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Phylogenetic Analysis and Molecular Diversity of *Capsicum* Based on rDNA-ITS Region

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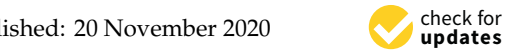

Abstract: The genus *Capsicum* is comprised of 5 domesticated and more than 30 wild species. The region of nuclear ribosomal DNA internal transcribed spacers (rDNA-ITS) has widely been used for species identification, but has rarely been used in *Capsicum*. In this study, the evaluation of genetic diversity and a phylogenetic analysis were conducted using rDNA-ITS of 28 *Capsicum* accessions, including five domesticated and two wild species. We surveyed six conventional keys of domesticated species and another five traits in *Capsicum* accessions. Specific morphological characteristics were found in *C. annuum*, *C. baccatum*, and *C. pubescens*. Three subclones of each accession were sequenced, and rDNA-ITS polymorphisms were detected in all accessions excluding *C. annuum*, suggesting that incomplete concerted evolution occurred in rDNA-ITS of *Capsicum*. The genetic diversity was evaluated using nucleotide polymorphism and diversity. *C. annuum* had the lowest genetic diversity of all species in this study. The phylogenetic tree formed a species-specific clade for *C. annuum*, *C. baccatum*, and *C. pubescens*. The *C. chinense* clade existed in the *C. frutescens* clade, implying that it was a cultivated variant of *C. frutescens*. *C. chacoense* likely belonged to the *C. baccatum* complex according to its morphologic and genetic features. This study indicated that the rDNA-ITS region can be used for simple identification of domesticated *Capsicum* species.

Keywords: *Capsicum*; rDNA-ITS; phylogeny; genetic diversity; morphological traits

1. Introduction

The genus *Capsicum* has been cultivated since at least 6000 B.C. by Native Americans [1], and is now produced at over 40 million tons per year worldwide [2]. Fruits of the genus have good health properties such as stress relief and fat breakdown [3,4]. Capsaicin, which is the main pungent component of *Capsicum*, has attracted much attention because of beneficial health properties [3,5].

The genus has five domesticated species, *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*, and more than thirty wild species [6,7]. The origin of the *Capsicum* genus is postulated to be along the Andes of western to north-western South America [8]. The most commonly cultivated *Capsicum* species is *C. annuum*, which is domesticated in northern Latin America [9,10]. *C. chinense* and *C. frutescens* are domesticated in tropical northern Amazonia, while *C. baccatum* and *C. pubescens* are more prevalent in Latin America and mid-elevation southern Andes, respectively [9]. The domesticated species can be classified by morphological traits: seed color, corolla yellow spot, number of flowers per axil, calyx annular constriction, and flower position [11].

There are three genetic complexes based on the degree of genetic proximity and cross compatibility among the five domesticated species and closely related wild species. The complexes are the *C. annuum* complex (*C. annuum*, *C. chinense,* and *C. frutescens*), *C. baccatum* complex (*C. baccatum*, *C. praetermissum*, and *C. tovarii*), and *C. pubescens* complex (*C. pubescens*, *C. cardenasii*, and *C. eximium*) [12–14]. Additionally, *C. chacoense* is sometimes assigned to the *C. annuum* complex [15] or *C. baccatum* complex [8] depending on the phylogenic analysis method.

In recent years, phylogenetic studies have been conducted to elucidate the relationship between *Capsicum* species based on molecular markers, including isozyme [16], random amplified polymorphic DNA [15], amplified fragment length polymorphism [17], simple sequence repeat [18,19], and single nucleotide polymorphism [20]. These studies analyzed the relationships between cultivated species, and indicated the species-specific clades formed; the species belonging to the *C. annuum* complex were genetically close to each other in phylogenetic trees.

DNA sequencing of genes or specific regions is often performed in phylogenetic studies. The internal transcribed spacers from nuclear ribosomal DNA (rDNA-ITS) is the most commonly used region for DNA barcoding [21]. For plant DNA barcoding, *rbcL*, *psbA-trnH* spacer regions, and *matK* on plastid DNA are proposed in addition to rDNA-ITS [21]. In the phylogenic analysis of *Capsicum* species, the *psbA-trnH* spacer region and *matK* on plastid DNA, and the partial sequence of *waxy* on nuclear DNA have been used for DNA barcoding [8]. On the other hand, rDNA-ITS has been used for the *Capsicum* species identification of 'Bhut Jolokia' [22] and genetic diversity evaluation [23,24]. Besides, it has been used for phylogenetic analysis of *Capsicum* species, although it is unclear whether rDNA-ITS can be used for the identification of domesticated *Capsicum* species, as the number of lines surveyed was one for most species surveyed [25].

