ING	OV5	FR719088	FR726133	–	FR720563
	MIB:Zpl:03795	*Origanum pseudodictamnius* Sieber	–		BGB	OP	FR719089	FR726137	FR720500	FR720564
	MIB:Zpl:03796	*Origanum heracleoticum* L.	–		BGB	OH	FR719090	FR726138	FR720501	FR720565
	MIB:Zpl:03797	–	–	Marjoram	CP1	MAC1	FR719091	FR726126	FR720502	FR720566
	MIB:Zpl:03300	–	–	Marjoram	CP2	MAC2	FR719092	FR726127	FR720503	FR720567
	MIB:Zpl:03798	–	–	Marjoram	CP4	MAC3	FR719093	FR726128	FR720504	FR720568
	MIB:Zpl:03302	–	–	Oregano	CP1	OC1	FR719094	FR726134	FR720505	FR720569
	MIB:Zpl:03799	–	–	Oregano	CP2	OC2	FR719095	FR726135	FR720506	FR720570
	MIB:Zpl:03302	–	–	Oregano	CP3	OC3	FR719096	FR726136	FR720507	FR720571
Group IV	MIB:Zpl:03800	*Salvia officinalis* L.	Albiflora		MBG	SO1	FR719097	FR726139	FR720508	FR720572
	MIB:Zpl:03801	*Salvia officinalis* L.	Albiflora		BGB	SO2	FR719098	FR726140	FR720509	FR720573
	MIB:Zpl:03297	*Salvia* officinalis L.	Albiflora		VIR	SO3	FR719099	FR726141	FR720510	FR720574
	MIB:Zpl:03802	*Salvia rutilans*	–		BGB	SR	FR719100	FR726142	FR720511	FR720575
	MIB:Zpl:03803	*Salvia sclarea*	–		BGB	SS	FR719101	FR726143	FR720512	FR720576
	MIB:Zpl:03804	*Salvia uliginosa*	–		BGB	SU	FR719102	FR726144	FR720513	FR720577
	MIB:Zpl:03304	–	–	Sage	CP2	SC1	FR719103	FR726145	FR720514	FR720578
	MIB:Zpl:03305	–	–	Sage	CP1	SC2	FR719104	FR726146	FR720515	FR720579
	MIB:Zpl:03306	–	–	Sage	CP3	SC3	FR719105	FR726147	FR720516	FR720580
Group V	MIB:Zpl:03307	*Thymus vulgaris* L.	Vulgaris		MBG	TV1	FR719106	FR726148	FR720517	FR720581
	MIB:Zpl:03308	*Thymus vulgaris* L.	Vulgaris		VIR	TV2	FR719107	FR726149	FR720518	FR720582
	MIB:Zpl:03309	*Thymus vulgaris* L.	Vulgaris		ING	TV3	FR719108	FR726150	FR720519	FR720583
	MIB:Zpl:03298		–	Thyme	CP1	TC1	FR719109	FR726151	FR720520	FR720584
	MIB:Zpl:03305		–	Thyme	CP2	TC2	FR719110	FR726152	FR720521	FR720585
	MIB:Zpl:03810		–	Thyme	CP4	TC3	FR719111	FR726153	FR720522	FR720586
Group VI	MIB:Zpl:03295	*Rosmarinus officinalis* L.	Arp		MBG	RO1	FR719112	FR726154	FR720523	FR720587
	MIB:Zpl:03296	*Rosmarinus officinalis* L.	Arp		ING	RO2	FR719113	FR726155	FR720524	FR720588
	MIB:Zpl:03811	*Rosmarinus officinalis* L.	Arp		VIR	RO3	FR719114	FR726156	FR720525	FR720589
	MIB:Zpl:03812	–	–	Rosemary	CP1	RC1	FR719115	FR726157	FR720526	FR720590
	MIB:Zpl:03303	–	–	Rosemary	CP4	RC2	FR719116	FR726158	FR720527	FR720591
	MIB:Zpl:03813	–	–	Rosemary	CP3	RC3	FR719117	FR726159	FR720528	FR720592

DNA was isolated using the DNeasy Isolation and Purification kit (Qiagen, Milan, Italy) to obtain high-quality DNA, free of polysaccharides or other metabolites that might interfere with DNA amplification. Purified DNA concentration of each sample was estimated both fluorometrically and by comparison of ethidium bromide-stained band intensities with λ DNA standard.

