RESEARCH NOTE

Upgrade of *Castanea sativa* (Mill.) genetic resources by sequencing of barcode markers

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Introduction

The *Castanea* genus belongs to the family Fagaceae. The first scientific evidence of its existence traces back to the late Eocene (Giordano 1993; Xiang *et al.* 2000; Bounous 2001; Donoghue *et al.* 2001). In the past two decades, historical, morphological, palynological, archaeobotanical and molecular analyses were performed to clarify evolutionary history and domestication of *Castanea* species, but in all cases, the results were limited or inconclusive. Different data demonstrate how this taxon probably originated in Asia, although contrasting opinions are reported in the literature about the exact place of its inception and the pathway of its geographical diffusion worldwide (Villani *et al.* 1994; Huang *et al.* 1998; Stanford 1998; Manos *et al.* 2001; Manos and Stanford 2001; Dane *et al.* 2003). In particular, two theories are essentially supported by scientists. The principal hypothesis identifies Turkish region as the original site of *Castanea* genus that later would have spread to Europe and America by two independent ways, a westward and an eastward expansion, respectively (Jaynes 1975; Bounous 2002). On the other hand, the second supposition suggests a Chinese origin, a unique westward migration via Europe to North America (Lang *et al.* 2007). During the worldwide distribution, several *Castanea* species were evolved, but among them only *C. sativa* (Mill.), the sweet chestnut, and *C. latifolia* (Blume) were native to Europe (Paganelli 1997). The complexity of this genus and the impossibility to obtain clear details about its phylogeny is probably due to Pleistocene glaciations. In fact, during the tertiary period, *Castanea* trees were extensively and continuously spreading in continents. On the contrary, in the quaternary era, two ice ages determined the extinction of some *Castanea* species, such as the European *C. latifolia*, and the discontinuity of plant natural arrangement and dispersal, produced evolutionary gaps. The plants which survived these climatic changes were those that were able to individuate specific geographical refugia characterized by a favourable microenvironment, for e.g. the Iberian peninsula (Krebs *et al.* 2004; Lang *et al.* 2007; Fernández-López and Monteagudo 2010; Martín *et al.* 2012). The extreme plasticity of *Castanea* genetics is another important element that should be considered in this context. On an average, indeed, these botanical species present, in their plastidial genomes, an elevated percentage of (A+T) rich regions that are well known to be subjected to replication slippage, a phenomenon that enhances nucleotide transversions during DNA synthesis. This mechanism could further justify the adaptability of *Castanea* species to the adverse climates of past geological eras but it would also explain their hypervariable genetic profiles (Lang *et al.* 2006). These days, *C. sativa* pattern of parentage and gene flow in Europe and in Italy are still confused and ambiguous, probably, because in the past millenniums, the diffusion of this species in the Mediterranean area was not spontaneous but strongly influenced by human impact. In fact, from ancient Romans and Greeks, and until today, this species is highly used for food and wood production and, as reported by Latin literature, also for medicinal purposes (Fineschi *et al.* 2000; Casasoli *et al.* 2001; Conedera *et al.* 2004; Mattioni *et al.* 2008). Because of the important ecological and commercial role of this genus, different projects were financed to realize germplasm collections (i.e. Chestnut Regional Repository in Chiusa Pesio, Cuneo Province, Italy; National Chestnut Germplasm Repository of China) of chestnut ecotypes for the preservation of their biodiversity (Guo-Tian *et al.* 2009; Mellano *et al.* 2012). However, similar successful results are not obtained for the creation of specific *C. sativa* molecular databases that are absent, or still in progress, in the scientific world. According to these forewords, the aim of the present study was to sequence

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five nuclear and plastidial barcode genes (rbcL, matK, trnH-psbA, nITS and 18S rDNA, see 'Results and discussion' section for details) of *C. sativa* with the aim to: (i) increase the genetic knowledge of this species; (ii) upgrade the scientific databases by registering obtained nucleotide sequences; (iii) compare our results with other literature data to extrapolate significant molecular features of these barcode genes in *Castanea* genus; (iv) promote the application of the barcoding technique for new and further studies to shed light on *Castanea* phylogeny, history, geographical distribution and origin.

