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Genomic Fingerprinting of *Camelina* Species Using cTBP as Molecular Marker

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Abstract

Interest on the genus *Camelina* has recently increased due to the biofuel, or jet fuel, potential of the oil extracted from seeds of the cultivated species *Camelina sativa* (L.) Crantz. While our knowledge on *C. sativa* is constantly augmenting, only few studies have been performed on the other species of the genus, which could be a potentially useful material for the genetic improvement of *C. sativa*. The genus *Camelina* consists of 11 species, but only six (*C. sativa*, *C. microcarpa*, *C. alyssum*, *C. rumelica*, *C. hispida* and *C. laxa*) could be retrieved from germplasm banks to carry out genomic fingerprinting studies based on the use of the cTBP molecular marker. Each species, with the exception of *C. alyssum* that is proposed to be a subspecies of *C. sativa*, shows a distinct cTBP profile resulting from multiple DNA length polymorphisms present in the second intron of the members of the *β*-tubulin gene family. In contrast to the high level of genetic diversity detected among the six *Camelina* species, low variability is observed among and within the accessions of the same species, except for *C. hispida* that is characterized by an intra-accession high number of cTBP polymorphic bands. In addition, cTBP is also able to identify incorrectly classified accessions and provide information on the ploidy level of each species.

Keywords

Genetic Diversity, Polymorphism, *β*-Tubulin Gene Family, False Flax, Chromosome Number

1. Introduction

The genus *Camelina* belongs to the tribe Camelineae of the *Brassicaceae* family which contains about 338 genera and over 3700 species distributed throughout the world [1]. In this last decade, interest in this genus has increased rapidly due to the biofuel or jet fuel potential of the oil extracted from the seeds of *Camelina sativa* (L.) Crantz [2]-[5]. Several authors report that the biofuel produced from *Camelina* oil can cut greenhouse gas emis-

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sions (GHG) by up to 75% compared to that of petroleum-based jet fuel [6] [7]. In addition to its use for biofuel production, a broad range of nutritional, medicinal and industrial applications of the oil have been also described [8] [9]. *Camelina* oil contains a high amount of unsaturated fatty acids (more than 90%), low concentration of erucic acid and high levels of natural antioxidants (tocopherols) [10]-[13].

Recently, many studies have been undertaken on the genetic and genomic characterization of *C. sativa*. In 2006, a preliminary genetic map of *C. sativa* has been constructed using 157 Amplified Fragment Length Polymorphism (AFLP) markers and 3 *Brassica* Simple Sequence Repeats (SSRs) [14]. Since then, the level of genetic diversity present in *Camelina* germplasm collections has been further assessed with the use of many others dominant or codominant molecular markers such as RAPDs (Random Amplification Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphisms), h-TBP (horse Tubulin-Based Polymorphysm) and SSRs (Simple Sequence Repeats) [15]-[18]. Nearly all of them have revealed the occurrence of a limited level of genetic diversity among the different *Camelina* accessions and strongly support the hypothesis of a polyploid origin for the *Camelina* genome [14] [18]. A polyploid origin is also suggested by Hutcheon *et al*. [19], who study the number and the genomic organization of genes involved in the fatty acid biosynthesis pathway, and by Galasso *et al*. [17] who similarly analyse the *β*-tubulin multigene family. Recently, a genome draft of *C. sativa* has been published and the sequence analysis confirmed that *Camelina* is a polyploid species constituted by three genome complements [20].

While our knowledge on *C. sativa* is progressively increasing, few studies have been so far performed on the taxonomy and the genetic and genomic characterization of the other species belonging to the genus *Camelina*, a source of genes potentiallly useful for widening the genetic base of *C. sativa* in the effort of improving it through classical or molecular-assisted breeding. According to Warwick *et al.* [1], the genus *Camelina* consists of 11 species. However, only the following six species are currently stored in the germplasm banks of IPK (Plant Genetics and Crop Plant Research, Germany) and USDA (United Stated Department of Agricultural, USA): *C. sativa*, *C. alyssum* (Mill.) Thell., *C. microcarpa* Andrz. ex DC., *C. rumelica* Velen, *C. hispida* var. *grandiflora* (Boiss.) Hedge and *C. laxa* C. A. Mey. According to Plessers *et al.* [21] *C. sativa*, which is the only cultivated species, comprises three subspecies: ssp. *pilosa* D.C., ssp. *sativa* s. str. Fr. and ssp. *foedita* Fr. In particular, *C. sativa* ssp. *pilosa* is a winter type and requires vernalisation to promote stem elongation and flowering, while ssp. *sativa* and ssp. *foetida* are defined as spring types since they do not require vernalisation [21]. Presently, only *C. sativa* ssp. *sativa* and ssp. *pilosa* are stored in IPK and USDA germplasm banks while no accessions of the ssp. *foetida* can be found in either of the two.

Given all these premises, we have set up experiments to fingerprint the genome of all the available *Camelina* species, based on the use, as molecular markers, of the introns of the *β*-tubulin gene family [22] [23]. The technique, named TBP (tubulin-based polymorphism) or cTBP or hTBP, depending on which intron or combination of introns is used as a marker [23] [24], relies on an exon-primed intron-crossing (EPIC) PCR reaction. Several papers have previously demonstrated that any of these TBP techniques is well suitable for genotyping new or neglected species, often characterized by poor if any genomic information [17] [24] [25].

2. Material and Methods

2.1. Plant Material

Seeds of *C. sativa* (46 accessions), *C. alyssum* (3 acc.), *C. microcarpa* (13 acc.), *C. rumelica* (2 acc.), *C. hispida* var. *grandiflora* (1 acc.) and *C. laxa* (1 acc.) were kindly provided by the IPK (http://gbis.ipk-gatersleben.de/), USDA (http://www.ars-grin.gov/) and the Arche Noah (Austrian Seed Savers Association, Austria) genebanks (**Table 1** and **Table 2**). All accessions were sown at the end of winter (10 March 2009) in order to define their life form (*i.e.* winter or spring form).

2.2. DNA Extraction, cTBP PCR Amplification and Fingerprinting

Genomic DNA was extracted from young leaf tissue of all *Camelina* accessions using the ‘‘GenElute Plant Genomic DNA Miniprep Kit’’ (SIGMA-Aldrich) in accordance with the manufacturer’s instructions. Intron2 of the *β*-tubulin gene family (**Figure 1**), chosen as the most informative molecular marker, was PCR amplified, as already described by Breviario *et al*. [23], using 10 ng of template genomic DNA and the following forward and reverse oligonucleotide primers combination: TBPfin2 (5’-GARAAYGCHGAYGARTGYATG-3’) and TBPrin2

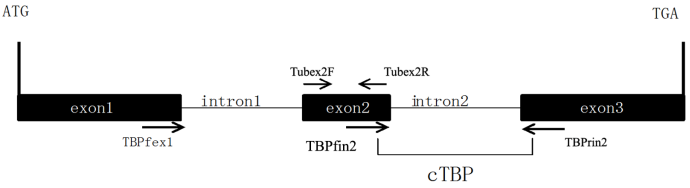

Figure 1. Schematic representation of a typical plant *β*-tubulin genomic locus. Arrows indicate different primers in their respective position and orientation. ATG and TGA indicate the start and stop codon, respectively. The bracket encompasses the region amplified by the cTBP method.

