DISTINCTION BETWEEN CRINUM LATIFOLIUM AND CRINUM ASIATICUM (AMARYLLIDACEAE) USING MOLECULAR DATA

VAN HONG THIEN, TRAN THỊ THU NGAN, LUU THAO NGUYEN, TRINH NGOC NAM

Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City;

vanhongthien@iuh.edu.vn, congchuahappy.thungan@gmail.com, luuthaonguyen@iuh.edu.vn, trinhngocnam@iuh.edu.vn

Abstract. *Crinum latifolium* and *C. asiaticum* are the two species of genus *Crinum*, Amaryllidaceae family and they have highly medicinal values. Distinction between two species is challenging due to many similar morphological characteristics. In this study, we used molecular makers to distinguish the two species *Crinum latifolium* and *C. asiaticum*. The phylogenetic tree was constructed based on the DNA sequence data of the ITS (internal transcribed spacer) nuclear ribosomal DNA (nrDNA) sequences of *Crinum latifolium* and *C. asiaticum*. As a result, *C. latifolium* and *C. asiaticum* were sorted into two different groups in the phylogenetic tree. Therefore, *C. latifolium* and *C. asiaticum* were carried out to solve the taxonomic ambiguity.

Keywords. *Crinum asiaticum, Crinum latifolium,* ITS nrDNA, molecular markers, phylogeny.

PHÂN BIỆT HAI LOÀI CRINUM LATIFOLIUM VÀ CRINUM ASIATICUM (HỌ AMARYLLIDACEAE) DỰA TRÊN DỮ LIỆU PHÂN TỬ

Tóm tắt. *Crinum latifolium* và *C. asiaticum* là hai loài có giá trị dược liệu thuộc chi *Crinum*, họ Amaryllidaceae. Tuy nhiên, *Crinum latifolium* và *C. asiaticum* hiện đang bị nhầm lẫn trong phân loại vì chúng có nhiều đặc điểm hình thái tương tự. Trong nghiên cứu này, chúng tôi sử dụng maker phân tử nhằm mục đích phân biệt 2 loài có đặc điểm hình thái tương tự và dễ gây nhầm lẫn là *Crinum latifolium* và *C. asiaticum. C*ây phả hệ được xây dựng dựa trên dữ liệu trình tự DNA của vùng trình tự ITS nrDNA của loài *Crinum latifolium* và *C. asiaticum* kết hợp với các loài *Crinum* có trên dữ liệu GenBank. Sử dụng phương pháp phân tích dữ liệu đã cho thấy 2 loài *C. latifolium* và *C. asiaticum* xếp ở 2 nhóm khác nhau trên cây phả hệ.

Từ khóa. *Crinum asiaticum, Crinum latifolium,* ITS nrDNA, molecular markers, phylogeny.

1 INTRODUCTION

The genus *Crinum* was first established by Linnaeus in 1737 [5] and there are about 130 species widely distributed in and around the tropical and sub-tropical regions of the world. In Vietnam, the genus *Crinum* was known to include eight species: *Crinum asiaticum, Crinum amabile, Crinum giganteum, Crinum moorei, Crinum ensifolium, Crinum latifolium* and *Crinum zeylanicum* [11]. *Crinum* species in Vietnam are widely used inindigenous medicine for many diseases, particularly, *Crinum latifolium.* However, *Crinum latifolium* is now confused with *C. asiaticum* because of many similar morphological characteristics, such as: geophytic plant, globose tuber, lanceolate to narrowly elliptic leaf blade, cylindrical inflorescence and emerging from sympodium, white petal (figure 1) [11]. Normally, the plant identification in general as well as the identification of species with similar morphology in particular requires the full reproductive organs of plants, especially flowers, which are not practically always collected at field.

Nowadays, the application of molecular markers to support the classification as well as to distinguish the plant species with similar morphologies is widely used all over the world and even applicable to the family Amaryllidaceae [2, 9, 10, 12]. ITS (internal transcribed spacer) shows greatest utility for generating gene phylogenies at the rank of family and below because they are comprised both of sequences that were highly conserved during evolution, and highly variable sequences among species and even within species [1]. In this study, we conducted the amplification and sequencing of ITS (Internal

Transcribed Spacer) nuclear ribosomal DNA of *C. latifolium* and *C. asiaticum* to distinguish the two species.