Materials and methods

Plant material and genetic analysis

Different accessions of *C. sativa* species were collected in four different areas, namely, Caserma Palazzo, Accettura, Castel Mezzano and Montepiano, of the regional Natural Park 'Gallipoli Cognato Piccole Dolomiti Lucane' in Basilicata region, south of Italy (Loc. Palazzo, Accettura, Matera District). In springtime, 10 young leaves were sampled and analysed for each accession (a total of 40 samples) to obtain significant data and to verify the repeatability of the results. DNA was extracted from plant material according to Gismondi and Canini (2013) method. PCR amplifications, gel electrophoresis and sequencing protocol were performed as reported in Gismondi *et al.* (2013a). In particular, 20 μM of each primer was employed for all the PCR reactions, while a specific annealing temperature was used for each primer pairs, as reported in table 1. Sequences were visualized and aligned by BioEdit ver. 7.0.5 software. No difference was detected during the genetic analysis of leaves collected from the same accession. Nucleotide sequence identification, comparison and similarity percentage with the other accessions registered in the scientific database of GenBank was performed using BLAST and in particular MEGABLAST (blastn), a function that optimizes the results according to 'highly similar sequences'. All the sequences identified in this study were registered in GenBank (accession no. KP096452, KP096453, KP096454, KP096455, KP096456; as reported in table 2).

Table 1. Primer pairs, sequences and melting temperatures used in this study.

Primer pair	Sequence* (5′−3′)	T_m (°C)
rbcL	F: ATGTCACCACAAACAGAGACTAAAGC	57.2
	R: CTTCTGCTACAAATAAGAATCGATCTC	
matK	F: GTTCTAGCACAAGAAAGTCGA	57.2
	R: CCTATCCATCTGGAAATCTTAG	
trnH-psbA	F: CGCGCATGGTGGATTCACAAT	56.5
	R: GTTATGCATGAACGTAATGCTC	
nITS	F: TCCTCCGCTTATTGATATGC	58.5
	R: CCTTATCATTTAGAGGAAGGAG	
18S rDNA	F: CGCATCATTCAAATTTCTGC	58.5
	R: TTCAGCCTTGCGACCATACT	

*Kress *et al.* 2005; Gambino and Gribaudo 2006; Kress and Erickson 2007.

Results and discussion

The high economic value of *C. sativa* and the risk of its extinction because of parasites, pathogens, climatic changes and human activities have made the scientific community to build new projects aiming at studying the genetic variability of this species and stopping the loss of its biodiversity. Different molecular markers (i.e. STR) and methods (i.e. AFLP, RAPD) were developed to characterize Italian and European chestnut varieties and to get new insights into *Castanea* evolutionary history (Fineschi *et al*. 2000; Marinoni *et al*. 2003; Mattioni *et al*. 2008; Martin *et al*. 2010; Mellano *et al*. 2012). However, no complete and unambiguous data were obtained in cited studies. Therefore, since barcoding technique has recently obtained a large consensus in biology, the first objective of this study was the suggestion of the use of this approach to definitely clarify *Castanea* phylogeny. In fact, it uses standard DNA sequence analysis for species identification and classification (Kress *et al.* 2005; Kress and Erickson 2007). According to our expertise in this field (Gismondi *et al.* 2012, 2013b), the best candidates as plant barcode sequences are the following three plastidial genes used in combination: ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCO large subunit, rbcL) gene; maturase K (matK) gene; intergenic spacer between tRNA-HisGUG gene and photosystem II thylakoid membrane protein of Mr 32,000 gene (trnH-psbA). Moreover, we think that the supplementation to this list of two other nuclear barcode sequences (internal transcribed spacer 1, 5.8S ribosomal RNA gene; internal transcribed spacer 2, nITS; 18S ribosomal RNA gene, 18S rDNA) could also be very useful. In reality, a few literature works (Stanford 1998; Fineschi *et al*. 2000; Lang *et al*. 2006, 2007) tried to investigate *Castanea* genetics using the barcoding technology but they did not produce interesting or informative results, probably because they performed the phylogenetic analyses: (i) just studying one marker at a time and not in a cumulative way; (ii) considering inappropriate or insufficiently variable barcode genes; (iii) sequencing too much short genetic regions; (iv) carrying out experiments on less amount of samples, even geographically very close. In the scientific literature, 156 articles meet to the keywords '*C. sativa*' and among them only about 25 works are based on the genetics of this species. On the other hand, in the GenBank database, 210 accessions are reported for *C. sativa* but only 45 of them describe barcoding sequences; however, a lot of these data are redundant or very short. Because of all these reasons, in this study, we sequenced five barcode genes (rbcL, matK, trnH-psbA, nITS and 18S rDNA) starting from *C. sativa* accessions collected in a Natural Park in the South of Italy (Basilicata region), to get a genetic integration of the molecular knowledge of this species (table 2). Obtained sequences are registered in the nucleotide database