Table 1. List of 46 accessions of *Camelina sativa* used in this study with their life form, accession number, donor and country of origin.

	Life form (a)	Life form (b)	Accession number	Donor	Country of origin
C. sativa subsp. *sativa*	Spring type	Spring type	CAM108	IPK*	Poland
C. sativa subsp. *sativa*	Spring type	Spring type	CAM110	IPK	Poland
C. sativa subsp. *sativa*	Spring type	Spring type	CAM111	IPK	URSS^
C. sativa subsp. *sativa*	Spring type	Spring type	CAM116	IPK	Belgium
C. sativa subsp. *sativa*	Spring type	Spring type	CAM123	IPK	Poland
C. sativa subsp. *sativa*	Spring type	Spring type	CAM136	IPK	Poland
C. sativa subsp. *sativa*	Spring type	Spring type	CAM137	IPK	Denmark
C. sativa subsp. *sativa*	Spring type	Spring type	CAM170	IPK	Poland
C. sativa subsp. *sativa*	Spring type	Spring type	CAM171	IPK	Unkn
C. sativa subsp. *sativa*	Spring type	Spring type	CAM173	IPK	URSS
C. sativa subsp. *sativa*	Spring type	Spring type	CAM174	IPK	Unkn
C. sativa subsp. *sativa*	Spring type	Spring type	CAM175	IPK	Sweden
C. sativa subsp. *sativa*	Spring type	Spring type	CAM187	IPK	Spain
C. sativa subsp. *sativa*	Spring type	Spring type	CAM266	IPK	URSS
C. sativa subsp. *sativa*	Spring type	Spring type	CAM268	IPK	Bulgaria
C. sativa subsp. *sativa*	Spring type	Spring type	CAM265	IPK	Italy
C. sativa subsp. *sativa*	Spring type	Spring type	CAM58	IPK	Germany
C. sativa subsp. *sativa*	Unkn	Spring type	CAM7	IPK	Kyrgyzstan
C. sativa subsp. *sativa*	Unkn	Spring type	CAM8	IPK	URSS
C. sativa subsp. *sativa*	Unkn	Spring type	CAM29	IPK	Ukraine
C. sativa subsp. *sativa*	Unkn	Spring type	CAM25	IPK	URSS
C. sativa subsp. *sativa*	Unkn	Winter type	CAM31	IPK	Poland
C. sativa subsp. *sativa*	Unkn	Spring type	CAM34	IPK	URSS
C. sativa subsp. *sativa*	Unkn	Spring type	CAM35	IPK	URSS
C. sativa subsp. *sativa*	Unkn	Winter type	CAM37	IPK	URSS
C. sativa subsp. *sativa*	Unkn	Spring type	CAM38	IPK	Austria
C. sativa subsp. *sativa*	Unkn	Spring type	CAM40	IPK	Unkn
C. sativa subsp. *sativa*	Winter type	Spring type	CAM172	IPK	URSS
C. sativa subsp. *sativa*	Unkn	Spring type	CAM134	IPK	Germany
C. sativa subsp. *sativa*	Spring type	Spring type	CAM120	IPK	Poland
C. sativa subsp. *pilosa*	Unkn	Spring type	CAM39	IPK	Austria
C. sativa subsp. *pilosa*	Winter type	Winter type	CAM76	IPK	URSS

Continued

C. sativa subsp. *pilosa*	Winter type	Winter type	D9952	IPK	Unkn
C. sativa subsp. *pilosa*	Winter type	Winter type	CAM132	IPK	Unkn
C. sativa subsp. *pilosa*	Spring type	Spring type	CAM270	IPK	Swiss
C. sativa subsp. *pilosa*	Unkn	Spring type	CAM180	IPK	Germany
C. sativa ssp.	Spring type	Spring type	CAM269	IPK	United kingdom
C. sativa ssp.	Spring type	Spring type	CAM45	IPK	URSS
C. sativa ssp.	Spring type	Spring type	CAM46	IPK	Unkn
C. sativa ssp.	Unkn	Winter type	PI650168	USDA[±]	United States
C. sativa ssp.	Unkn	Spring type	PI650146	USDA	Sweden
C. sativa ssp.	Unkn	Spring type	PI650142	USDA	Denmark
C. sativa ssp.	Unkn	Spring type	FF084	Arche[∞]	Austria
C. sativa ssp.	Unkn	Spring type	FF006	Arche	Austria
C. sativa ssp.	Unkn	Spring type	FF004	Arche	Austria
C. sativa ssp.	Unkn	Winter type	PI650167	USDA	Poland

(a) Life form as reported in the genebanks; (b) Life form as verified in this work. Unkn= unknown life form or country of origin. [*]IPK, Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany; [±]USDA, United States Department of Agriculture, USA. [∞]Arche Noah, The Austrian Seed Savers Association, Austria; [^]URSS, Union of Soviet Republics.

Table 2. List of *Camelina* species used in this study with their accession number, donor and country of origin.

Camelina species	Accession number	Origin	Donor
C. microcarpa	PI650135	France	USDA[±]
	PI633191	Montana, USA	USDA
	PI650134	Spain	USDA
	PI633190	Germany	USDA
	PI633188	Poland	USDA
	PI633186	Hungary	USDA
	CAM6	Germany	IPK[*]
	CAM47	Germany	IPK
	CAM48	Germany	IPK
	CAM51	unknown	IPK
	CAM60	unknown	IPK
	CAM71	unknown	IPK
	CAM75	Germany	IPK
C. alyssum subsp. *alyssum*	CAM176	unknown	IPK
C. alyssum	CAM21	Germany	IPK
C. alyssum	PI650132	Germany	USDA
C. rumelica	PI650138	Iran	USDA
C. rumelica	CAM244	URSS	IPK
C. hispida var. *grandiflora*	PI650133	Turkey	USDA
C. laxa	PI633185	Turkey	USDA

[*]IPK Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany. [±]USDA United States Department of Agriculture, USA.

(5'-CRAAVCCBACCATGAARAARTG-3'). For each accession, a total of 3 up to 15 individual plants were

analysed. Amplified products were separated on a 6% non-denaturing acrylamide gel and bands were visualized by silver nitrate staining as reported in Breviario *et al*. [23]. Experiments were independently repeated two or three times, to guarantee the consistency of the genomic profile attributable to each species.