The objectives of this study were to conduct phylogenetic analyses of *Capsicum* species using rDNA-ITS, verify whether rDNA-ITS can identify *Capsicum* species, especially domesticated ones, and describe their morphological characteristics. The rDNA-ITS sequences and morphological traits of domesticated and wild *Capsicum* species were examined. Moreover, genetic diversity within species was evaluated using rDNA-ITS sequences.

2. Materials and Methods

2.1. Plant Materials

Twenty-six *Capsicum* accessions, including five domesticated and two wild species, were provided by the National Agriculture and Food Research Organization Genebank (Tsukuba, Japan) or the USDA/ARS *Capsicum* germplasm collection (Griffin, GA, USA) (Table 1). After seeds were germinated on moistened filter papers in petri dishes, all seedlings were transplanted to pots (9 cm diameter, 10 cm depth) filled with culture soil (Sakata Super Mix A, Sakata Seed Co., Yokohama, Japan). The seedlings were cultivated in constant conditions (25 °C, 12/12 h light/dark, 85 μmol m^{-2} s^{-1}). At 30 days after germination, plants were transferred to bigger pots (21 cm diameter, 15 cm depth) and placed in a greenhouse (natural day length; Osaka Prefecture University, Sakai, Osaka, Japan). Only *C. pubescens* was kept at room temperature because it is not suitable for cultivation at a high temperature.

For morphologic investigations, five key traits (seed color, corolla yellow spot, number of flowers per axil, calyx annular constriction, and flower position) were described for the classification of domesticated *Capsicum* species according to the International Board for Plant Genetic Resources [11], in addition to other five traits (plant growth habit, anther color, calyx margin, fruit shape, and fruit pungency) (Figures 1 and 2). Morphologic surveys, excluding plant growth habit, seed color, fruit shape, and pungency, were conducted when each plant flowered. Plant growth habits were evaluated 5 months after germination when plant growth habits no longer change. Seed color was evaluated before sowing, and fruit shape and pungency were evaluated when each plant's fruits ripened. Fruit shapes were visually classified based on the illustration described by the International Plant Genetic Resources Institute (IPGRI) [26]. Fruit pungency was evaluated as presence or absence of pungency by

sensory test. Three plants per accession were investigated to determine each trait and whether the traits were uniform within each accession.