Table 2. Nucleotide sequences of *C. sativa* barcode genes are reported. In particular, in nITS sequence, the position of three polymorphisms detected in *Accettura* accessions (C375T, C412A and C418G) are reported as bold and underlined nucleotides.

Barcode gene	Sequence (5′–3′)	Type of genome (bp) GenBank accession no.
rbcL	CCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAA GATTATAAATTGACTTATTATAGTCCTGACTATCAAACCAAAGATACTG ATATCTTGGCAGCCTTCCGAGTAACTCCTCAACCCCGAGCTCCGCCGGA GGAAGCAGGGGCCGCGGTAGCTGCTGAATCTTCCACTGGGACATGGAC AACTGTGTGGACTGACGGGCTTACCAGTCTTCATCGTTACAAAGGACGA TGCTACCACATCGAGCCGGTTGCTGGAGAAGAAAATCAATCTATCGCTT ATCTACCTTACACATTACACCTCTTTGAAGAAGGTTCTGTTACTAACAT GTTTACTTCCATTGTGGGTAATGTATTTGGATTCAAGGCCATGCGCGCT CTACGTCTGGAGGATTTGCGAATCCCTACTTCTTATTCTAAAACTTTCCA AGGTCCGCCTCATGGCATCCAAGTTGAGAGGGATAAATTACACAAGTA TGGCCGCCCCCTATTACGATGTACTATTAAACCTAAATTGGGATTATCC GCTAAGAATTACGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGGCTTG ATTTTACCAAAGATGATGAGAACGTTAATTCCCAACCATTTATGCGTTG GAGAGACCGTTTCCTATTTTGTGCCGAAGCAATTTA	Plastidial (670) KP096455
matK	CATATTCTTTTTTTTTGAGGATCCGCTGTAATAATGAGAAAGATTTCTAC ATATATGCAAAAATCGATTGATAATCTCAAAATCCGATAAATCGGCCC GAGTCAGCTTACTAATTGGATGCCCTACTGCGTTACAAAATTTCGCTTT AGCCAATGATCCAATCAGAGGAATAATTGGAACTATTGTATCGAGTTTA TTGGGAGCATTATTTAGTGGAAATGAATTTTCTAGCATTTGATTCCGCA CCACTGCAGGATTTCGTCGAACACTTGAAAAGTAACTCAAAAAATCGA GGGAATGCTTGGATAATTGGTTTATACGGATACTTGCCGCGTGAGACCA TACATCAAAATGACATTGCCATAAATTGACAAGGTAAGATTTCCATTTA TTCATTAGAAGAGGTGTGTCTTTGGAAGCCAGAATTGATTTTCCTTGAT ATCTAACATAATGCATGAAAGGATCCTTGAGAAAGCATGGGATGACCG GAAAATCATTAGCAAAGACTTCGGCAAAATGTTCTATTTTTCTATATAA ACAGAGTCGTTCAAAAAGGACTCCAGAAGATGTTAATCGTAAATGAGA AGATTGGTTACGGAGAAAAAGGAAGATGGA	Plastidial (615) KP096452