To assess the comparative performance of different DNA markers, each sample was analysed with four candidate DNA barcoding genomic regions. These included three coding (rbcL, rpoB and matK) and one non-coding (trnH-psbA intergenic spacer) plastidial DNA regions.

A PCR amplification for each candidate marker was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Italy) in a 25 μL reaction according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation step for 7 min at 94 °C, followed by 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at different temperature; see Table 2) and extension (1 min at 72 °C), and, hence, a final extension at 72 °C for 7 min. Details of primers used for amplification are provided in Table 2. The heavy DNA strands were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea. Manual editing of raw traces and subsequent alignments of forward and reverse sequences allowed us to assign edited sequences for most species. The 3′ and 5′ terminals were clipped to generate consensus sequences for each taxon.

2.3. Data analysis

The first step of the work was to provide evidence for the universality of the four DNA candidate markers. For this reason we evaluated which were the DNA markers routinely amplified and sequenced in the highest number of analysed samples, including commercial spices (powder or chopped leaves). To facilitate the interpretation of successes and failures in the amplification and sequencing of the candidate DNA regions, only the most universal primer combinations for each candidate DNA marker were tested (Table 2). For all taxa and loci, we conducted PCR amplification in a two-stage trial. In the first stage, we used the standard PCR conditions described above, starting from 10 ng of DNA template. The second stage was performed only on those samples that did not generate any amplicons or that produced multiple and/or aspecific PCR products. The samples that failed to amplify were repeatedly amplified at lower stringency conditions: with a reduction of 5 °C in the annealing temperature (as described in Table 2) and using 40 PCR cycles. In case of a new failure, PCR products belonging to both stages were reamplified using 1 and 20 ng of DNA template. Only in cases of negative amplification with all conditions the PCR reaction was considered a failure and the related samples were removed from the dataset.

According to the guidelines provided by the Consortium for the Barcoding of Life (http://www.barcoding.si.edu/protocols.html), the evaluation of comparative levels of variability and discrimination power for the four markers were undertaken using MEGA 4.0 (Tamura, Dudley, Nei, & Kumar, 2007). In particular Kimura 2-parameter (K2P) distance matrices were generated for each locus and for each taxonomic group (as listed in Table 1) using as a reference only those samples identified by morphology during sampling collection therefore excluding commercial processed spices samples. Intra and interspecific comparisons were calculated in an attempt to define the levels of molecular variability within each group. Finally, the same approach was conducted using differential combinations of the markers considered.

To give a schematic view of the relationships among the reference species considered and to track the correctness of the taxonomic assignment of commercial spices samples, a phenetic tree was generated for the most variable markers. Each tree has been obtained using MEGA 4.0 — options = tree inference method: neighbor-joining; phylogeny test and options: bootstrap (500 replicates); gaps/missing data: pairwise deletion; codon positions: 1st + 2nd + 3rd + non-coding; substitution model: K2P; substitutions to include: transitions + transversions; pattern among lineages: same (homogeneous); rates among sites: uniform rates.

Table 2
Primer and PCR annealing temperatures used in the present study for the four selected DNA barcoding markers.

Locus	Primer name	Sequences (5′–3′)	Annealing temperature	Ref.
matK	matK 390	CGATCTATTCATTCAATATTC	48 °C	Cuènoud et al., 2002
	matK 1326	TCTAGCACACGAAAGTCGAAGT		
trnH-psbA	psbA	GTTATGCATGAACGTAATGCTC	53 °C	Newmaster & Ragupathy, 2009
	trnH	CGCGCATGGTGGATTCACAATCC		
rpoB	rpoB 1F	AAGTGCATTGTTGGAACTGG	55 °C	Fazekas et al., 2008
	rpoB 4R	GATCCCAGCATCACAATTCC		
rbcL	rbcL 1F	ATGTCACCACAAACAGAAAC	48 °C	Fay, Bayer, Alverson, de Bruijn, & Chase, 1998
	rbcL 724R	TCGCATGTACCTGCAGTAGC		

3. Results

3.1. Amplification and sequencing success

A total of 64 spices samples (Table 1) were used for DNA extraction. High DNA quality and good yield (from 30 to 50 ng/μl) were obtained from all the analysed samples, with the exclusion of some processed commercial mint (MEC1 and MEC3) and basil (BC1) samples. In these cases, electrophoretic analysis showed partially degraded DNA in the 100–1000 bp range and low yield of DNA extraction (about 20 ng/μl), (data not shown).