Figure 1. Phenotype of *Crinum latifolium* (a) and *C. asiaticum* (b)

2 MATERIALS AND METHODS

2.1 Materials

Plant specimens from two taxa of *Crinum latifolium* and *C. asiaticum* were collected from Thu Thua District, Long An Province and Cam My District, Dong Nai Province, respectively.

2.2 Methods

Total genomic DNA was extracted from fresh leaf tissues using a Genomic DNA Purification Mini Kit (Thermo, USA). The target ITS region was amplified by polymerase chain reaction (PCR) using primers as mentioned by White *et al.* (1990) [17]. The PCR reactions were performed in an Eppendorf Mastercycler Gradient using a volume of 25µl reaction mixture: 12.5 µl go taq green master mix (Promega, USA), 1.25µl of each forward and reverse primers (10 µM), 9.5µl HPLC water and 0.5 µl DNA template (25 µg/ml). PCR cycles consisted of an initial denaturation for 5 min at 95°C; 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55^{o}C) and extension (1:30 min at 72°C); and a final extension at 72°C for 10 min. The PCR products were purified and sequencing by Nam Khoa Biotek Company Ltd. (Vietnam) using ABI 3130 XL Sequencer.

For multiple alignments, the software ClustalW [15] was used to recognise the homology between sequences. ITS sequences of additional species from GenBank were also used in our phylogenetic analysis (table 1). Phylogenetic analysis was carried out with PAUP* ver. 4.0 Beta [13] using pasimony and neighbor joining methods with *Amaryllis belladonna* (table 1) as the outgroup [12]. The statistical support for phylogenetic trees was calculated using the bootstrap method [4] with 1,000 replicates. Bootstrap values of 50% or higher were performed to obtain cluster supports. The pairwise genetic distance [6] was calculated using MEGA6 [14].

Table 1. Sequences from GenBank database used in this study

Taxa	Accession Number	References
Crinum pedunculatum	AY139143	[9]
Crinum firmifolium	EU836632	[1]
Crinum ligulatum	AY139138	[9]
Crinum razafindratsiraea	AY139145	[9]
Crinum defixum	AY139128	[9]
Crinum mauritianum	AY139139	[9]
Crinum subcernuum	AY139150	[9]
Crinum macowanii	AF373094	[8]
Crinum bulbispermum	JX464263	[11]
Crinum crassicaule	AY139126	[9]
Crinum yemenense	AY139151	[9]
Crinum abyssinicum	AY139117	[9]
Crinum moorei	AY139141	[9]
Crinum politifolium	AY139144	[9]
Crinum kirkii	AY139136	[9]
Crinum humile	AY139134	[9]
Crinum campanulatum	AF373088	[8]
Crinum papillosum	DQ386444	[1]
Amaryllis belladonna	JX464257	[11]

3 RESULTS

3.1 Feature of ITS nrDNA region of *Criun asiaticum* and *C. latifolium*

The length of the obtained ITS region sequences ranged from 580 to 795 bp. The entire aligned length of ITS region was 576 bp. In four *Crinum* species, the ITS alignment contained 576 positions of which 35 were variable. The average A+T content in the ITS region is 40%. Insertion and deletion (indels) ranged in size of *C. asiaticum* and *C. latifolium* (in comparision with outgroup) between 1 and 2 bp, excluding the long indel of 2 bp found in *C. asiaticum* (table 2).

Table 2. Indels of the ITS region, posision and size

Number	Range (bp)	Posision	Type of indel	Taxa
1	1	89	Deletion	*C. latifolium*
2	2	90-91	Insertion	*C. asiaticum*
3	1	131	Insertion	*C. latifolium*
4	1	520	Insertion	*C. asiaticum*

The variable nucleotide positions between *C. latifolium* and *C. asiaticum* was shown in table 3. These variation were larger than among other *Crinum* species. *Crinum asiaticum* shared 34 mutations with *C. latifolium* whereas *C. asiaticum* and *C. latifolium* shared 1 mutation with *C. pedunculatum* and *C. abyssinicum*, respectively.