trnH-psbA	CATACCCCCCAGTCTCAAGAGGAATACAAAAATTTCAATTTCTACCATT CCTCTTGTTTTATTTCCCTTTCTTATAAGATAAAAGACAAGAGGCAGAA AATAAATTGAAAGCTTTATCTTTTTTATTTAAATAAATAAATAAAAACT TCACCGATAAAAACATATATAAAATAAAAATTACTACATAAGTCAAAT AGGATACTCAAATACTTTTTCGTATCTTGAGTTAAAATAAAAAAAAGCT TATATTATATTCTATTAAGTAAAAAAGTAAAAGAAAAGACGACTAAAT AGTAATTACAATTATATAATAATTAAAGGGCTAATAAATAAGAAAGGA GCAATAATCAACTTCTTGTTTTATCAAGAGGGTTGACATTGCTCCTTTCT TATTTATTTTCAATAACTCATATATACTAAGACCTAAGTCTTATCCATTT GTAGATGGAGCTTCGACAGAAGCTAAGTCTAGAGGGAAATTATGAGCA TTACGTTCATGCATAAC	Plastidial (505) KP096456
nITS	AAGCGCCTGACTGGGGTCGCGTTGGGAGCGCCGCCGAGGCGACGCGTT AGGGTCTCGAGAGCGCGTTCGGGCGACGGGGCGCGCACGACGGGGAA CGAGGGTCGAAGAACCACCGATTGTCGTGGCGCTCGTCGCCGAGGACT CGCTTTTGGGCTAACCGCGCGCGCGGGCGCGCACGGGAGGCCAACTTC CGCCCCGCCCCGAACCGGAGTTGGGGGGGGCAACGATGCGTGACACCC AGGCAAACGTGCCCTCGGCCGAATGGCTTCGGGCGCAACTTGCGTTCA AAAACTCGATGATTCGCGGGATTCTGCAATTCACACCAAGTATCGCATT TCGCTACGTTCTTCATCGATGCGAGAGCCTAGATATCC**C**TTGCCGAGAG TCGTTTTGGATAATTCGTAAGACGCC**C**ACCCCC**C**GGGGC̲GCACCGTGTCC GGGGCCTCCGGCGTGGCTCTCTTGGT̲T̲TTGATT̲TCCTTGGCGCGTTCCGC GCCGGGGTTCGTTTTGCGGGCGAGAGACGCGGGGTCCCCGCCCGCAGT GGG	Nuclear (535) KP096453
18S rDNA	TCATTCAAATTTTTGCCCTATCAACTTTCGATGGTAAGATAGTGGCCTA CTATGGTGGGGACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGG GAGGCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCA AATTACCCAATCCTGACACGGGGAGGTAGTGACAATAAATAACAATAC CGGGCTCTCACGAGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTT AACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT TCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTA GTTGAACCTTGGGTTGGGCAGAGCGGTCCGCCCCTGGTGTGCACCGGTC TGCTCGTCCCTTCTACCGGCGATGCGCTCCTGGCCTTAACTGGCCGGGT CGTGCCTCCGGTGCTGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAG CCTACGCTCTGGATACATTAGCATGGGATAACATCATAGGATTTCGGTC CTATTCTGTTGGCCTTCGGGATCGGAGTAATGATTAACAGGGACAGTAG GGGGCATTCGTATATCATAGTCAGAG	Nuclear (609) KP096454