Results of our tests among the six selected groups showed good amplification success. All the analysed loci exhibited high PCR success with standard primers reaching a success rate near to 100%. Few cases of failed PCR amplifications occurred only in the *Group I.* One of these cases is related to the commercial mint (MEC3): despite the amplification with matK primers was repeated three times, starting from different DNA concentrations (1, 10 and 20 ng) and at low stringency conditions (see Material and methods), the results were negative in all cases. The same situation occurred for *Mentha acquatica* L. samples amplified with the rpoB primers.

All the PCR products corresponding to the four DNA markers were successfully sequenced and high quality bidirectional sequences were obtained. Some sequencing problems were encountered with the trnH-psbA for some Basil cultivar samples (i.e. OBcv2, OBcv4; OBcv7, Table 1), in part attributable to a high frequency of mononucleotide repeats that disrupt individual sequencing reads. This problematic was solved with a strong and careful manual editing of the obtained sequences.

The same sequence length was observed in all the analysed group for matK (810 bp), rbcL (551 bp) and rpoB (491 bp), while substantial differences were observed in the trnH-psbA alignment length among the different groups as well as within each group. In particular, for this barcode region, sequence length ranged from 348 to 421 bp (data not shown).

The accession numbers of the obtained sequences are listed in Table 1.

3.2. Discrimination performances of the four candidate DNA loci

In order to identify the best DNA barcode markers for spices identification and traceability, the values of genetic divergence for all the four tested loci were computed in each analysed group at different taxonomic levels (intra and interspecific comparisons) and by considering only fresh-morphologically identified samples (Table 3). In addition a phenetic tree showing the relationship among analysed samples was generated for the most efficient tested markers (Fig. 1a–c).

Table 3
Comparative performances and variability of different DNA barcoding markers. For each groups (G), the average K2P distance (transformed into percent), the standard error (S.E.) and the range of K2P variation are given for each tested marker and for three different markers combinations. For each group, the number of species (N) and the Mean Number (MN) of Barcode sequences per species with Standard Deviation (ND = Not Determinable). Both the intraspecific and the interspecific (when two or more species are available) comparisons for each group are considered.

				Single marker								Marker combination					
				matK		trnH-psbA		rpoB		rbcL		matK + trnH-psbA		matK + rbcL		trnH-psbA + rbcL	
G	N	MN (S.E.)	Comparison	Mean % variation (S.E.%)	range %	Mean % variation (S.E.%)	range %	Mean % variation (S.E.%)	range %	Mean % variation (S.E.%)	range %	Mean % variation (S.E.%)	range %	Mean % variation (S.E.%)	range %	Mean % variation (S.E.%)	range %
1	3	2.3 (0.6)	Between sp.	0.72 (0.60)	0.08–1.08	2.31 (1.70)	0.30–3.33	0 (0)	–	0.04 (0.03)	0–0.06	2.31 (1.74)	0.3–3.33	0.44 (0.33)	0.07–0.67	0.97 (0.70)	0.16–1.40
			Within sp.	0.03 (0.05)	0–0.08	0.20 (0.18)	0–0.34	0 (0)	–	0.04 (0.07)	0–0.12	0.20 (0.18)	0–0.34	0.03 (0.06)	0–0.1	0.11 (0.11)	0–0.21
2	3	4.3 (5.8)	Between sp.	1.76 (0.40)	1.26–2.09	2.75 (0.70)	2.12–3.51	0 (0)	–	0.48 (0.20)	0.36–0.73	2.07 (0.50)	1.54–2.54	1.23 (0.30)	0.89–1.43	1.40 (0.29)	1.08–1.63
			Within sp.	1.05 (0)	–	0.72 (0)	–	0 (0)	–	0 (0)	–	0.95 (0)	–	0.62 (0)	–	0.30 (0)	–
3	4	2.8 (2.1)	Between sp.	0 (0)	–	1.05 (0.53)	0–1.37	0 (0)	–	0.05 (0.03)	0–0.08	0.35 (0.18)	0–0.46	0.02 (0.01)	0–0.03	0.47 (0.24)	0–0.63
			Within sp.	0 (0)	–	1.37 (0.55)	0.98–1.76	0 (0)	–	0.10 (0.01)	0.09–0.11	0.46 (0.18)	0.33–0.59	0.04 (0)	–	0.64 (0.24)	0.47–0.81
4	4	1.5(1)	Between sp.	3.39 (1.70)	0.74–4.86	4.90 (2.47)	1.03–7.05	1.03 (0.43)	0.41–1.65	1.54 (0.85)	0.36–2.41	3.88 (1.94)	0.84–5.40	2.63 (1.32)	0.59–3.55	2.91 (1.45)	0.64–4.01
			Within sp.	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–
5	1	3 (ND)	Between sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			Within sp.	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–
6	1	3 (ND)	Between sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			Within sp.	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–