Table 3. Variable nucleotide positions of the ITS region in the four Crinum species

Posision	*C. pedunculatum*	*C. asiaticum*	*C. latifolium*	*C. abyssinicum*
13	T	T	C	C
26	C	C	T	T
30	T	T	C	C
35	A	A	G	G
86	G	G	A	A
87	T	T	-	-
88	G	G	-	-
89	T	T	-	-
97	C	C	T	T
104	A	A	G	G
110	T	T	C	C
134	A	A	-	-
138	G	G	A	A
141	T	T	C	C
147	G	G	A	A
162	G	G	A	A
214	G	G	A	A
217	C	C	T	T
300	T	T	C	C
341	T	T	C	C
350	C	C	T	T
380	G	G	C	C
392	T	T	C	C
399	T	T	C	C
406	G	G	T	T
408	T	T	C	C
419	C	C	T	T
424	G	A	A	A
460	A	A	G	A
464	C	C	T	T
485	T	T	C	C
496	T	T	C	C
518	A	A	-	-
547	T	T	C	C
550	G	G	C	C

3.2 The parsimonious tree

The consensus parsimony phylogenetic tree using PAUP* ver. 4.0 Beta softwere show that *Crinum latifolium* and *C. asiaticum* were sorted into two different groups in the phylogenetic tree (figure 2). *C. latifolium* was grouped together with *C. abyssinicum, C. yemense* and *C. kirkii* with the bootstrap values in the pasimony and neighbor joning methods of 65%. Meanwhile, *C. asiaticum* was grouped with *C. pedunculatum, C. defixum, C. subcernuum, C. fimbriatulum, C. razafindratsiri* and *C. mauritianum* with the bootstrap values in the pasimony and neighbor joning methods of 97%. In addition, the pairwise genetic distances between *C. latifolium* and *C. asiaticum* is larger than in comparision with other *Crinum* species (table S1). The pairwise genetic distances between *C. latifolium* and *C. asiaticum* were up to 0.054, while the pairwise genetic distances between *C. latifolium* and *C. abyssinicum*; *C. asiaticum* and *C. pedunculatum* were 0.002.