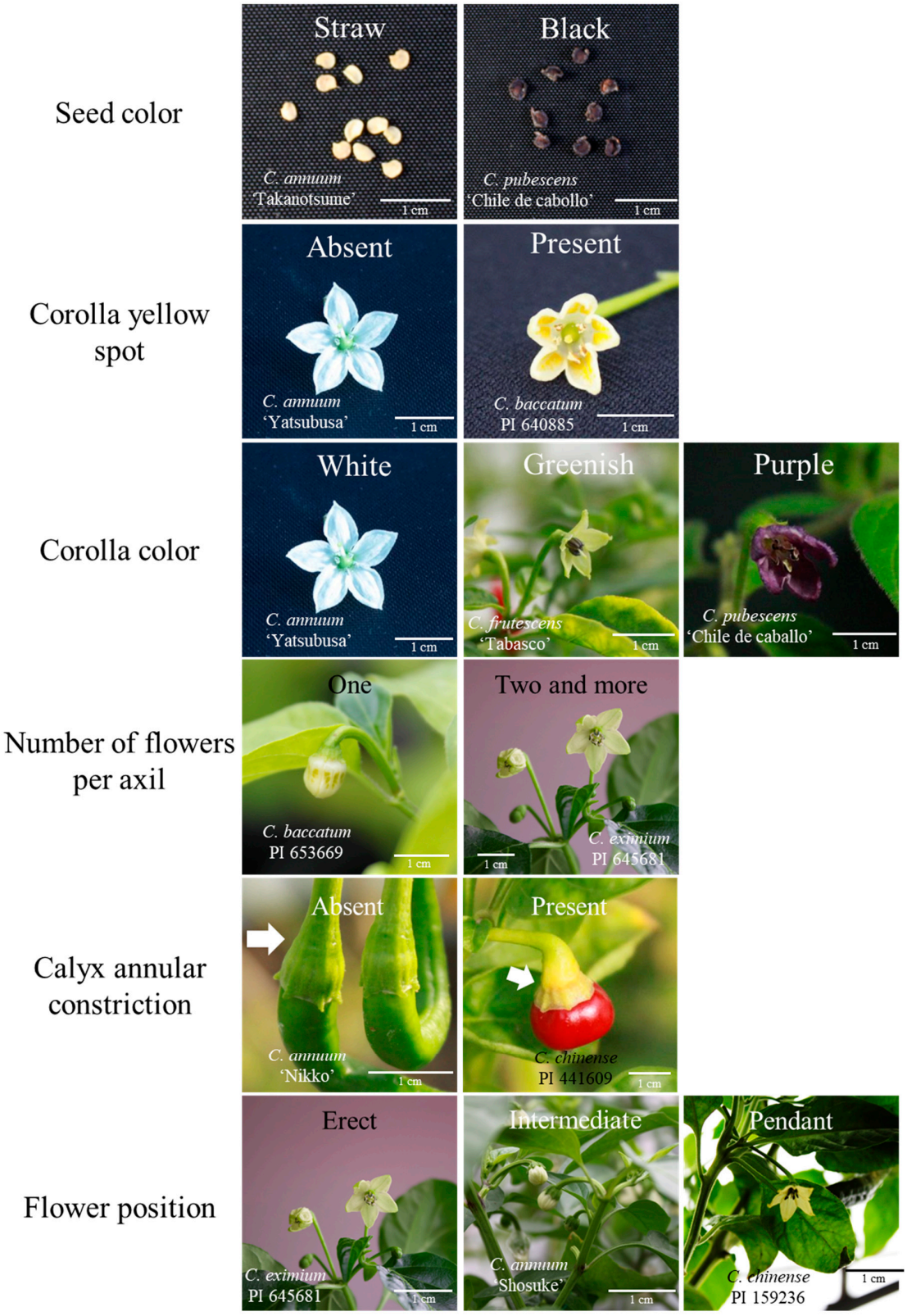

Figure 1. Traits for classification of domesticated species.

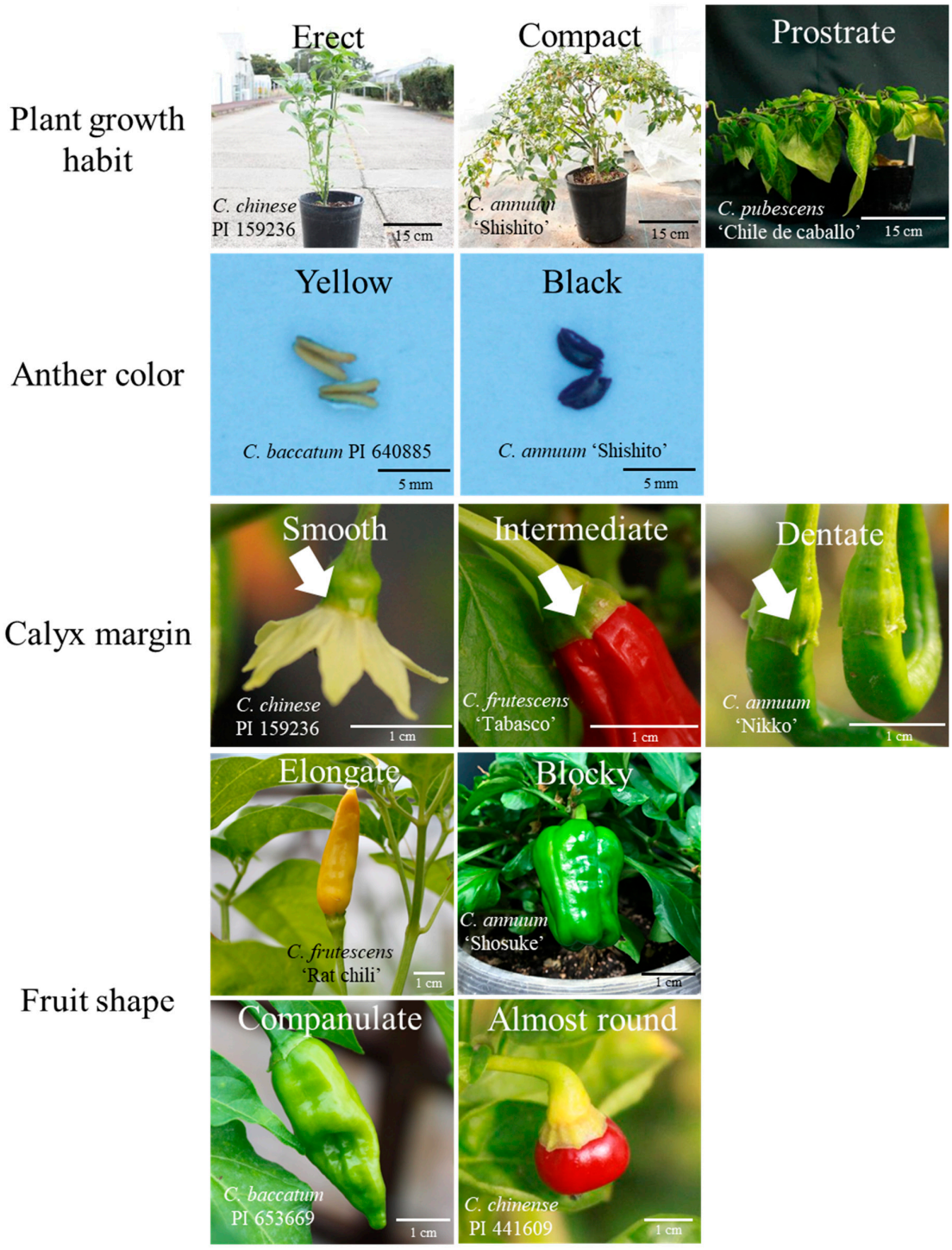

Figure 2. Other traits surveyed in this study.