of GenBank and are available. Sequencing analysis was independently repeated at least five times for sample and nodifference was detected. Moreover, all the samples did not show polymorphisms among their barcode sequences, except for nITS. In fact, all the *C. sativa* samples presented the reported nITS sequence, while *Accettura* accessions showed the same sequence but with three nucleotide mutations (C375T, C412A and C418G) (table 2). This observation deserves attention because it confirms that this hypervariable barcode gene, usually adopted for interspecific taxonomic identification could also be applied to intraspecific and population studies, as suggested in Gismondi *et al.* (2013a). The five amplicons obtained from *C. sativa* samples were then compared with other nucleotide sequences of *Castanea* genus registered in GenBank (table 3). The E-value, equal or very close to zero indicated the high significance of all the matches extrapolated by BLAST. In particular, we found that:

- rbcL gene was abundantly sequenced in *C. sativa* (nine accessions); however, we observed that the nucleotide sequence of our samples possessed 2–3% of variability with respect to those deposited in the scientific database;
- matK region was identified as an highly conserved gene, as revealed by the percentage of identity (100%) of the matches between sample and *C. sativa* registered accessions;
- trnH-psbA sequence found a correspondence with two *C. sativa* accessions (FN687510.1 and HE966544.1) reported in GenBank even if the query coverage was not complete (95 and 58%, respectively); moreover, since we collected our samples in the south of Italy and we also observed that their match identity was of 99% with FN687510.1 Latial (central Italy) accession and 97% with HE966544.1 Friulian (north Italy) accession, these results suggested the possibility that in this barcode gene the nucleotide variability could be associated to the geographical distance of the specimens compared, as proposed yet by Martín *et al.* (2010);
- by this work, *C. sativa* nuclear ITS sequence, the only registered in the database (accession number EU016360.1), was extended from 361 bases to 535 nucleotides;
- *C. sativa* 18S rDNA (609 bases), for the first time was sequenced and deposited in GenBank; in fact, BLAST comparison did not find any accession corresponding to *C. sativa* 18S rDNA but it just individuated a similarity with the unique 18S ribosomal RNA gene sequence codified starting from a member of *Castanea* genus (*C. sequinii*, accession AY263902.1).

In conclusion, matK gene was identified as well-conserved plastidial region while rbcL, trnH-psbA and nITS markers were suggested as good candidates for intraspecific and population analyses. According to the results obtained from the present work, the genes identified as the best barcoding candidates for *C. sativa* phylogenetic discrimination were rbcL, for the plastid genome and nITS for the nuclear one. Moreover, since sample sequences showed interesting nucleotide

Table 3. Comparison between sample barcode genes (queries) and GenBank nucleotide sequences (subjects) obtained by BLAST application.