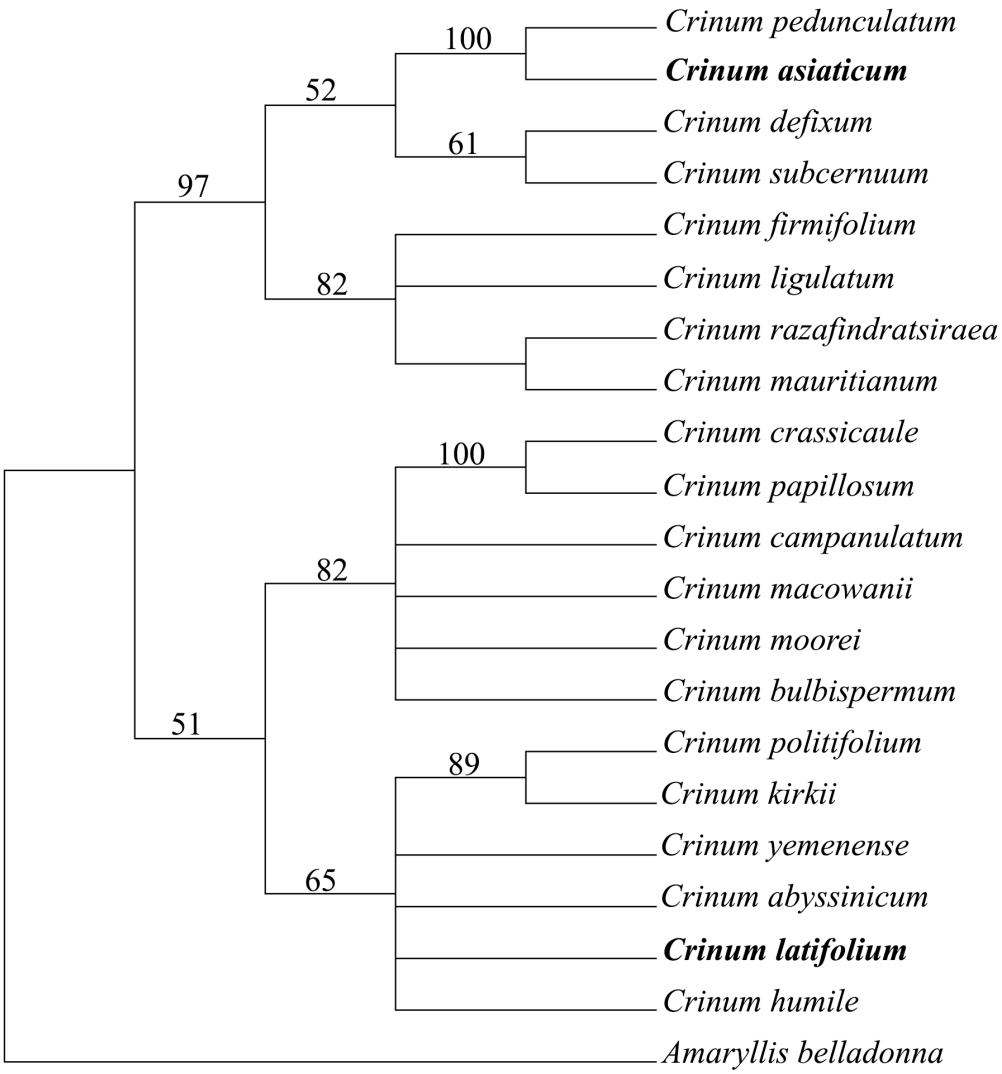

Figure 2. One of most-parsimonious tree obtained based on combined data set of ITS nrDNA of *Crinum latifolium*, *C. asiaticum* and 19 taxa from GenBank database. Gaps treated as missing data. The bootstrap values of 50% or more than from 1000 replicates are shown above the nodes. Tree length= 141 steps, CI (Consistency index) = 0.84, RI (Retention index) = 0.87, and RC (Rescaled consistency index) = 0.73.

3.3 The neighbor joining tree

The neighbor joining phylogenetic tree using PAUP* ver. 4.0 Beta softwere (figure 3) show that *Crinum latifolium* and *C. asiaticum* were sorted into two different groups in the phylogenetic tree. *C. latifolium* was grouped together with *C. abyssinicum, C. yemense* and *C. kirkii* with the bootstrap values in the pasimony and neighbor joning methods of 70%. Meanwhile, *C. asiaticum* was grouped with *C. pedunculatum, C. defixum, C. subcernuum, C. fimbriatulum, C. razafindratsiri* and *C. mauritianum* with the bootstrap values in the pasimony and neighbor joning methods of 99%.