2.3. DNA Cloning, Sequence Comparison and Phylogenetic Analysis

Tubulin nucleotide sequences identification and isolation was carried out as reported in Galasso *et al*. [17]. For each species only one accession was chosen, selected as the most representative when not the only one available. Selected accessions were: *C. sativa* CAM134, *C. microcarpa* CAM47, *C. rumelica* CAM244, *C. hispida* PI650133 and *C. laxa* PI633185. The procedure is briefly described from here to follow. First, the *β*-tubulin gene family of each accession was PCR amplified from target genomic DNA using the primers combination TBPfex1 (5'-AACTGGGCBAARGGNCAYTAYAC-3') and TBPrin2 (5'-CRAAVCCBACCATGAARAARTG-3). TBPfex1 anneals at the end of the first exon while TBPrin2 matches the complementary target sequence at the beginning of the third exon (**Figure 1**). The PCR products resulting from this amplification step were purified and cloned into the pGEM-T easy vector (Promega). About 100 clones for each accession were sequenced in both directions by Macrogen (Seul, Korea). Search on nucleotide sequence homologies was carried out consulting the National Center Biotechnology Information (NCBI) database, with the use of the BLAST algorithm (http://www.ncbi.nlm.nih.gov). A search for tandem repeat motifs and microsatellite sequences was performed using the Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html) and Sputnik (http://www.cbib.u-bordeaux2.fr/pise/sputnik.html) programs. Nucleotide sequence has been deposited in the EMBL (European Molecular Biology Laboratory) Nucleotide Database with its own specific accession number (from LN811270 to LN811335) (**ESM-Table 1**). Nucleotide sequences of all the investigated *Camelina* species were multialigned with the Clustal Omega programme (http://www.ebi.ac.uk/Tools/msa/clustalo/) using default parameters. The evolutionary genetic distance was estimated using Tamura-Nei method and a Maximum Likelihood phylogenetic tree was inferred using the Nearest-Neighbour-Interchange method using MEGA version 5 [26].

2.4. Southern Analysis

Genomic DNA was extracted from fresh leaf tissue, according to the method reported by Doyle and Doyle [27]. For Southern hybridisation, 5 µg of *C. sativa* CAM134, *C. alyssum* CAM21, *C. microcarpa* CAM47, *C. rumelica* CAM244, *C. hispida* PI650133 and *C. laxa* PI633185 genomic DNA were digested with the restriction enzyme *Eco*RI, size-separated on a 1% agarose gel and transferred onto a nylon membrane. The DNA sequence of exon2, amplified by PCR using the primers Tubex-2F: 5'-GATTCCAAGTGTGTCACTCGTTG-3' and Tubex-2R: 5'-TTACAGCTAGGAGTGGTGAGCTT-3', designed on the beginning and the end of exon2, was used as probe (**Figure 1**). Once labelled with α-[^{32}P]-dCTP, using a random primer DNA labelling kit (Fermentas, Life Sciences), the probe was used for filter hybridisation. Filters were washed in 1.5 mM sodium citrate pH 7.0, 15 mMNaCl, 0.5% SDS (0.1xSSC), at 65˚C, before exposure to X-ray films (Biomax XAR, Kodak).

2.5. Chromosome Counts

Flower buds were used for chromosome preparations according to Schwarzacher and Heslop-Harrison [28]. Briefly, after a treatment with 2 mM 8-hydroxyquinoline for 1 h at room temperature followed by 1 hour at 4˚C, the buds were fixed in ethanol-acetic acid (3:1, v/v) and stored at −20˚C until use. Before squashing, flower buds were washed twice for 10 min in 1 x enzyme buffer solution (0.01 M citric acid-sodium citrate buffer, pH 4.8) and then digested at 37˚C for 45 min with an enzyme solution containing 1% (w/v) cellulase (Calbiochem), 1% (w/v) Onozuka R-10 cellulase (Serva) and 20% (v/v) pectinase (Sigma). Chromosome preparations were stained with DAPI (4', 6-diamidino-2-phenylindole) and counted under a fluorescence microscope (Axiovert 200, Zeiss) in twenty complete metaphases of somatic cells of *C. rumelica*, *C. hispida*, *C. laxa* and *Camelina* spp.

3. Results

3.1. Life Form Identification

In order to verify their life form, all *Camelina* accessions (**Table 1** and **Table 2**) were sown in small plots of 1 square meter in an open field at the end of winter. Among the 46 *C. sativa* accessions analysed, 39 showed a

spring life form (spring type) because they started stem elongation after 44 - 50 days from sowing. Accordingly, they have all been classified as *C. sativa* ssp. *sativa*. On the contrary, the remaining 7 accessions (CAM31, CAM37, CAM76, D9952, CAM132, PI650168 and PI650167) showed a winter life form (winter type) since they remained in a prolonged vegetative stage, producing many leaves, with no stem elongation. In fact, these accessions require a vernalization period to start stem elongation and flowering. According to Plessers *et al.* [21] these seven accessions can thus be classified as *C. sativa* ssp. *pilosa*. With reference to the *C. sativa* accessions (CAM269, CAM45, CAM46, PI650168, PI650146, PI650142, FF084, FF006, FF004 and PI650167), obtained from the germplasm banks as *C. sativa* ssp., they all turned out to be of the spring type with the exception of PI650168 and PI650167 that resulted winter types. Finally, all the accessions of the species *C. microcarpa*, *C. rumelica*, *C. hispida* and *C. laxa* (**Table 2**) exhibited a strict winter life form while *C. alyssum* showed both winter (CAM176) and spring types (PI650132 and CAM21).

3.2. cTBP Fingerprinting on *Camelina* Species

The cTBP method was applied to all the *Camelina* species and accessions listed in **Table 1** and **Table 2**. cTBP amplifies the genomic region that contains only the second intron of the different members of the *β*-tubulin gene family. After PCR amplification, performed with the combination of primers TBPfin2 and TBPrin2 (**Figure 1**), the products were analysed on non-denaturing acrylamide gels (**Figure 2** and **Figure 3**). The cTBP profiles detected for each species are described in more detail from here to follow.

C. sativa: forty-six accessions of different origin were genotyped using cTBP. With the exception of PI650167, all *C. sativa* accessions showed a very similar cTBP pattern with few polymorphic bands detected at about 500 bp, 750 bp and 800 bp (**Figure 2**). These polymorphisms were found in both *C. sativa* ssp. *sativa* and ssp. *pilosa*. Accession PI650167 showed a cTBP profile remarkably different compared to all the others *C. sativa* accessions, strongly resembling that of the majority of the accessions of *C. microcarpa* (such as PI633186 or CAM60, see **Figure 3**). This suggests that accession PI650167 has most likely been misclassified.