In general, the trnH-psbA ranked first in divergence values among species, followed by matK (Table 3). On the contrary, rpoB sequences showed the lower sequence divergence and for this reason the phenetic tree related to this marker was not generated.

In the first group (*Group I*), all the tested markers showed the same sequences for *M. piperita* L. and commercial mint samples. Moreover, only trnH-psbA and matK revealed differences among the three species considered: *M. piperita–M. aquatica* mean K2P distance 3.33% (s.e. 0.91%) for trnH-psbA and 1.08% (s.e. 0.32%) for matK and *M. spicata–M. aquatica* mean K2P distance 3.29% (s.e. 0.92%) for trnH-psbA and 1.0% (s.e. 0.32%) for matK (data not shown). A negligible genetic diversity was revealed between *M spicata* and *M. piperita*, and was based on 6 single base mutations for trnH-psba and only one for matK (Fig. 1a and b).

Analyses carried out on samples belonging to *Group II* showed that the sequence divergences of marker trnH-psbA, matK and rbcL clearly distinguish *O. gratissimum* L., and *O. tenuiflorum* L. from common basil (*O. basilicum* L.) with the following mean K2P distance values: *O. gratissimum–O. basilicum* 2.61% (s.e. 0.76%) for trnH-psbA, 1.92% (s.e. 0.43%) for matK and 0.73% (s.e. 0.35%) for rbcL; *O. tenuiflorum–O. basilicum* (OB1) 3.51% (s.e. 0.88%) for trnH-psbA, 2.09% (s.e. 0.45%) for matK and 0.36% (s.e. 0.25%) for rbcL. Only the sequences of the first two markers (trnH-psbA and matK) showed appreciable differences among the analysed basil cultivars (Fig. 1) with mean K2P distance values of 0.82% (s.e. 0.5%) for trnH-psbA and 1.21% (s.e. 0.6%) for matK. In both cases the observed differences were attributable to SNP — Single Nucleotide Polymorphisms (10 and 27 SNP for trnH-psbA and matK, respectively).

Analyses carried out with matK and rpoB on *Origanum* samples (*Group III*) did not show any sequence polymorphism (Table 3). Only two single nucleotide polymorphisms in the rbcL region were detected between the three commercial oregano and the other analysed samples. Sequences of trnH-psbA marker showed several genetic differences among samples and surprisingly, the intraspecific genetic diversity was higher than the interspecific one, both for trnH-psbA and rbcL (Table 3 and Fig. 1a and c). Finally, a clear distinction between commercial oregano and the morphologically identified *Origanum* samples used as reference samples was observed for this marker (10.8%; SE 1.78%).

The analyses performed on *Group IV* clearly show the identity between *S. officinalis* L. and commercial sage samples for all the tested markers. Moreover, all DNA markers clearly distinguished these from the other tested samples, belonging to the genus *Salvia*.

Analyses performed on samples of *Group V* and *Group VI* suggested that all the commercial spices share the same genetic constitution of original spontaneous plants (*Thymus vulgaris* L. and *Rosmarinus officinalis* L); however, only matK and trnH-psbA were able to clearly distinguish these groups from the other tested spices (Fig. 1).

To evaluate potential benefits of multilocus barcodes over a single-marker we examined multiple combinations of the three plastidial markers that showed appreciable genetic diversity levels in the previous analyses: trnH-psbA, matK and rbcL. Comparative K2P variability results for the tested combinations are provided in Table 3. The most suitable combination was the matK + trhH-psbA; however, the observed K2P distance values were very similar to the performance of the best single locus (trnH-psbA) for all of the analysed groups.