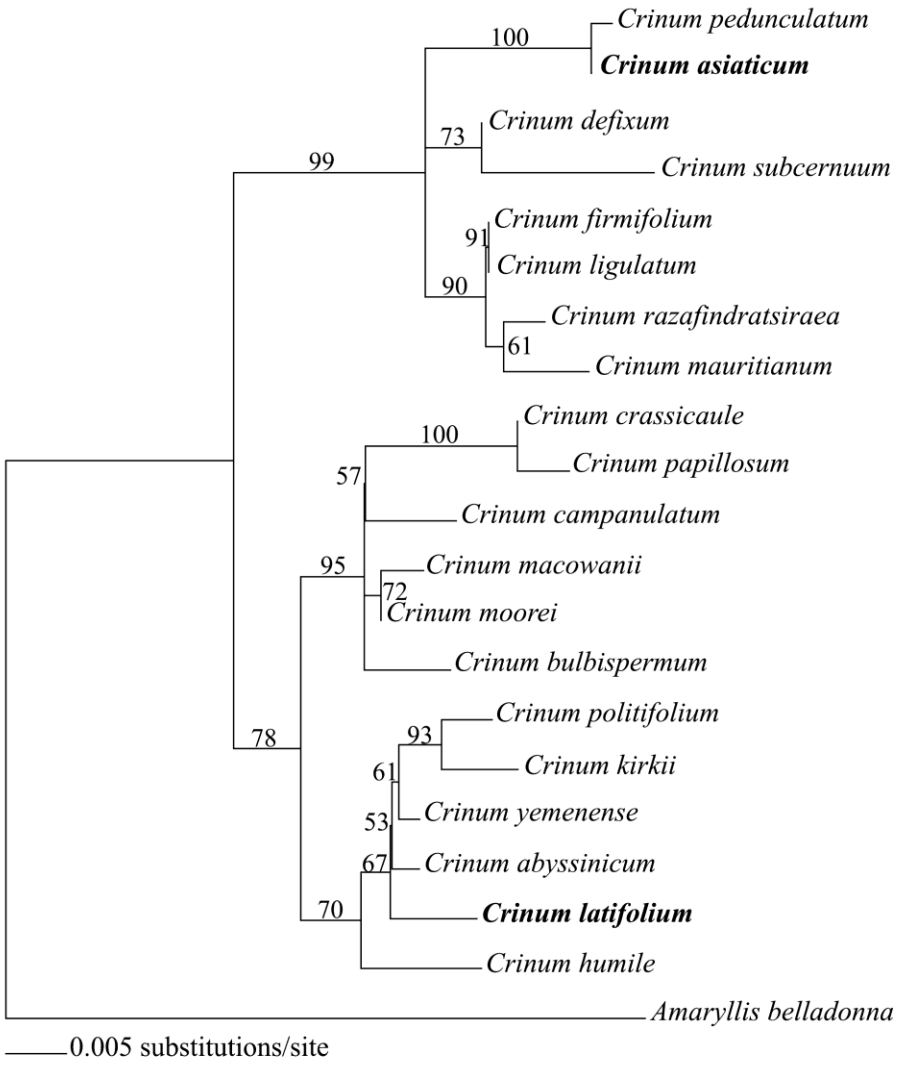

Figure 3. The neighbor-joining tree obtained from combined data set of ITS nrDNA of Crinum latifolium, C. asiaticum and 19 taxa from GenBank database. The bootstrap values of 50% or more than from 1000 replicates are shown above the nodes.

4 DISCUSION

There have been many confusions in the classification using morphological comparison method. For instance, Linnaeus recognised four species, including *Crinum latifolium, C. asiaticum, C. americanum* and *C. africanum* when established the group *Crinum* in 1737 [8]. However, there were approximately 130 new species added into the genus as time passed. Later on, *C. africanum* was then included in the genus *Agapanthus*. There were a number of mistakes in the identification of *Crinum* species conducted by Linnaeus in 1781 [5].

Between 1800 and 1830, a competition in naming the newly collected species of genus *Crinum* was initiated when a number of *Crinum* species were collected from India, West Africa, the Caribbean, mostly from the British colonies, which led to many misinterpretations such as the broad petalled *Crinum* species like *C. zeylanicum* being added into the genus *Amaryllis (Amaryllis zeylanica*). During these times, forms with narrow petals like *C. Asiaticum* were even identified as *Crinum* [5]. A few years later, different ideas in grouping *Crinum* species led to the confusions in their classification and nomenclature, such as the same *Crinum* species being identified as different species by different authors with different names. Consequently, attempts at identification and classification of *Crinum* and the numbers of synonyms increased at the same time [5].

The application of molecular markers for the purpose of accurately determining the positional classification of species which are currently confusing in classification is very necessary. The results obtained in the study have shown that *C. latifolium and C. asiaticum* were clearly distinguished using the nrDNA sequence, which was difficult to be done using the conventional morphological comparison method, especially when the flowers of these two species were not obtained. This classification was similar to many recent studies in the family *Amaryllidaceae* in particular and in plants in general, for example, Kwembeya *et al.* (2007) [7] and Meerow *et al.* (2003) [10] used *trn*L-*trn*F markers IGS and ITS nrDNA to demonstrate that *C. latifolium and C. asiaticum* were found in two different groups in the phylogenetic tree. Similarly, Van *et al.* (2016) [16] used marker *trn*L-*trn*F IGS and *mat*K of chloroplast DNA to show that *Alocasia rivularis (Araceae)* had distinct characteristics in t*rn*L-*trn*F IGS and *mat*K region compared to species with similar morphology characteristics.

5 CONCLUSIONS

Molecular data analysis clearly distinghuished *Crinum latifolium* and *C. asiaticum* as two separate species.

REFERENCES

[1] B. G. Baldwin. Nuclear ribosomal DNA phylogenetics of the tarweeds (Madiinae: Asteraceae). *American Journal Botany* vol. 78, pp. 165–166, 1991.

[2] C. S. Bjora, E. G. Kwembeya, J. Bogner and I. Nordal, Geophytes diverging in rivers-a study on the genus Crinum, with two new rheophytic taxa from Cameroon, Taxon, vol. 58, no. 2, pp. 561-571, 2009.

[3] C. S. Bjora, G. Ezekeil, G. Kwembeya and I. Nordal, Crinum jasonii (Amaryllidaceae): A New Endemic Pan Species of the Luangwa Valley in Zambia with Notes on Different Seed Structures in the Genus. Kew Bulletin, vol. 61, no. 4, pp. 569-577, 2006.