Table 1. *Capsicum* and rDNA-ITS accessions used in this study.

Species	Accession No.	Cultivar and Line Name	Origin	Sources	rDNA-ITS Accession No.	Reference of rDNA-ITS
C. annuum	JP 32511	Sapporo Onaga Nanban	Japan	NARO [a]	LC510550	This study
	JP 32520	Mie Midori	Japan	NARO [a]	LC510551	This study
	JP 32523	Akashi	Japan	NARO [a]	LC510552	This study
	JP 32549	Yatsubusa	Japan	NARO [a]	LC510553	This study
	JP 32555	Zairai	Japan	NARO [a]	LC510554	This study
	JP 32562	Nikko	Japan	NARO [a]	LC510555	This study
	JP 32566	Fushimiamanaga	Japan	NARO [a]	LC510556	This study
	JP 82498	Takanotsume	Japan	NARO [a]	LC510557	This study
	JP 123787	Shosuke	Japan	NARO [a]	LC510558	This study
	JP 124339	Murasaki	Japan	NARO [a]	LC510559	This study
	PI 640723	Shishito	Japan	NARO [a]	LC510560	This study
C. chinense	PI 159236		USA	USDA [b]	LC510561-510563	This study
	PI 315008	Scarlet Lantern	Peru	USDA [b]	LC510564-510566	This study
	PI 438614	Habanero	Mexico	USDA [b]	LC510567-510568	This study
	PI 441609		Brazil	USDA [b]	LC510569-510570	This study
C. frutescens	PI 439512	Rat chili	Mexico	USDA [b]	LC510571-510573	This study
	PI 586675	Tabasco	USA	USDA [b]	LC510574-510576	This study
	PI 634826	Greenleaf Tabasco	USA	USDA [b]	LC510577-510579	This study
C. baccatum	PI 640882		Peru	USDA [b]	LC510580-510582	This study
	PI 640885		India	USDA [b]	LC510583-510585	This study
	PI 653669		Colombia	USDA [b]	LC510586-510588	This study
C. pubescens	PI 593624	Chile de caballo	Guatemala	USDA [b]	LC510589-510591	This study
	Grif 1613		Unknown	USDA [b]	LC510592-510594	This study
	Grif 1614		Mexico	USDA [b]	LC510595-510597	This study
C. chacoense	PI 273419		Argentina	USDA [b]	LC510598-510600	This study
C. eximium	PI 645681		Australia	USDA [b]	LC510601-510603	This study
			Mexico		AY665841	[27]
C. lycianthoides			USA		DQ314158	[28]
Solanum nigrum			China		FJ980391	[29]

[a] National Agriculture and Food Research Organization Genebank (Tsukuba, Japan), [b] USDA/ARS *Capsicum* germplasm collection (Griffin, GA, USA).

2.2. DNA Sequence of rDNA-ITS Region

Total DNA was extracted from individual leaves using the cetyltrimethylammonium bromide (CTAB) method [30] with minor modifications. Each leaf was ground in a mortar with liquid nitrogen. The ground leaf was mixed with CTAB isolation buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v β-mercaptoethanol, 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris-HCl, pH 8.0) preheated at 60 °C, and the mixture was incubated at 60 °C for 60 min. The suspension was extracted twice with chloroform/isoamyl alcohol (24:1) and centrifuged for 15 min at 500 g. The aqueous phase was transferred to a new tube; nucleic acids were precipitated by the addition of isopropanol (2/3 volume) and centrifuged for 20 min at 1,000 g. The pellet was washed with 70% ethanol and dissolved in 40 µL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

To enhance the specificity, the rDNA-ITS region was amplified by touchdown PCR using forward primer (5′-CTGCGGAAGGATCATTGTCG-3′) and reverse primer (5′-TAAACTCAGCGGGTAATCCC-3′), which were designed for *Capsicum* [31]. Touchdown PCR was performed in 40 µL reaction mixture containing 0.2 mM of dNTP, 0.2 µM primers, 1 U of KAPATaq EXtra DNA Polymerase (Kapa Biosystems Inc., Wilmington, MA, USA), 5×KAPATaq EXtra Buffer, and approximately 50 ng of DNA as template. The first step started with 94 °C for 3 min, followed by the touchdown phase, and PCR phase. The touchdown phase started with 94 °C for 30 s, annealing for 50 s, followed by elongation at 68 °C for 15 s. The annealing step of the touchdown phase had a temperature ramp from 67 to 64 °C in seven cycles (0.5 °C per cycle). The PCR phase had 33 thermal cycles, and each cycle had melting at 94 °C for 30 s, annealing at 64 °C for 50 s, and elongation at 72 °C for 15 s. The amplicons were verified on a 2% agarose gel in single band pattern. The amplicons were purified with Plus Gel Elution Kit (GMbiolab Co., Ltd., Taichung, Taiwan) following the manufacturer's protocol.