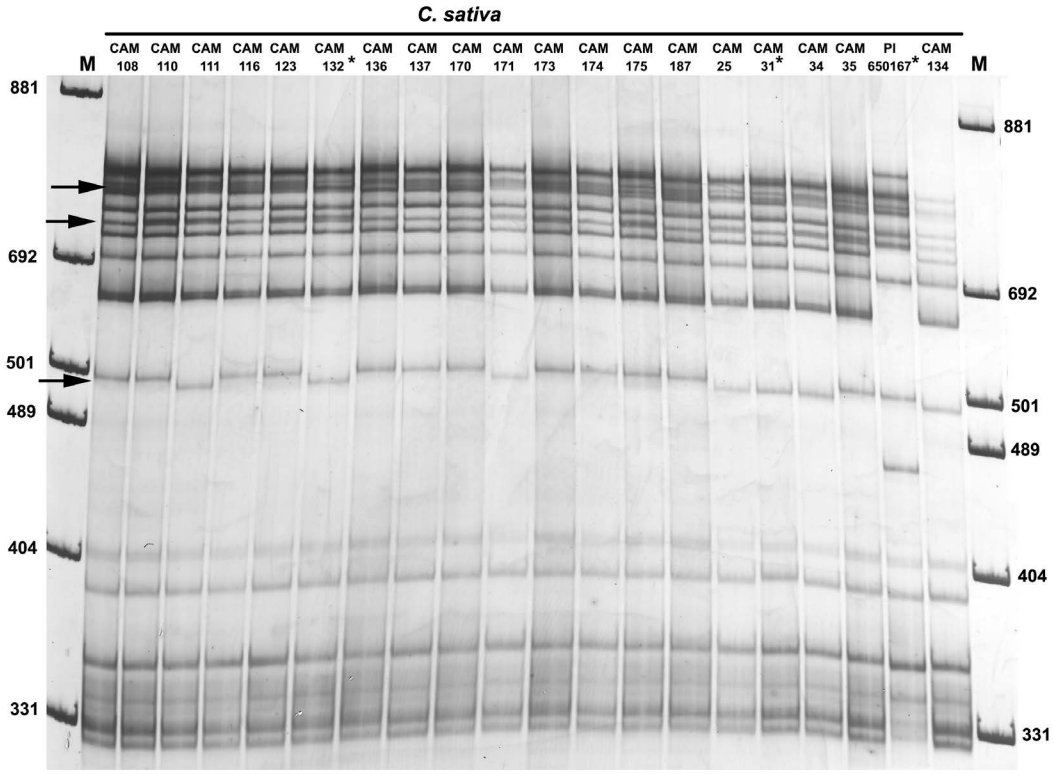

Figure 2. cTBP amplification profile of intron2 of 20 out 46 different *C. sativa* accessions. Accession numbers are reported on top of the cTBP profile. Asterisks next to the accession numbers indicate the *C. sativa* winter types. Arrows indicate polymorphic bands. M = Molecular marker size in bp.

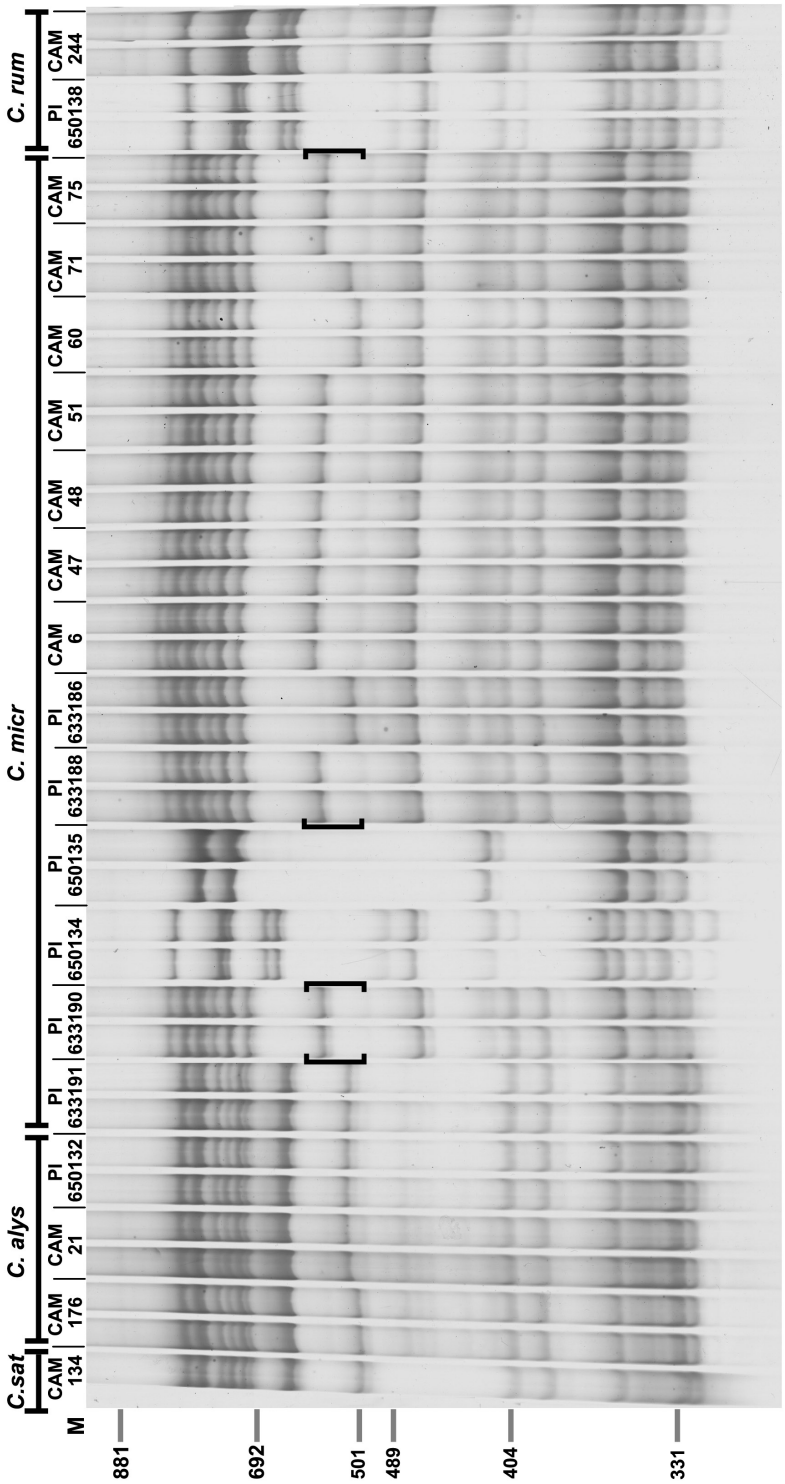

Figure 3. cTBP amplification profile of intron2 of *C. sativa* (*C. sat*), *C. alyssum* (*C. alys*), *C. microcarpa* (*C. micr*) and *C. rumelica* (*C. rum*). Accession numbers are reported on the top of the cTBP profile. For each accession, except *C. sat* CAM134 used as reference, the cTBP profile of two individuals is reported. The polymorphic bands among the *C. microcarpa* accessions are boxed. The accessions PI633191, PI650134 and PI650135 were not included between the boxes because they showed a different cTBP profile from the remaining *C. microcarpa* accessions. M = Molecular marker size in bp.

C. alyssum: all three accessions showed a cTBP fingerprint substantially identical to *C. sativa* (**Figure 3**). As observed in *C. sativa* also the three *C. alyssum* accessions show a polymorphic band around 500 bp sizes. This band appears slightly higher in CAM21 and PI650132 respect to CAM176 (**Figure 3**).

C. microcarpa: thirteen accessions with different provenience were analysed (**Table 2**). According to the cTBP profiles, some of the analysed *C. microcarpa* accessions appear to have been misclassified. More specifically, accessions PI633191 and PI633134 revealed a cTBP profile that is, respectively, much more similar to *C. sativa* and *C. rumelica* than to *C. microcarpa* (**Figure 3**), whereas the accession PI650135 showed a unique cTBP profile, very different from any other *Camelina* species. Besides these three, all the remaining *C. microcarpa* accessions shared a very similar cTBP profile, characterized once more by the presence of a pronounced DNA polymorphism around 500 bp. The sizes of this polymorphic band is of 550 bp in the majority of the *C. microcarpa* accessions, but PI633186, CAM60 and one individual of accession CAM71 showed a lower size fragment (**Figure 3**). Comparison of the cTBP profile of the majority of the *C. microcarpa* accessions with that of *C. sativa* and *C. alyssum*, reveal many bands in common. This suggests that these three species might share one common progenitor, at the least (**Figure 3**). Accession PI650135, of French origin (**Table 2**) stored at the IPK genebank as *C. microcarpa*, turned out to be a mixture of genetically distinguishable individuals. In fact, cTBP analysis carried out on 15 single individual plants demonstrated that only 9 showed a cTBP profile characteristic of *C. microcarpa* (data not shown), while 6 individual plants showed a distinct profile with a low number of cTBP bands, as that shown in **Figure 3**. Because of the distinctiveness of their profile we will refer to this group of samples as *Camelina* spp. In addition, the low number of cTBP amplified bands is suggestive of a diploid status.