[4] J. V. Freudenstein and M. W. Chase, Analysis of mitochondrial nadIb-c intron sequences in Orchidaceae: utility and coding of leghth-change characters, Systematic Botany, vol. 26, pp. 643-657, 2001.

[5] L. S. Hannibal and A. Williams, The Genus Crinum (Amaryllidaceae). http://www.crinum.org, 1998.

[6] M. Kimura, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., vol. 16, pp. 11-120, 1980.

[7] E. G. Kwembeya, S. Charlotte, C. S. Bjora, B. Stedje and I. Nordal, Phylogenetic relationships in the genus Crinum (Amaryllidaceae) with emphasis on tropical African species: evidence from trnL-F and nuclear ITS DNA sequence data, Taxon, vol. 56, no. 3, pp. 801–810, 2007.

[8] I. Nordal, Revision ofthe East African taxa of the genus Crinum (Amaryllidaceae). Norwegian Journal of Botany, vol. 24, pp. 179-194, 1977.

[9] A. W. Meerow and D. A. Snijman, Phylogeny of Amaryllidaceae tribe Amaryllideae based on nrDNA ITS sequences and morphology, Am. J. Bot., vol. 88, no. 12, pp. 2321-2330, 2001

[10] A. W. Meerow, D. J. Lehmiller and J. R. Clayton, Phylogeny and biogeography of Crinum L. (Amaryllidaceae) inferred from nuclear and limited plastid non-coding DNA sequences. Bot. J. Linn. Soc., vol. 141, no. 3, pp. 349-363, 2003.

[11] H. Pham-Hoang, Amaryllidaceae. In:Pham-hoang, H. (Ed.) Cây cỏ Việt Nam: An Illustrated Flora of Vietnam, Youth Publishing House, Ho Chi Minh City, vol. 3, pp. 499-500. [in Vietnamese with English summary].

[12] N. Ronsted, M.R. Symonds, T. Birkholm and B. Christensen, phylogeny predict chemical diversity and potential medicinal activity of plants? A case study of Amaryllidaceae, Evol. Biol., vol. 12. No. 1, pp. 182, 2012.

[13] D. L. Swofford, PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods)". Version 4.0 Beta. Sinauer Associates, Sunderland, Massachusetts, 2002.

[14] K. Tamura, G. Stecher, D. Peterson, A. Filipski and S. Kumar, MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Bio. Evol., vol. 30, pp. 2725-2729, 2013.

[15] D. J. Thompson, D. G Higgins, T. J Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res, vol. 22, pp. 4673-4680, 1994.

[16] H. T. Van, P. N. Nguyen, N. T. Tran, H. T. Luu, Morphological and molecular data reveal a new species of Alocasia (Araceae) from Vietnam, Vietnam Science & Technology, vol. 59, no. 2, pp. 76-82, 2017.

[17] T. J. White, T. Bruns, S. Lee, J. Taylor, Ampli cation and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in PCR Protocols: a guide to methods and applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. Academic Press, New York, USA, 1990, pp. 315–322.

Received on August 18 – 2017

Revised on December 04 – 2017

SUPPLEMENTARY DATA

Table S1. Mean pairwise genetic distances between *Crinum* species based on the combined data set of ITS nrDNA

	1. *C. pedunculatum*	2. *C. asiaticum*	3. *C. defixum*	4. *C. subcernuum*	5. *C. firmifolium*	6. *C. yemenense*	7. *C. kirkii*	8. *C. abyssinicum*	9. *C. latifolium*	10. *C. humil*
1. *C. pedunculatum*	-									
2. *C. asiaticum*	0.002	-								
3. *C. defixum*	0.020	0.018	-							
4. *C. subcernuum*	0.027	0.025	0.014	-						
5. *C. firmifolium*	0.020	0.018	0.014	0.022	-					
6. *C. yemenense*	0.050	0.048	0.037	0.052	0.037	-				
7. *C. kirkii*	0.056	0.054	0.046	0.062	0.046	0.011	-			
8. *C. abyssinicum*	0.050	0.048	0.037	0.052	0.037	0.004	0.013	-		
9. *C. latifolium*	0.056	0.054	0.042	0.054	0.042	0.009	0.018	0.002	-	
10. *C. humile*	0.052	0.050	0.039	0.054	0.039	0.013	0.022	0.013	0.018	-