4. Discussion

4.1. Detection of the best DNA barcoding marker to spices identification

The globalization of the food trade requires the development of integrated approaches, such as traceability of origin, quality and authenticity of commercialized products, to ensure food safety (Dalvit, De Marchi, Targhetta, Gervaso, & Cassandro, 2008; Barbuto et al., 2010). The main goal of this work was to define a system for the traceability of commercial spices using a 'DNA barcoding' approach. A first result of our work is that in almost all the cases considered, the industrial processes that have been conducted on the commercial spices samples collected (e.g. crumbling, drying) do not affect the success rate of DNA extraction, amplification and sequencing therefore allowing the analysis through a DNA barcoding approach.

Since an ideal DNA barcode should be applied to a large number of species with standard PCR conditions (Hebert et al., 2003; Chase et al., 2007), our work showed that all the tested markers generated positive results among the analysed groups with a single step of PCR conditions. This is an impressive performance if the wide range of genera encompassed by our analyses is considered. However, the

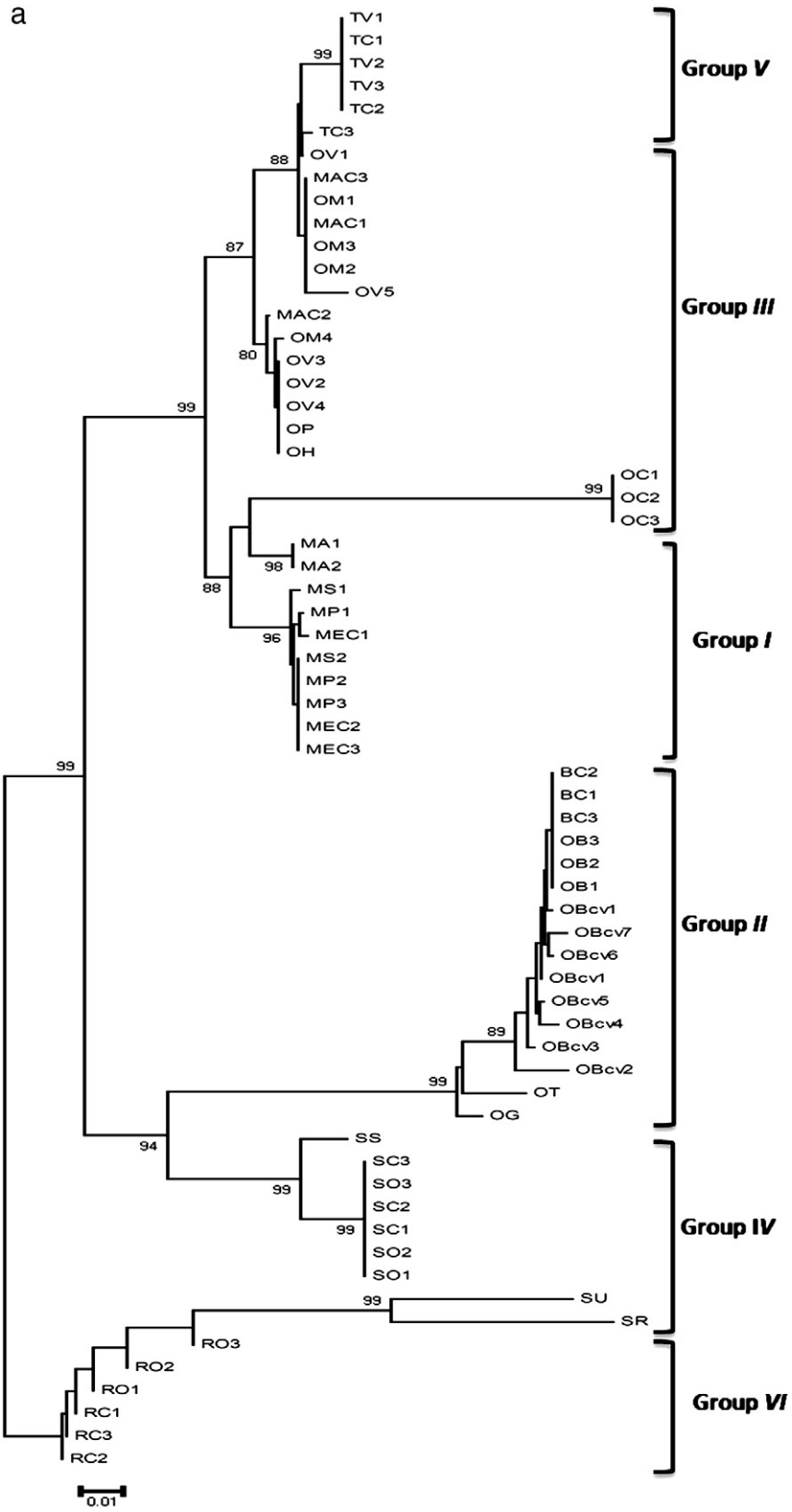

Fig. 1. Neighbor-joining reconstructions obtained with MEGA 4.0 for three out of the four molecular datasets produced in this study. Each tree encompasses all the samples analysed for the six taxonomical group considered: a) trnH-psbA, b) matK, c) rbcL. Bootstrap values lower than 70% not showed. Details on samples, species, cultivar, provenance and accession numbers for each marker can be retrieved from Table 1. Each taxonomic group has been shown on the tree with squared brackets.

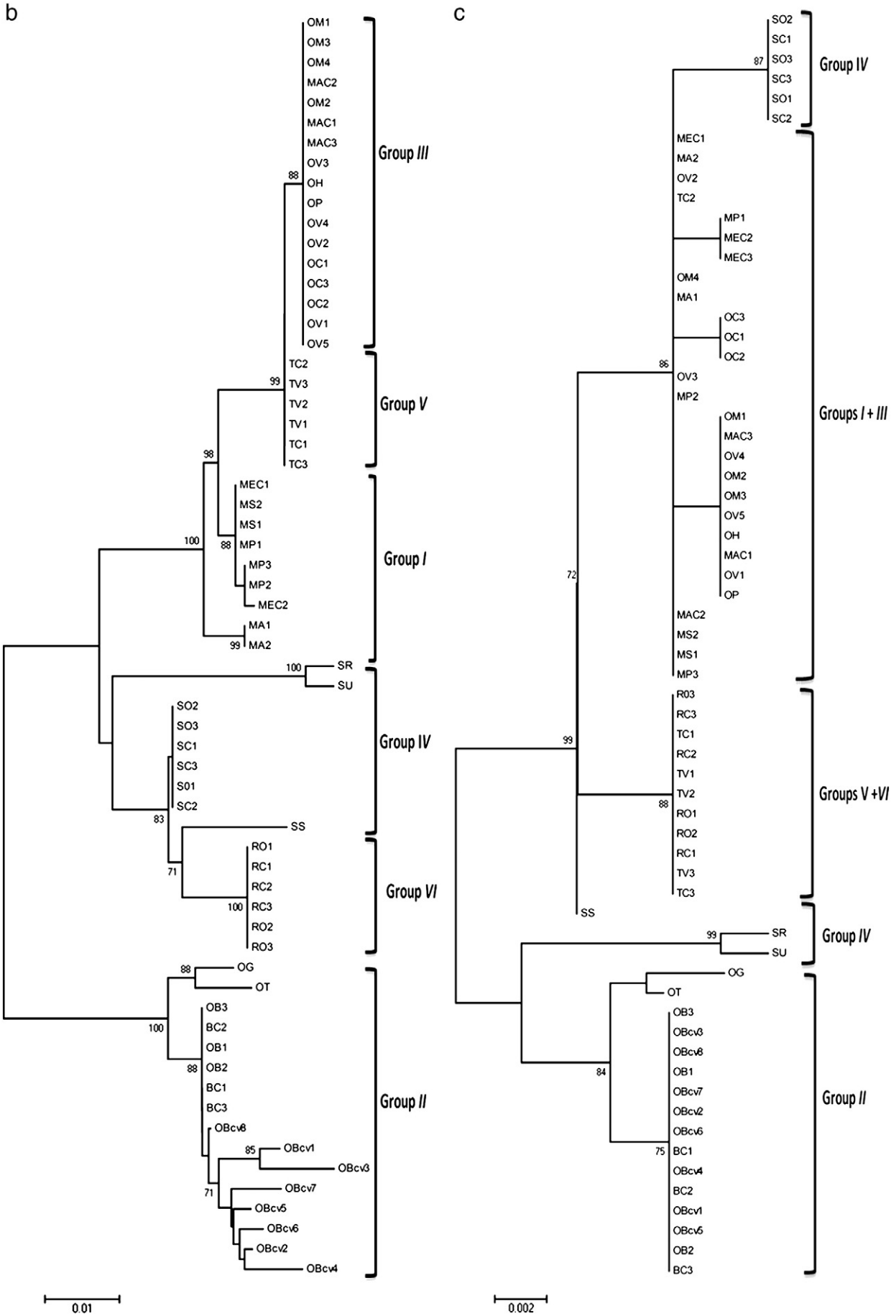

Fig. 1 (*continued*).