The preliminary attempt to use direct nucleotide sequencing for rDNA-ITS amplicons frequently failed to obtain good electropherograms, suggesting the possibility that each band contained different sequences derived from rDNA-ITS paralogs within individuals. Therefore, PCR products were cloned using the pGEM®-T Easy Vector System I cloning kit (Promega Co. Ltd., Madison, WI, USA) with competent cells of *Escherichia coli* strain DH5α. The sequences of three cloned amplicons from one plant per *Capsicum* accession were determined using BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems Co. Ltd., Waltham, MA, USA), M13 forward (5′-GTAAAACGACGGCCAGT-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers, and an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). Nucleotide sequences of rDNA-ITS determined are available in DDBJ/EMBL/GenBank (accession No. LC510550– 510603).

2.3. Sequence Alignment and Phylogenetic Analysis

The obtained rDNA-ITS sequences were aligned, using MUSCLE in the MEGA6 program [32], with other rDNA-ITS sequences downloaded from GenBank, including three sequences from *C. eximium*, *C. lycianthoides*, and *Solanum nigrum* (Table 1). The sequence of *S. nigrum* was used as the outgroup. The evolutionary history was inferred using MEGA6, with the maximum-likelihood method based on the general time reversible model [33]. Branch support was assessed by bootstrap resampling with 1000 replications.

2.4. Evaluation of Genetic Diversity

Based on the rDNA-ITS sequences, the number of nucleotide mutations, haplotype number and diversity, average number of nucleotide differences within population, and nucleotide polymorphism (θw) and diversity (π) were calculated using the DnaSP package ver. 6.0 [34].

3. Results

3.1. Morphology Characters of Each Capsicum Species

The morphological traits of 26 *Capsicum* accessions, including 11 *C. annuum*, 4 *C. chinense*, 3 *C. frutescens*, 3 *C. baccatum*, 1 *C. pubescens*, 1 *C. chacoense*, and 1 *C. exmium*, were studied (Table 1, Supplemental Table S1). In addition to six key traits used for domesticated species classification, five traits were also surveyed (Figures 1 and 2, Table S1). The results showed that seed color was black in *C. pubescens*, while straw in others. The corolla color was yellow in *C. baccatum*, white or purple in *C. annuum*, white or greenish in *C. chinense*, greenish in *C. frutescens* and *C. eximium*, purple in *C. pubescens*, and white in *C. baccatum* and *C. chacoense*. Some accessions in *C. annuum* and *C. chinense* had two or more flowers per axil, whereas others only had one. Two out of four *C. chinense* accessions had calyx annular constriction. Flower position was erect in *C. frutescens*, *C. chacoense*, and *C. eximium*, erect or intermediate in *C. annuum*, erect, intermediate, or pendant in *C. chinense*, intermediate or pendant in *C. baccatum*, and intermediate in *C. pubescens*. All accessions of five domesticated species had been correctly classified according to morphological traits. However, the *C. eximium* accession was likely to be misclassified because it is characterized by a purple corolla [35]; this misclassification was also inferred from the following phylogenetic analysis based on rDNA-ITS in the present study. The morphological traits of the *C. chacoense* accession were in line with those described in literature [36].

Plant growth habit was compact in *C. annuum* and *C. baccatum*, erect or compact in *C. chinense*, erect in *C. frutescens* and *C. eximium*, and prostrate in *C. pubescens* and *C. chacoense*. Anther color was yellow in *C. baccatum* and *C. chacoense*, while black in others. Calyx margin was dentate in *C. annuum*, *C. pubescens* and *C. chacoense*, smooth in *C. chinense*, and smooth or intermediate in *C. frutescens* and *C. baccatum*. Fruit shape was elongated or blocky in *C. annuum*, elongated, campanulate, or almost round in *C. chinense*, elongated in *C. frutescens*, *C. eximium*, and *C. chacoense*, elongated or campanulate in *C. baccatum*, and almost round in *C. pubescens*. Fruit pungency was present or absent in *C. annuum*, while present in others.