C. rumelica: two accessions were analysed, with origin from Iran and Union of Soviet Republics (IPK genebank), respectively. Both showed a very similar cTBP pattern with no evidence for polymorphic bands (**Figure 3**).

C. hispida: only one accession of this species could be recovered from the genebanks. This accession resulted contaminated with seeds of other species, in particular *C. microcarpa*. (**Figure 4(A)**). Once cleaned from the

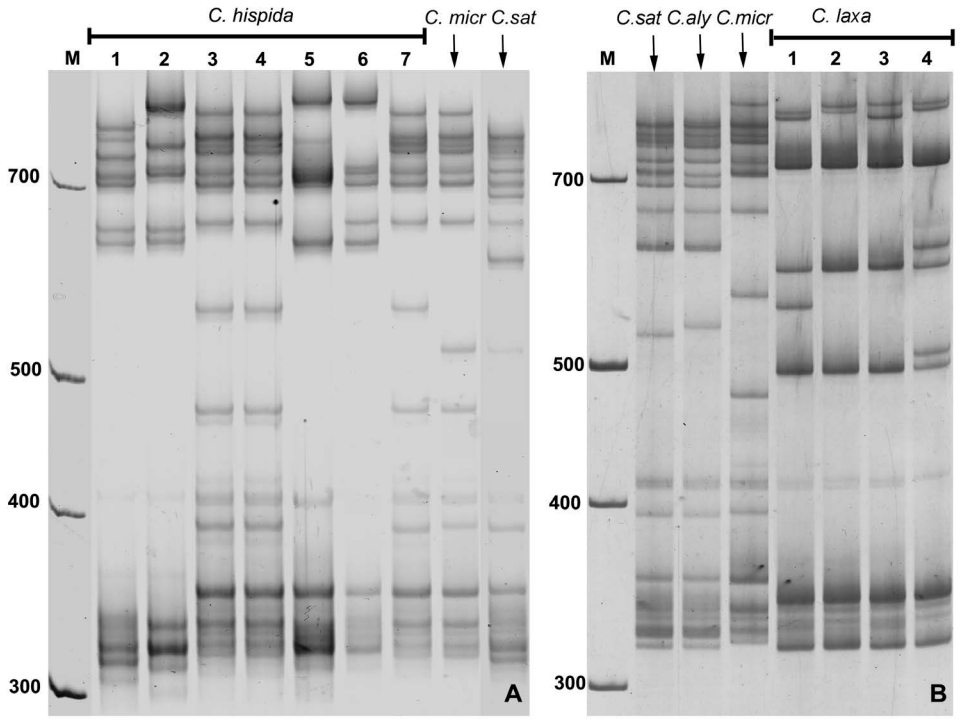

Figure 4. (A) The cTBP amplification profile of seven out of 15 individuals of *C. hispida* is compared with that of *C. microcarpa* (*C. micr*) and *C. sativa* (*C. sat*). Three individuals (3, 4 and 7) show a cTBP profile similar to *C. microcarpa* while individuals 1, 2, 5 and 6 show a high number of polymorphic bands. (B) cTBP amplification profile of 4 individuals of *C. laxa* showing intra-accession genetic variability. *C. sativa* (*C. sat*), *C. alyssum* (*C. alys*) and *C. microcarpa* (*C. micr*) were used for comparison. M = Molecular marker size in bp.

contaminating seeds, C. *hispida* was nevertheless characterised by a high number of intra-accession polymorphic bands. This is in contrast with the other *Camelina* species which showed very few polymorphic bands between the single individuals of the same accession.

C. laxa: only one accession of this species was retrievable from the genebanks. Four single plants were analysed, showing some cTBP polymorphic bands (**Figure 4(B)**). *Camelina laxa* showed an intra-accession variability lower than *C. hispida* but higher than that of the other *Camelina* species.

3.3. Sequence Comparison and Phylogenetic Analysis

In order to isolate and sequence the complete *β*-tubulin gene family from each *Camelina* species only one accession was chosen and analysed. The accessions selected were those that showed the most representative cTBP profile of the species. In accordance, the *β*-tubulin gene families from *C. microcarpa* CAM47, *C. rumelica* CAM244, *C. hispida* PI650133 (individual number 2), *C. Laxa* PI6331858 (individual number 2) and a sample of *Camelina* spp., were PCR amplified and cloned. Nucleotide sequences of the C. *sativa* (CAM134) *β*-tubulin gene family, already available at the EMBL Database [17] were retrieved for sequence comparison and phylogenetic analysis. By using the forward TBPfex1 and the reverse TBPrin2 primers, located at the end of the first exon and at the beginning of the third exon (**Figure 1**) respectively, the following numbers of partial *β*-tubulin gene sequences were isolated from the different *Camelina* species: 19 from genomic DNA of *C. microcarpa* (CmTUB1..CmTUB19), 15 from *C. rumelica* (CrTUB1..CrTUB15), 12 from *C. hispida* (ChTUB1..ChTUB12), 11 from *C. laxa* (ClTUB1..ClTUB11) and 9 from *Camelina* spp. (CameTUB1..CameTUB9). As predicted, each of the camelina *β*-tubulin nucleotide sequence contained 97 bp of the coding exon1 (partial), 270 bp of exon 2 (complete) and 147 bp of exon3 (partial) together with the two full-length introns, 1 and 2 (**ESM-Table 1**). Intron length varied in all the *β*-tubulin isotypes of the *Camelina* species. The shortest (80 bp) and the longest (936 bp) intron1 nucleotide sequence were both found in *Camelina* spp. (CameTUB1 and CameTUB9, respectively), whereas the shortest (82 bp) and longest (592 bp) intron2 sequences were detected in *C. laxa* (ClTUB3) and *C. hispida* (ChTUB12), respectively (**ESM-Table 1**). A search for tandem repeat motifs and microsatellite sequences revealed the presence in all species, with the exception of *C. laxa*, of one direct head-to-tail tandem repeat located in either the intron1 or the intron2 of some *β*-tubulin isotypes. *Camelina microcarpa* is the only species that showed the presence of one tandem repeat motif in both introns (CmTUB19). The tandem repeat located in intron2 was the longest detected (189 bp) (**ESM-Table 1**). Microsatellites such as trinucleotides $(CTT)_6$ and $(CTT)_7$ and dinucleotide stretches $(TA)_5$ and $(TA)_6$ were detected in introns 1 and 2, respectively. Compound microsatellites $(TA)_5$ $(TC)_3$ and $(TA)_7$ $(TC)_3$ were observed only in intron1 of *Camelina* spp. CameTUB9 and *C. laxa* ClTUB11, respectively. The multialigned nucleotide sequences of all the camelina and *Arabidopsis thaliana β*-tubulins were used to infer a Maximum Likelihood phylogenetic tree. As shown in **Figure 5**, the 95 different *β*-tubulin genes (86 from *Camelina* and 9 from *A. thaliana*) are grouped in two main clusters (I and II) which are further subdivided in sub-clusters (A, B, C and D). With the exception of the sub-cluster D, all the others are further subdivided in two groups (A1-A2; B1-B2; C1-C2). The phylogenetic tree spreads the analysed sequences throughout the clusters, sub-clusters and groups without distinction between *Camelina* species and *A. thaliana*. The only exception is represented by group C2 that contains only *β*-tubulins from *Camelina* species (**Figure 5**). Furthermore, each group includes at least one *β*-tubulin sequence per analysed species, except B1 that do not contain any *β*-tubulin of *C. hispida* and *C. microcarpa*.

