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Article

Intraspecific Variation in *Commiphora wightii* Populations Based on Internal Transcribed Spacer (ITS1-5.8S-ITS2) Sequences of rDNA

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Abstract: *Commiphora wightii* is an endangered, endemic species found in the Thar Desert of Rajasthan, India and adjoining areas of Pakistan. The populations of this plant are rapidly dwindling due to overexploitation for their medicinally important resin. Analysis of nucleotide sequences of the internal transcribed spacer of rDNAs revealed low genetic diversity (π = 0.03905; θ_w = 0.05418) and high population structure (Φ_{ST} = 0.206). Parsimony based assessment and Bayesian analyses were conducted on the dataset. Mantel's test showed a statistically significant positive correlation between genetic and geographic distance (r^2 = 0.3647; p = 0.023). Anthropogenic overexploitation of *C. wightii* for its natural resources has resulted in population fragmentation. Initiatives should be taken immediately to preserve the diversity of this important plant species.

Keywords: internal transcribed spacer; rDNA; genetic diversity; guggul; endangered; endemic

1. Introduction

Commiphora wightii (Arn.) Bhandari, of the family Burseraceae [1], is a small tree that has a thick main stem with crooked knotty branches. The plant is primarily found in the arid to semi arid regions of the Thar Desert (in the state of Rajasthan, India) and adjoining parts of Pakistan. It grows on plains and hilly areas with preference for dry rocky terrains. The plant is reported to be apomictic [2]. Mature plants (5–7 yrs old) produce a medicinally important resin which is called 'guggulu' in Sanskrit. Guggulu is mentioned in the classical medical treatises Charaka Samhita (1000 BC) and Shushruta Samhita (600 BC); in Western texts it is known as 'guggul' [3]. The major bioactive components of guggul resin are *Z*-guggulsterone and *E*-guggulsterone [4] that are used for the treatment of hypercholesterolemia and cardiovascular diseases [5-6]. Recent studies have shown that guggulsterones also possess anti-cancer activity [7-9]. Due to its medicinal importance the resin has high commercial value. Those seeking to obtain guggulu, resort to inflicting severe and often fatal wounds to the main stem of the plant for quick extraction of the resin. Due to the presence of resin, the main stem burns well and hence it is also used as fuel wood. These anthropogenic activities have resulted in a drastic decrease in the number of populations. In response to these situations the Government of India has placed *C. wightii* in the RET (Rare, Endangered and Threatened) category. If such exploitation continues without sustainable and appropriate management of the genetic resources, the diversity and natural abundance of this species will be eroded further [10]. Conserving and managing a species without regard to its population structure may fail to conserve the full spectrum of functions provided by that species, thus it becomes critical to understand the nature of its population structure.

The nuclear ribosomal transcription unit (NRTU) is comprised of 18S, 5.8S and 28S genes, two internal transcribed spacers (ITS-1 and ITS-2), and an intergenic spacer (IGS). After transcription, the NRTU is processed to produce mature rRNAs that are key components of cytoplasmic ribosomes. NRTU are found in hundreds to thousands of tandem copies and usually several NRTU clusters are present within plant genomes. The conserved regions (18S and 28S genes) of NRTU are used to infer phylogenetic relationships at higher taxonomy levels, whereas the more rapidly evolving segments (ITS and IGS) are used for studies at the genic or population levels [11-12]. For over a decade, sequences of internal transcribed spacers (ITS) of NRTUs have been widely used to infer phylogenetic relationships, genetic diversity and to unravel evolution in a wide range of complexes in plants [12-17]. Although NRTUs are found in thousands of copies within a genome, intra-genomic diversity is generally low [14]. This homogeneity among NRTUs is attributed to concerted evolution [14,18], a process that acts through gene conversion and unequal crossing over. Despite the fact that homogenization is a norm among NRTUs in a genome, extensive intra-individual and intra-specific variation has been observed in various plant species [19-20]. Evidence is accumulating that suggests that intra-individual variation of nuclear ribosomal ITS regions should not be considered as exceptional [21]. Because of the influence of concerted evolution, the occurrence of ancestral polymorphisms is not the most likely ultimate cause for intra-genomic variability in this marker. Instead, a more frequent origin is the merging of different ITS copies within the same genome as a consequence of gene flow. Once the two copies meet, the fate of the polymorphism depends on genetic, reproductive and population-level factors: specifically, the number and location of ribosomal loci (on the same or different chromosomes), the occurrence of polyploidy and/or apomixis [15,19,22], and the relative abundance of different ITS copies in the breeding populations [21].

Molecular phylogenetic analysis using NRTU sequence data of Mexican *Bursera* spp. [23-25] revealed *Commiphora* spp. and *Bursera* spp. to be closely related and resolved them as monophyletic sister groups, with considerable differentiation between them. *C. wightii* was not included in the study.

Weeks *et al.* [26] included *C. wightii* in their attempt to reassess biogeographical studies of 48 species belonging to Burseraceae of Gondwanan origin, but the sample was acquired from the arid lands greenhouse, Arizona, USA. Moreover, these studies did not capture genetic variation among *Commiphora* spp. Our earlier studies to genetically dissect *C. wightii* populations by RAPD markers revealed low diversity and high population differentiation [27]. An important prerequisite for development of an effective conservation strategy is the proper evaluation of the distribution and level of genetic variation [28]. Information on population structure and genetic variation of *C. wightii* is lacking, which is surprising, considering the endangered status of the plant. The principal objectives of our present study was to utilize the nucleotide data of the ITS1-5.8S-ITS2 sequences to (a) determine the genetic variability within *C. wightii* (b) evaluate the degree of differentiation among *C. wightii* populations and its relationship with geographical distribution, and (c) offer genetic information that can be used in development of a *C. wightii* conservation strategy.