3.2. Variations of Sequence Length and GC Content, and Genetic Diversity in rDNA-ITS

We obtained the rDNA-ITS sequences from three subclones for each of the 26 *Capsicum* accessions. In all accessions of *C. annuum*, each had the same sequence among three subclones. *C. chinense* 'Habanero' and PI 441609 had the same sequence in two of the three subclones. In other accessions, three subclones from the same individual were different from one another. Additionally, rDNA-ITS sequences of *C. eximium*, *C. lycianthoides*, and *Solanum nigrum* were obtained from the NCBI database. Lengths of ITS1 were from 140 to 245, 138 to 161 for 5.8S rDNA, and 200 to 233 for ITS2 (Table S2). The GC percentages of ITS1 were from 51.9% to 72.0%, 43.8% to 55.0% for 5.8S rDNA, and 52.7% to 70.0% for ITS2 (Table S2).

The genetic diversity of rDNA-ITS in each species was evaluated (Table 2). In *C. annuum*, 7 of 11 sequences were the same haplotype, and 2 of the 10 sequences were the same haplotype in *C. chinense*, whereas sequences of other species were all different haplotypes. Therefore, haplotype diversity was lower in *C. annuum* and *C. chinense* than in others. We used two indicators of genetic diversity: nucleotide polymorphism (θw) [37], which reflects the number of mutation nucleotides in a population, and nucleotide diversity (π) [38], which reflects the average number of nucleotide differences between two sequences selected at random. The genetic diversity of rDNA-ITS in *C. annuum* was lowest among all species used in this study according to nucleotide polymorphism and diversity (Table 2).

3.3. Phylogenetic Relationship Between Capsicum Species Based on rDNA-ITS

We constructed a phylogenetic tree using the maximum-likelihood method based on the rDNA-ITS sequences (Figure 3, Figure S1). The phylogenetic tree showed that in-group species were divided into two clades (*C. pubescens* clade vs. the others) with 50% support value (Figure 3). The *C. annuum* clade then formed a monophyletic group with 71% support value. In addition to the *C. annuum* clade and the *C. pubescens* clade, the phylogenetic tree formed the *C. chinense* and *C. frutescens* clade and the *C. baccatum* clade, although the bootstrap value was less than 50%. The *C. chinense* clade was derived from the *C. chinense* and *C. frutescens* clade. *C. eximium* PI 645681 existed in the *C. chinense* and *C. frutescens* clade, while *C. chacoense* PI 273419 existed in the *C. baccatum* clade.

Table 2. Evaluation of genetic diversity within species based on rDNA-ITS.

Species	Number of Plant Materials	Number of Sequences [a]	Number of Haplotypes	Haplotype Diversity	Number of Mutation Nucleotides	Nucleotide Polymorphism (θw)	Average Number of Nucleotide Differences	Nucleotide Diversity (π)
C. annuum	11	11	4	0.49091	9	0.00808	1.78182	0.00476
C. chinense	4	10	9	0.95556	46	0.03661	14.55556	0.03239
C. frutescens	3	9	9	1	150	0.13385	40.47222	0.09922
C. baccatum	3	9	9	1	110	0.10795	34.11111	0.09187
C. pubescens	3	9	9	1	74	0.06188	18.52778	0.04449
C. chacoense	1	3	3	1	84	n.d.	56	n.d.
C. eximium	1	3	3	1	49	n.d.	32.66667	n.d.
Total	26	57	48	0.981	279	0.14439	34.323	0.08192

[a] Number of sequences indicates the number of subclones, excluding overlapped subclones in each accession. "n.d." indicates that the calculations could not be performed due to insufficient numbers of sequences.