3.4. Southern Hybridization Analysis

To obtain additional information on the genomic organization and the number of the *β*-tubulin genes present in the different *Camelina* species, we performed a Southern blot analysis on the *Eco*RI restricted genomic DNA of *C. sativa*, *C. alyssum*, *C. microcarpa*, *C. rumelica*, *C. hispida*, *C. laxa* and *Camelina* spp. A radioactively-labelled exon2 fragment was used as a probe. *Eco*RI endonuclease was chosen since no restriction sites for this enzyme are present in any of the exon2 sequences of all *β*-tubulins. This implies that each of the detected hybridization fragments should correspond to one gene. As shown in **Figure 6** the number of hybridization bands is higher in *C. sativa*, *C. alyssum*, *C. microcarpa* and *C. rumelica* compared to that of *C. hispida*, *C. laxa* and *Camelina* spp. This is in agreement with the number of the isolated *β*-tubulin genes that was lower in *C. hispida*, *C. laxa* and *Camelina* spp. than in the other species. As already observed comparing the cTBP profile of *C. sativa* and *C. alyssum* (see **Figure 3**) also the Southern blot hybridization showed very few differences between these two species (**Figure 6**).

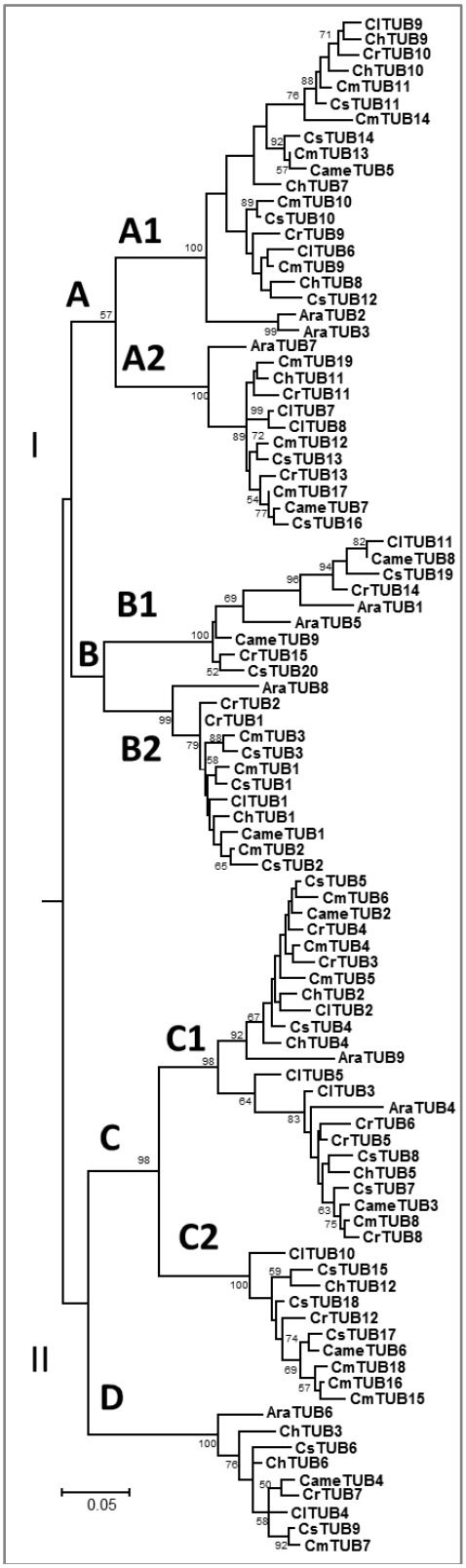

Figure 5. Phylogenetic tree constructed using MEGA software. Abbreviation CsTUB, CmTUB, CrTUB, ChTUB, Came TUB, ClTUB and AraTUB indicate the *Camelina* and the *A. thaliana* β-tubulin genes, respectively. On each node the bootstrap values out of 1000 replicates are indicated. The scale bar of genetic distance is shown at the bottom.

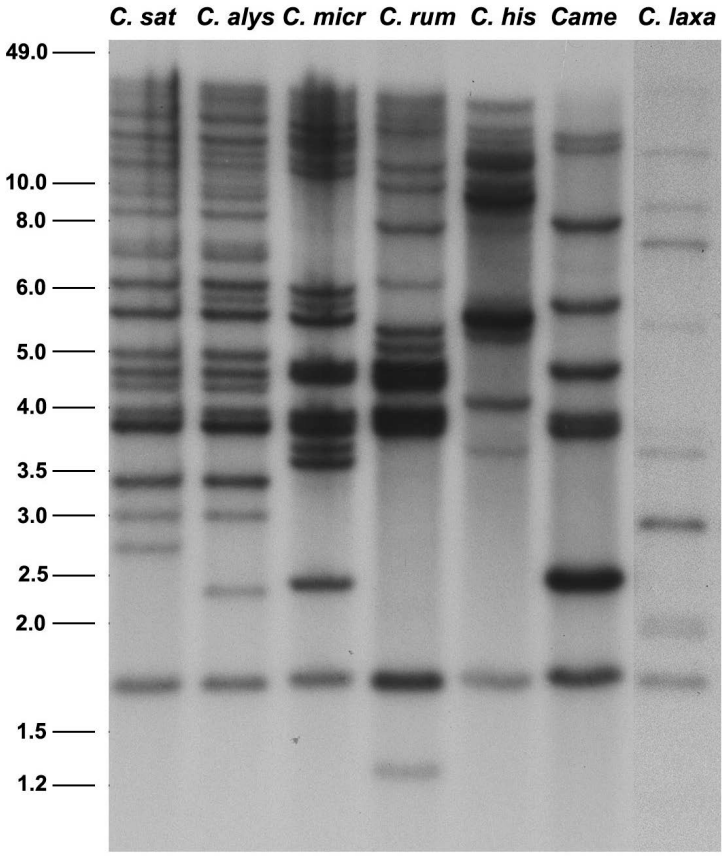

Figure 6. Southern blot analysis of genomic DNA extracted from *C. sativa* (*C. sat*), *C. alyssum* (*C. alys*), *C. microcarpa* (*C. micr*), *C. rumelica* (*C. rum*), *C. hispida* (*C. his*), *Camelina* spp. (*Came*) and *C. laxa*. Genomic DNAs were digested with *Eco*RI and probed with an exon2 fragment labelled with α-[32P]-dCTP. Molecular size markers are indicated in kbp.