trade-off in the universality of the rpoB is its relatively low discrimination power at the species level as showed in all the analysed group. For this reason, we considered this marker unsuitable for the aromatic plants identification. Similar data were observed for rbcL that showed low discrimination values at cultivars level (i.e. *Group II*) as well as among different spices taxonomic groups (Fig. 1). Similar results were obtained in previous investigations (Fazekas et al., 2008; Hollingsworth, Clark, et al., 2009) carried out on large plant datasets in which this marker showed a low mutation rate and discrimination values.

The non-coding trnH-psbA intergenic spacer is the most viable candidate in all six analysed groups. In particular, the presence of highly conserved PCR priming sites combined with a non-coding region that exhibits high numbers of substitutions, make the trnH-psbA spacer highly suitable as a plant barcode. Previous researches suggested that during the alignment of sequences of this intergenic spacer, several problems can occur, especially in monocots (Chase et al., 2007) and conifers (Hollingsworth, Clark, et al., 2009). Some difficulties in obtaining bidirectional sequences of good quality were also encountered in our dataset in the case of some basil samples. However, we consider this technical problem easily solvable with the use of appropriate polymerases as suggested by Fazekas, Steeves, and Newmaster (2010a). Based on the patterns of genetic diversity detected in the tested spices groups and given the comparative performances of the analysed markers (Fig. 1), we concur with Kress and Erickson (2007) that trnH-psbA is the most suitable marker for DNA barcoding of plants. Concerning non-coding sequences, also the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (18S–5.8S–26S) could be a good candidate for plant DNA barcoding; however, this marker shows divergent paralogues that require cloning of multiple copies, and secondary structure problems resulting in poor-quality sequence data (Kress et al., 2005; Bruni et al., 2010). The matK gene showed easy amplification and alignment in the analysed taxa, however a good level of discrimination based on this marker was observed only in some spices groups (*Group II* and *Group IV*, see Table 3). Although, CBOL Plant Working Group (www.barcoding.si.edu/plant_working_group.html) identified this gene as one of the universal DNA barcode for flower plants, our analysis suggests that matK is not the best DNA barcode for spices identification.

The key foundation step in the context of plant DNA barcoding is to reach an international agreement on the definition of a standardized set of loci showing sufficient levels of variability to enable large-scale sequencing and the development of a global plant barcoding database. The CBOL suggests the use of the plastidial matK as a standard barcode combined with another coding gene such as rbcL (Hollingsworth, Forresta, et al., 2009). Although our results clearly showed that a multilocus barcoding approach does not increase the species discrimination rate, according to the CBOL, we suggest the combination of the matK gene with another marker, but we propose the trnH-psbA spacer instead of rbcL. This non-coding region, initially excluded by CBOL due to the problems in obtaining high quality bidirectional sequences, offers resolution values in Lamiaceae species identification that are higher than the other tested makers (Fig. 1). In addition recent improvements in DNA amplification largely reduced the occurrence of sequence quality problems caused by the presence of mononucleotide repeats (Fazekas, Steeves, Newmaster, & Hollingsworth, 2010b).

4.2. Spices traceability from field to table

The food traceability process has been indicated as a production action to improve reliability of labelling, to certify the origin and quality of products on the market, and to prevent fraudulent or deceptive labelling (European Commission, 2002). Our tests carried out on six groups of Lamiaceae largely used as spices for cooking showed that in some cases spices are characterized by consistent traceability problems. In the case of mint group (*Group I*), trnH-psbA and matk markers clearly distinguish *M. aquatica* L. from the other two *Mentha* species; however, trnH-psbA and matK sequences showed low genetic differences and complete genetic identity between *M. piperita* L. and *M. spicata* L. We emphasize that the genus *Mentha* is characterized by a large number of species and hybrids (Gobert, Moja, Colson, & Taberlet, 2002) and that peppermint (*M. piperita* L.) is a sterile hybrid of *M. aquatica* L. × *M. spicata* L. (Tucker, 1992). The chloroplast uniparental markers used in this study, confirm that *M. spicata* L. is the maternal parental of *M. piperita* L. because both species showed the same plastidial DNA profile. However, to confirm definitively the hybrid origin of *M. piperita* L. and identify both parental species, co-dominant markers should be considered (Bruni et al., 2010; Trindade, 2010). Natural interspecific hybridization occurs with high frequency in section Mentha, both in wild populations and in cultivation. Most commercial hybrids are sterile or subfertile, but vegetative propagation enables them to persist. Complex hybrid populations may arise, and if they are subfertile, they may cross with parental or nonparental species. This situation leads to large genetic diversity and to several taxonomic problems, further complicated by polyploidy and vegetative propagation phenomena. These events may have generated the genetic differences detected by trnH-psbA among different analysed mints (Fig. 1a). Based on these data we can therefore conclude that the DNA barcoding approach cannot be considered as a good traceability tool for mint group, because it is not able to distinguish different hybrids and these from their parents. It should be considered that this is not a problem of DNA barcoding only, but a clear limitation of all the molecular approaches based on plastidial markers in plant kingdom (Bruni et al., 2010).