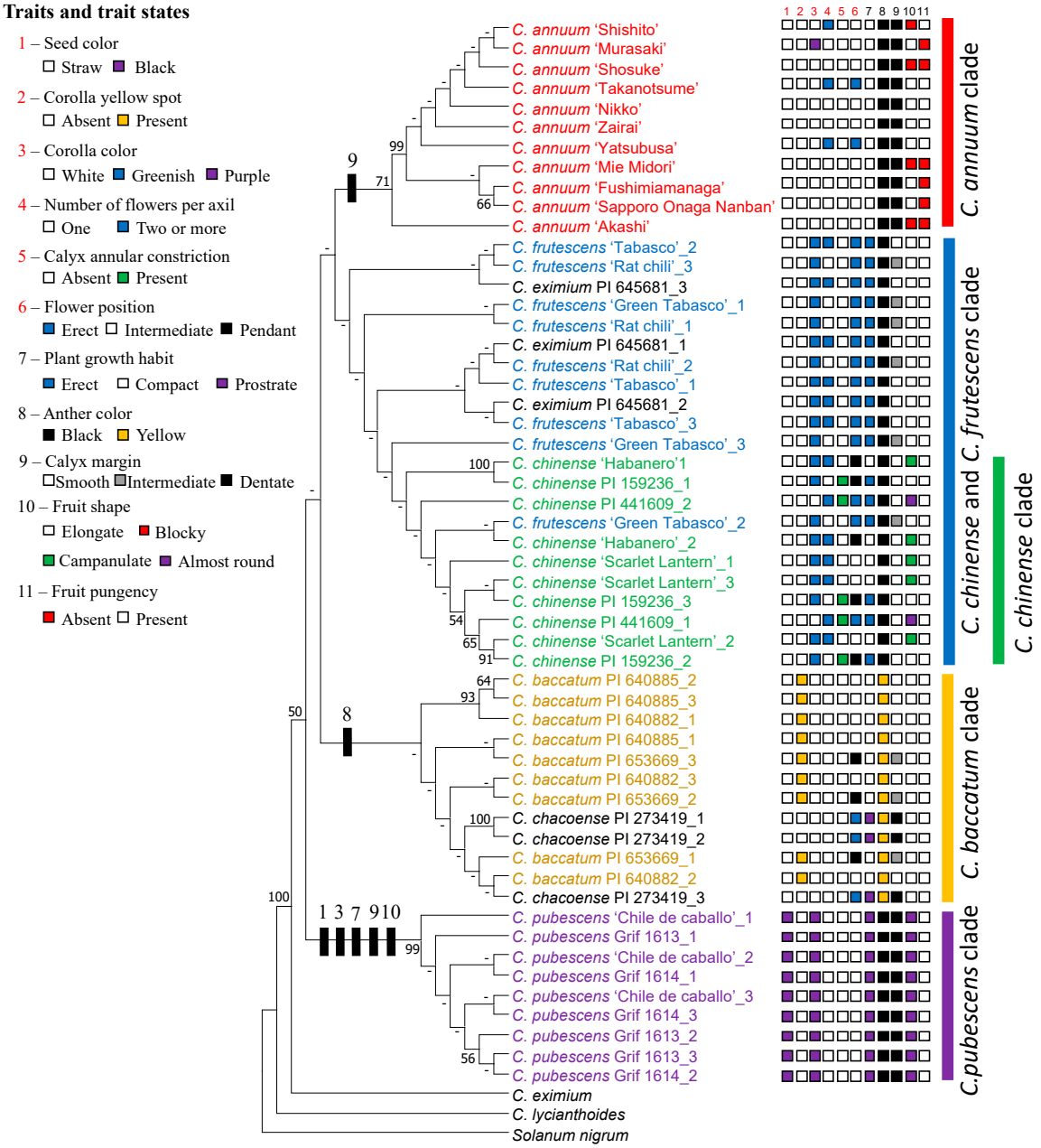

Figure 3. Molecular phylogenetic tree based on a maximum-likelihood analysis of rDNA ITS sequences, and evolution of morphological traits in *Capsicum*. Bootstrap = 1000 replicates (any clade with a hyphen has a bootstrap < 50). Trait state changes are indicated by black rectangles with the number of the trait on the phylogenetic tree. Traits 1 to 6 (written in red) are the key morphological traits of domesticated species.

4. Discussion

All accessions of five domesticated species had been correctly classified by key morphological characteristics for domesticated *Capsicum* species classification [11]. *C. chacoense* PI 273419 would be correctly classified because the morphological traits were not in conflict with those described in the literature [36]. However, *C. eximium* PI 645681 may be misclassified, because the corolla color of PI 645681 was greenish, while it is purple in general [35]. According to the key morphological characteristics of domesticated *Capsicum* species [11], *C. eximium* PI 645681 should be classified as *C. frutescens*. Although *C. eximium* PI 645681 belonged to the *C. chinense* and *C. frutescens* clade, the

C. eximium (AY665841) obtained from NCBI did not belong to any clades in the phylogenetic tree on rDNA-ITS (Figure 3). The deviation of *C. eximium* (AY665841) in the tree seemed to reflect that *C. eximium* is not very close to domesticated *Capsicum* species [8]. Therefore, *C. eximium* PI 645681 seemed to be *C. frutescens* considering its morphological and molecular traits.

The rDNA-ITS is a tandem repeat unit of hundreds or thousands of copies [39]. Individual copies of rDNA-ITS are homogenized in the same sequence type via concerted evolution, which is thought to be induced by unequal crossing over and high frequency of gene conversion [40]. However, in this study, rDNA-ITS polymorphisms within individuals were detected in *Capsicum* species, except in *C. annuum*, suggesting that concerted evolution was incomplete. The polymorphism of rDNA-ITS paralogs within individuals is also observed in other plants [41,42]. Although the mechanism of incomplete concerted evolution has not been elucidated thoroughly, some reports have shown that it occurs in cases where hybridization is involved [43], or when paralogous rDNA-ITS sequences are present in a non-homologous locus [44]. Although further analysis is needed to determine the factors of incomplete concerted evolution, this study revealed that it occurs in rDNA-ITS of *Capsicum*, except for *C. annuum*.