3.5. Chromosome Number Counting

The chromosome number resulted 2n = 14 in *C. hispida* and 2n = 12 in *C. laxa* and *Camelina* spp. (**Figure 7**) suggesting a diploid status of these three species respect to *C. sativa*, *C. alyssum* and *C. microcarpa*, that are all polyploids with a chromosome number 2n = 40 [14] [29], and to *C. rumelica* characterized by a chromosome number 2n = 26 (**Figure 7**).

4. Discussion

The results presented here demonstrate that all *Camelina* species, with the exception of *C. alyssum*, can be easily distinguished at genomic level by using the cTBP method. In fact the multiple length polymorphism of β-tubulin intron2 gives rise to amplification profiles which resulted highly specific for each of the analysed species (see **Figure 2** and **Figure 3**). With regard to *C. alyssum*, the results that we have obtained with both cTBP fingerprinting and Southern blot analysis, question its proposed classification as an independent species [29] suggesting that it may instead be better categorized as a subspecies of *C. sativa* because of the strong resemblance of their reciprocal profiles (**Figure 3** and **Figure 6**).

The cTBP fingerprinting observed among the different accessions belonging to the same species indicates a high degree of internal genetic similarity. This result is consistent with most of the molecular studies carried out on cultivated *C. sativa* done with the use of different markers such as AFLP, RAPD and SSR [15] [16] [18]. In addition, no difference was substantially detected between the cTBP profiles of the two subspecies *C. sativa* ssp. *pilosa* (winter type) and ssp. *sativa* (spring type), while the consistency of the cTBP banding pattern observed across 46 different *C. sativa* accessions, collected from different countries, suggests that the PI650167 accession, characterized by a significantly different cTBP profile much more similar to *C. microcarpa*, has been probably

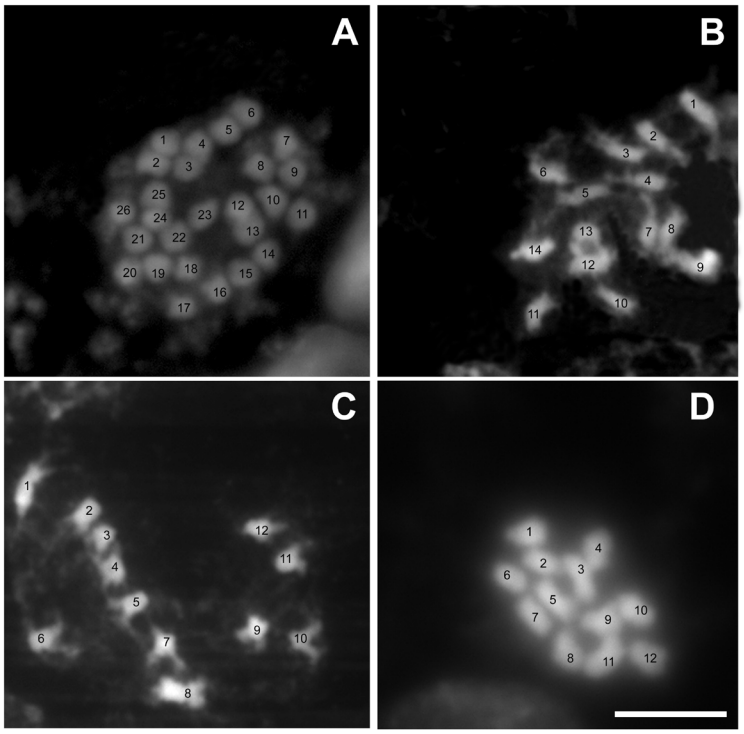

Figure 7. (A) *C. rumelica*; (B) *C. hispida*; (C) *Camelina* spp. and (D) *C. laxa* chromosomes stained with DAPI. Bar = 5 µm.

misclassified (**Figure 2** and **Figure 3**).

In contrast to all the other *Camelina* species, which showed almost no polymorphisms among individuals of the same accession, *C. hispida* presented a high degree of internal polymorphism (see **Figure 4(A)**). At least four different cTBP profiles were detected with very few bands in common. This unexpected finding is likely to result from cross-pollination of *C. hispida* with other *Camelina* species since we found no evidence of contamination, *i.e.* all four individuals showed a very similar morphology when grown in plots until flowering stage (data not shown). As seen for *C. sativa*, a high number of cTBP amplified bands was detected for *C. microcarpa* and *C. alyssum* suggesting a large size of the *β*-tubulin gene family, which is confirmed, for both *C. sativa* and *C. microcarpa*, by the number of the isolated *β*-tubulin genes and the number of hybridizing bands in the Southern blot analysis. The number of the cTBP bands in *C. rumelica* and *C. hispida* was lower. In *Camelina* spp. only 9 hybridization bands were detected by Southern analysis, a number that perfectly matches that of the identified *β*-tubulin genes (CameTUB1..CameTUB9).

Therefore, the cTBP marker and Southern hybridization analysis have highlighted a high variability among the different species which could be exploited to improve the genetic background of the cultivated species with respect to its utilization as a biofuel crop.

In addition to its discrimination power at plant species level, the cTBP method can also provide information on the ploidy status of a plant species. Breviario *et al*. [23] reported that the number of amplified bands in the genus *Eleusine* strongly correlates with the ploidy level of each taxon. Indeed, the three tetraploids *E. coracana* ssp. *coracana*, *E. coracana* ssp. *africana*, and *E. kigeziensis* show a consistently higher number of cTBP bands than that produced from each *Eleusine* diploid species. Similarly, the high number of cTBP bands detected in *C. sativa*, *C. microcarpa* and *C. alyssum* is fully consistent with their hexaploid status [19] [20]. Our results on *C. rumelica*, characterized by a chromosome number 2n = 26 and a reduced number of cTBP bands compared to *C. sativa*, *C. microcarpa* and *C. alyssum*, suggest a tetraploid status for this species. Finally, a diploid chromosomal status is suggested for *C. hispida*, *C. laxa* and *Camelina* spp. in view of the low number of the cTBP amplified bands and the small number of chromosomes (2n = 14 *C hispida*; 2n = 12 *C laxa* and 2n = 12 *Camelina* spp.), relative to that of *C. sativa*, *C. alyssum* and *C. microcarpa* (2n = 40) [14] [29].

The phylogenetic tree emerging from the analysis of the isolated *β*-tubulin genes demonstrate a distribution of

the different camelina *β*-tubulin isotypes that is spread throughout the clusters without separation from the *A. thaliana* orthologs. A similar distribution was reported in other studies that compared several *β*-tubulin genes of monocotyledonous and dicotyledonous plants [30]-[32]. Thus, our results provide further evidence that the angiosperm *β*-tubulin genes have likely originated from a single ancestral gene and that the only group of the tree (group C2), which does not include any *β*-tubulin counterpart from *A. thaliana*, might actually have originated by a duplication and a speciation event that took place after the separation of the two species. With regard to the lack of members of *C. hispida* and *C. microcarpa* in the B1 group this has most likely to be attributed to failure in cloning large size sequences.

5. Conclusion

In this study, we have shown that the cTBP molecular marker may indeed be a useful tool for the correct classification of *Camelina* germplasm species and, more generally, for unequivocal identification of any other plant species and accessions. In addition, cTBP might be very useful to track interspecies introgression events that may result from breeding programs aimed to widen the genetic base of the cultivated *C. sativa*.