A similar situation was observed for the *Origanum* L. (*Group III*). This genus contains two important spices commonly used as spices for cooking with different secondary metabolite content: marjoram and oregano. The aromatic quality of marjoram is generally found in one species in the section Majorana only (*O. majorana* L.). In contrast to marjoram, the quality of oregano arises from many different species, subspecies, varieties, and hybrids that can be distinguished individually, although extensive variation still exists. However, the best qualities of oregano come from different subspecies of *O. vulgaris*, *O. onites* and *O. syriacum* (Baser, Azek, Tümen, & Sezik, 1993; Azizi et al., 2009). Our molecular data, obtained using trnH-psbA marker, confirm these considerations: a moderate genetic variability was detected among the marjoram samples while large genetic differences were observed among *O. vulgaris* accessions and related species. We conclude that the DNA barcoding approach is not suitable for *Origanum* traceability because this genus seems to show a large genetic promiscuity. In particular, commercial accession often derive from hybridization events between different species (Gounaris et al., 2002) thus the intraspecific genetic diversity could be higher than the interspecific one.

The situation is different in the case of basil: both trnH-psbA and matK clearly distinguish the *Ocimum basilicum* L. from the other two species, as well as different analysed cultivars (Fig. 1). The genus *Ocimum* L., comprises 30–160 annual and perennial herbs and shrubs (Paton, Harley, & Harley, 1999) with a lot of these deriving from hybridization events; moreover, several species are commercial cultivars. Among the genus, *O. basilicum* L. is the most economically important species consisting of a large number of cultivars with different genetic constitution as detected by discontinuous markers such as AFLP — Amplified Fragments Length Polymorphism — (Labra et al., 2004; Carovic-Stanko et al., 2010). Our analyses confirmed these data by using universal DNA barcoding tools; considering that the analysed basil cultivars showed private DNA barcoding profiles, we can conclude the DNA barcoding is a suitable tool for tracking the basil from the field to the consumer's table.

In the case of sage, thyme and rosemary (*Groups IV*, *V* and *VI*), the commercial spices showed the same DNA barcoding profiles of the

related original plant species. In this preliminary work, for rosemary and thyme, congeneric species or cultivars were not analysed, while in the case of sage clear genetic differences were observed between *S. officinalis* — commercial sage and the other *Salvia* species for all the tested markers. These data suggest that a DNA barcoding approach can univocally identify edible sage from the other ornamental species which in some cases can be toxic for humans (Vohra, Seefeld, Cantrell, & Clark, 2009).

5. Conclusion

In conclusion, the European Union has considered the use of high-quality raw material in food production as a prerequisite to obtain a genuine and safe product of adequate nutritional value (Commission of the European Communities, 2000). Consequently molecular traceability is assuming a particular relevance in the global process of food production and marketing (Dalvit et al., 2008; Costa, Mafra, Amaral, & Oliveira, 2010). We consider DNA barcoding as a promising tool in providing a practical and standardized identification of aromatic plants (useful also for their traceability). The next step in this research would be the establishment of a dedicated aromatic plant DNA barcoding database in which all species and cultivars are described under the morphological and molecular approaches (based on trnH-psbA alone or in combination with matK). Based on this large database it will be possible to better evaluate the discrimination power of different DNA barcoding markers and the support of proper bioinformatic tools (Casiraghi, Labra, Ferri, Galimberti, & De Mattia, 2010) will lead to the development of an innovative tool suitable for rapid spices identification during the industrial production process.

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