The genetic diversity in *Capsicum* species was investigated using rDNA-ITS (Table 2). The results suggested that the genetic diversity in *C. annuum* was much lower than other *Capsicum* species. Moreover, the genetic differences between lines of *C. annuum* were small in the phylogenetic tree (Figure S1). Low diversity of *C. annuum* was also observed in the analysis of *C. annuum* lines worldwide using 746k polymorphic sites [45]. Only Japanese cultivars of the *C. annuum* accessions were used in this study. It is possible that the genetic diversity of *C. annuum*, especially in Japanese cultivars, has decreased due to factors like intensive selective breeding.

The phylogenetic tree based on rDNA-ITS formed the *C. annuum* clade, the *C. chinense* and *C. frutescens* clade, the *C. baccatum* clade, and the *C. pubescens* clade. Therefore, rDNA-ITS can distinguish domesticated *Capsicum* species, but this was difficult for *C. chinense* and *C. frutescens* (Figure 3). However, the clades supported by bootstrap values above 50% were just the *C. annuum* clade and the *C. pubescens* clade. Therefore, rDNA-ITS should be used only for a rough identification of domesticated *Capsicum*. The *C. chinense* clade might be divided into two groups: one consisting of mainly *C. frutescens* and another consisting of *C. chinense*, suggesting that *C. chinense* may have evolved from *C. frutescens*. Consequently, *C. chinense* may be a cultivated variant of *C. frutescens* [46]. It was unclear to which complex *C. chacoense* belongs [8,15], but according to the phylogenetic tree based on rDNA-ITS, it belonged to the *C. baccatum* complex (Figure 3); it also had yellow anther, the same morphological trait as *C. baccatum*.

The phylogenetic analysis using rDNA-ITS almost agreed with previous studies using molecular markers [15,16,18–20] and DNA barcoding [8] with sequences except rDNA-ITS in terms of the formation of species clades and phylogenetic relations, although rDNA-ITS could not differentiate between *C. chinense* and *C. frutescens*. The analysis using molecular markers or DNA barcoding except rDNA-ITS requires many *Capsicum* species for species identification, and thus takes a lot of effort. On the other hand, many sequences of the rDNA-ITS region in *Capsicum* species have been accumulated in the NCBI database. Therefore, phylogenetic analysis using the rDNA-ITS region would be a low-effort method for *Capsicum* species identification.

The evolution of the morphological traits of each species on a phylogenetic tree was also studied (Figure 3). In the key morphological traits for domesticated species classification, black seed and purple corolla were the characteristic traits of the *C. pubescens* clade [46]. Most accessions in the *C. chinense* and *C. frutescens* clade had a greenish corolla (Figure 3, Table S1) [46,47], although it was not a characteristic trait of *C. chinense* and *C. frutescens* clades. In the traits not used for domesticated species classification, common traits were found in each clade. In the *C. pubescens* clade, prostrate growth habit and almost-round fruit were the characteristic traits [46]. A dentate margin of the calyx was a *C. pubescens* characteristic trait (Figure 3, Table S1), although a large-scale survey is needed to confirm that. The phylogenetic tree in Figure 3 suggested that yellow anther in the *C. baccatum* clade

seemed to be a trait obtained after differentiating from a common ancestor of *C. annuum*, *C. chinense*, and *C. frutescens*. It was necessary to investigate the anther color of other species in the *C. baccatum* complex to verify yellow anther as the characteristic trait of the *C. baccatum* complex. *C. tovarii* and *C. praetermissum*, belonging to the *C. baccatum* complex, had blue and purple anthers, respectively [13,48]; therefore, yellow anther was not a characteristic trait of the *C. baccatum* complex, but it may be useful for the identification of some species belonging to the *C. baccatum* complex. In the *C. annuum* clade, dentate calyx margin was a trait distinct from the *C. chinense* and *C. frutescens* clades (Figure 3, Table S1). However, dentate calyx margin cannot be used as a characteristic trait because there were some lines in *C. annuum* that had smooth or intermediate calyx margin [49]. All *C. frutescens* accessions had erect flower position and growth habit, but these traits cannot distinguish *C. frutescens* from *C. annuum* and *C. chinense* (Figure 3, Table S1). No new traits were found to distinguish *C. annuum*, *C. chinense*, and *C. frutescens* through the key traits of domesticated species, but based on their morphological traits, they were closely related.

This study revealed the phylogenetic relationships between *Capsicum* species based on 11 morphological traits and rDNA-ITS sequences, and the low genetic diversity of *C. annuum* cultivars based on rDNA-ITS sequences. These results may be used for future studies on species differentiation and genetic resources in *Capsicum*.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-7524/6/4/87/s1, Figure S1: Molecular phylogenetic tree based on a maximum-likelihood analysis of rDNA-ITS sequences with evolutionary distances, Table S1: Morphological traits of Capsicum investigated in the present study, Table S2: Length range (bp) and GC content (%) analysis of ITS sequences.

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