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ESM-Table 1. *β*-tubulin isotypes isolated from *Camelina* species. For each isotype is reported the EMBL accession number, the complete size in base pair (bp), the length of intron1 (Int1) and intron2 (Int2). Microsatellites and tandem repeats present in the Int1 and/or Int2 are reported. Numbers in the tandem repeats column indicated the position of the repeat motif in the nucleotide sequence. Dash (-) is introduced for better alignment.

***C. rumelica* CAM244**	**Size bp**	**Int1 bp**	**Int2 bp**	**Microsatellites**	**Tandem Repeats**	**EMBL Accession Number**
CrTUB1	693	87	92			LN811270
CrTUB2	713	107	92			LN811271
CrTUB3	730	119	97			LN811272
CrTUB4	731	119	98			LN811273
CrTUB5	781	163	104			LN811274
CrTUB6	782	166	103			LN811275
CrTUB7	793	100	179			LN811276
CrTUB8	803	191	98			LN811277
CrTUB9	864	106	244	Int1$(CTT)_7$		LN811278
CrTUB10	1120	111	578		Int2 707 5’TGCTATTCTCTCTTGTTAAG 3’726 727 5’TGCTATACTTTCTTGTTAAG 3’746	LN811279
CrTUB11	1122	210	115		Int1 193 5’TCTGATTT-GAACTGGTAATT-GCGGATTAAGTTGCTTATG GTGGTT 3’237 238 5’TCTGATTTTGAACTGGTATTTTGGGGATTAGGTTGCTTATG GTGGTT 3’284	LN811280
CrTUB12	1154	171	469			LN811281
CrTUB13	1298	206	92		Int1 200 5’ATTTTGAACTGGTAATTGCGGATTAGTTTGCTTATGGTC 3’238 239 5’ATTTTGAACTGGTAATTGCGGATTAGGTTGCTTATGGTG 3’276	LN811282
CrTUB14	1324	718	495			LN811283
CrTUB15	1497	868	398			LN811284
***C. microcarpa* CAM47**						
CmTUB1	694	88	92			LN811285
CmTUB2	704	99	92	Int2$(TA)_5$		LN811286
CmTUB3	723	115	94	Int2$(TA)_5$		LN811287
CmTUB4	758	148	96			LN811288
CmTUB5	765	152	99			LN811289
CmTUB6	767	144	109			LN811290
CmTUB7	797	104	179			LN811291

Continued

CmTUB8	808	191	103			LN811292
CmTUB9	858	105	239	Int1(CTT)$_7$		LN811293
CmTUB10	926	101	311	Int1(CTT)$_6$	Int2 596 5’GTTTTGGTACTGTTTCTAGTGGCATTTGCTATTT 3’629 630 5’GTTTTGTTACTGTTTCCAGTGGCATTTGCTATTT 3’663 664 5’GTTTTGTTACTGTTTCCAGTGGCATTTGCTATTG 3’696	LN811294
CmTUB11	1114	111	489			LN811295
CmTUB12	1117	210	393		Int1 190 5’GGTTTCTGATTTTGAACTGGTAATTGCGG-ATTAGGTTGCTTATGGT 3’235 236 5’GGTTTCTGATTTTGAACTGGTAATTTGGGGATTAGGTTGCTTATGGT 3’282	LN811296
CmTUB13	1144	100	530	Int1(CTT)$_6$		LN811297
CmTUB14	1148	144	490			LN811298
CmTUB15	1168	182	472			LN811299
CmTUB16	1186	182	490			LN811300
CmTUB17	1194	161	519			LN811301
CmTUB18	1226	182	530			LN811302
CmTUB19	1305	206	585		Int1 200 5’ATTTTGAACTGGTAATTGCGGATGAGTTTGCTTATGGTC 3’238 239 5’ATTTTGAACTGGTAATTGCGGATTACGTTGCTTATGGTG 3’276 Int2 621 5’GTTGATCTGTCTCTGTGATTCTACTGTGTTTGTTATTTGATGCATAGTTATT CATGAGTACAATGTGTAATGCTATAATTTGGACTTGTGTCTGCTGATGTGATCAGAGC GTTAGTCTTCTCAATGTCTCAAATCATATCCCCTTGTTCAATCAATGTCACTATTGTT ACTGTCGTTGTGATTTTAAGG 3’809 810 5’GTTGATTTGTCTCTGTGGTTCTACAGTGTTTGTTATTTGATGCATAGTTTATT CATAAGTATGATGTGTAACGCTATAAATTGGACATGGTGCCTGCTGTTGTGATAGTCTT CTCAATGTTTCAAATCATATCCTTGTTCAATCAGTATCACTATTGTTACTCTGTCGTTG TGGTTTTAAGG 3’991	LN811303
C. hispida **PI650133**						
ChTUB1	696	88	94	Int2(TA)$_6$		LN811304
ChTUB2	732	120	98			LN811305
ChTUB3	740	95	131			LN811306
ChTUB4	756	146	96			LN811307

Continued

ChTUB5	779	165	100		LN811308
ChTUB6	792	100	178		LN811309
ChTUB7	1109	105	490	Int1(CTT)$_6$	LN811310
ChTUB8	1112	105	493	Int1(CTT)$_6$	LN811311
ChTUB9	1115	111	490		LN811312
ChTUB10	1122	107	500	Int1 802 5’TGATTGTCAAAATGCTTTTA 3’ 821 822 5’TGATTGTAAAACTGCTTTTA 3’ 841	LN811313
ChTUB11	1126	213	399	Int1 194 5’GGTTTCTGATTTTGAACTGGTAATT-GCGGATTAGGTTGCTTA TGGT 3’239 240 5’GGTTTCTGATTTTGAACTGGTAATTTGGGGATTAGGTTGCTT ATGGT 3’286	LN811314
ChTUB12	1210	104	592		LN811315
C.laxa PI633185					
ClTUB1	696	87	95		LN811316
ClTUB2	746	143	89		LN811317
ClTUB3	781	185	82		LN811318
ClTUB4	791	99	178		LN811319
ClTUB5	796	185	97		LN811320
ClTUB6	855	98	243	Int1(CTT)$_6$	LN811321
ClTUB7	1024	154	356		LN811322
ClTUB8	1033	163	356		LN811323
ClTUB9	1126	109	502		LN811324
ClTUB10	1231	174	543		LN811325
ClTUB11	1233	627	92	Int1(TA)$_7$(TC)$_3$	LN811326
***Camelina* spp.**					
CameTUB1	686	80	92	Int2(TA)$_5$	LN811327
CameTUB2	767	144	109		LN811328
CameTUB3	802	185	103		LN811329
CameTUB4	816	103	199		LN811330
CameTUB5	1151	103	534	Int1(CTT)$_6$	LN811331
CameTUB6	1163	174	475		LN811332
CameTUB7	1194	161	519		LN811333
CameTUB8	1295	689	92	Int1(TA)$_5$(TC)$_3$	LN811334
CameTUB9	1565	936	115	Int2 1339 5’CAAAATGTATCTAAAACTCAATCTTGATATTTT 3’ 1371 1372 5’CAAAATGTGTTTAAAACTAAATCTTCATATTGT 3’ 1404	LN811335