Species	Query cover	E-value	Identity	Accession
C. sativa rbcL region vs GenBank sequences				
C. mollissima	100	0.0	98	HQ336406.1
C. sativa	**100**	**0.0**	**98**	**FN689363.1**
C. sativa	**100**	**0.0**	**98**	**AY548965.1**
C. sativa	**100**	**0.0**	**98**	**M94936.1**
C. sativa	**97**	**0.0**	**97**	**AF500363.1**
C. henryi	97	0.0	97	KJ440003.1
C. crenata	95	0.0	97	AB060565.1
C. sativa	**94**	**0.0**	**97**	**HM849869.1**
C. sequinii	93	0.0	97	AY263937.1
C. mollissima	94	0.0	97	KF418893.1
C. sativa	**86**	**0.0**	**97**	**HE963387.1**
C. dentata	84	0.0	97	KF418892.1
C. dentata	82	0.0	97	KF613012.1
C. sativa	**80**	**0.0**	**97**	**JN891888.1**
C. sativa	**80**	**0.0**	**97**	**JN891651.1**
C. sativa	**80**	**0.0**	**97**	**JN892846.1**
C. sativa matK region vs GenBank sequences				
C. sativa	**100**	**0.0**	**100**	**JN895513.1**
C. sativa	**100**	**0.0**	**100**	**JN894680.1**
C. sativa	**100**	**0.0**	**100**	**JN894077.1**
C. mollissima	100	0.0	100	HQ336406.1
C. mollissima	100	0.0	100	EF057124.1
C. henryi	100	0.0	100	EF057123.1
C. mollissima	100	0.0	99	FJ185050.1
C. mollissima	100	0.0	99	U92862.1
C. crenata	100	0.0	99	AB107636.1
C. crenata	100	0.0	99	AB060056.1
C. sativa	**95**	**0.0**	**99**	**HE967367.1**
C. henryi	96	0.0	99	KJ510904.1
C. sativa	**95**	**0.0**	**99**	**HM850888.1**
C. sequinii	91	0.0	99	AY263920.1
C. mollissima	12	2e-33	100	AY042427.1
C. henryi	12	2e-33	100	AY042425.1
C. henryi	12	2e-33	100	AY042424.1
C. sativa	**12**	**2e-33**	**100**	**AY042423.1**
C. sativa	**12**	**2e-33**	**100**	**AY042422.1**
C. sativa	**12**	**2e-33**	**100**	**AY042421.1**
C. pumila	12	2e-33	100	AY042420.1
C. dentata	12	2e-33	100	AY042419.1
C. dentata	12	2e-33	100	AY042418.1
C. crenata	12	8e-32	99	AY042417.1
C. crenata	12	8e-32	99	AY042416.1
C. crenata	12	8e-32	99	AY042415.1
C. sativa trnH-psbA region vs GenBank sequences				
C. sativa	**95**	**0.0**	**99**	**FN687510.1**
C. dentata	93	0.0	99	JQ677925.1
C. pumila	93	0.0	99	JQ77926.1
C. dentata	93	0.0	99	JQ677921.1
C. dentata	93	0.0	99	JQ677922.1
C. pumila	93	0.0	99	JQ677932.1
C. pumila	93	0.0	99	JQ677933.1
C. pumila	93	0.0	99	JQ677931.1
C. mollissima	95	0.0	94	HQ336406.1
C. crenata	77	0.0	97	AB107687.1
C. sativa	**58**	**4e-138**	**97**	**HE966544.1**
C. sativa nITS region vs GenBank sequences				
C. pumila	96	0.0	98	AY040394.1
C. sequinii	96	0.0	98	AY040397.1
C. sequinii	96	0.0	98	AY040395.1
C. mollissima	96	0.0	97	AY040396.1
C. sativa	**68**	**0.0**	**98**	**EU016360.1**
C. sativa 18S rDNA region vs GenBank sequences				
C. sequinii	100	0.0	98	AY263902.1

Species name, % of query coverage, E-value (significance of the result, max value is 0.0), % of identity of the sequences and GenBank accession number of the subject are reported for each match. Comparison of sample sequences with those reported in literature belonging to *C. sativa* species are in bold.

differences with respect to GenBank registered genes (2–3% in rbcL; 0–1% in matK; 1–3% in trnH-psbA; 2% in nITS), we supposed that the chestnuts of the Natural Park could have antique origins or could have been subjected to a past genetic isolation. In fact, the samples used in this work were specifically chosen because they belong to very ancient *C. sativa* plantations of the Basilicata region. In fact, according to the literature, this area could represent a possible refugium for *Castanea* genus to Pleistocene glaciations (Krebs *et al.* 2004). Moreover, since both Romans and Greeks extensively used *C. sativa* plants, it is not clear how these populations contributed to the dispersal and the domestication, especially in the southern part of the Italian peninsula (Conedera *et al.* 2004; Krebs *et al.* 2004). According to these data and hypotheses, the present study, implemented with further molecular analyses, would represent and give new and very interesting insights about south Italian *C. sativa* origin, diffusion and genetics. Finally, the improvement and the upgrading of GenBank database with the *C. sativa* sequences obtained in this work will favour all the future genetic researches about this topic.

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