2. Material and Methods

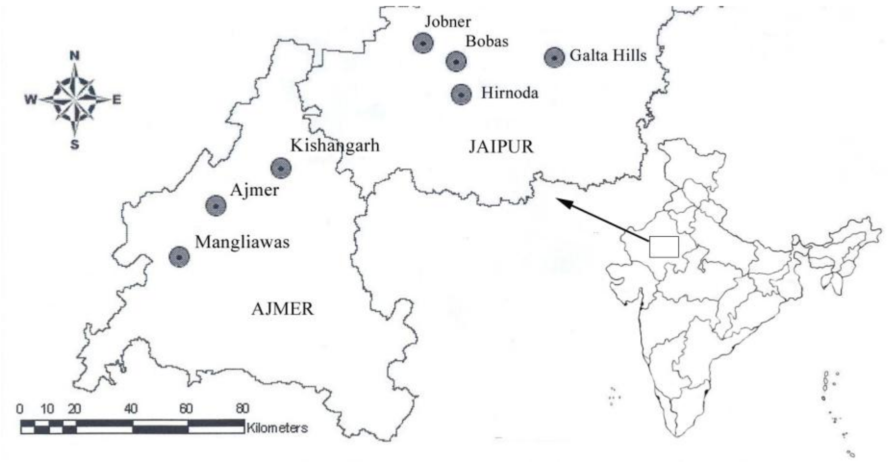
C. wightii plants only produce leaves during the short rainy season (July-October). Fresh young leaves showing no signs of necrotic lesions were sampled from 32 plants growing naturally, belonging to seven populations (Table 1), collected from the state of Rajasthan, India (Figure 1) during September 2007. Voucher specimens were deposited at the herbarium of SKN University College at Jobner, Rajasthan. For each specimen total genomic DNA was isolated from 0.4 g of leaves following a modified CTAB DNA extraction procedure [29] developed in this laboratory for plants having significant quantities of secondary metabolites. The DNA samples were quantified on a Fluorometer (VersaFluor, Bio-Rad Lab., Hercules, CA, USA) with Hoechst 33258 and using Calf thymus DNA as a standards. The quality of DNA was checked both spectrophotometrically and by agarose (0.8%) gel electrophoresis as well.

Table 1. Location of the sampled population of *C. wightii*.

Population	Terrain	Population Code	Sample size	Latitude	Longitude	Elevation (ft)
Mangliawas	Hilly	M	7	26.283	74.500	1,433
Ajmer	Hilly	A	4	26.450	74.633	1,548
Kishangarh	Hilly	KG	4	26.566	74.866	1,420
Jobner	Plains	J	8	26.966	75.383	1,243
Bobas	Plains	B	3	26.905	75.498	1,220
Hirnoda	Plains	H	3	26.885	75.333	1,116
Galta Hills	Hilly	GH	3	26.916	75.857	1,698

The internal transcribed spacers (ITS1 and ITS2) and the 5.8S-coding region were amplified by primers ITS4 and ITS5 [30]. Reaction conditions were according to Haque *et al.* [29]. Amplified products were resolved by electrophoresis on 1.4% agarose gels prepared with 1× TAE (Tris-acetate-EDTA) buffer system, run at 5 V/cm, and stained with ethidium bromide solution (0.5 µg L^{-1}). A 100 bp DNA ladder (Hyperladder IV, Bioline Ltd., London, UK) was used to estimate the molecular weights of the amplified products. The stained gels were viewed briefly and bands were excised and eluted from the gel using the MinElute gel extraction kit (Qiagen). The samples were directly sequenced on an automated sequencer ABI-3730xl at a commercial facility (Macrogen Inc., Seoul, South Korea). To obtain proper and reliable sequences, all the amplified samples were sequenced in both directions with two flanking (ITS5 and ITS4) and two internal (ITS2 and ITS3) primers which provided four overlapping sequences, two for the sense strand and two for the antisense strand for each sample selected for the study (Supplementary Data 1).

Figure 1. Location of the seven populations of *C. wightii* included in the study from the state of Rajasthan, India.



2.1. Data Analysis

Complimentary strands of the ITS region were assembled using Sequencher 4.8 (Gene Codes Corporation, MI, USA) and checked for homology using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide multiple alignments were performed with ClustalX [31] using the default parameters (Supplementary data 2). Nucleotide polymorphism, as measured by θ_w [32] and diversity, as measured by π [33], were calculated using DnaSP v4.5 [34].

Analysis of molecular variance (AMOVA) was performed using GenAlEx 6.1 [35] to assess genotypic variations across all the populations studied. This analysis, apart from partitioning of total genetic variation into within-group and among-group variation components, also provided a measure of intergroup genetic distance as the proportion of the total variation residing between populations. The program was also used for a subsequent Mantel's test [36] to evaluate the relationship between genetic and geographic distance. The significance of both the analysis was tested using 999 random permutations.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [37] and a Maximum Parsimony (MP) tree was constructed using the Close-Neighbour-Interchange algorithm. A bootstrap consensus tree was inferred from 1000 replicates, and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. To check for congruency, a Bayesian analysis using Metropolis-coupled Markov chain Monte Carlo (MCMCMC) was also implemented on the ITS dataset using MrBAYES software [38]. Prior to phylogenetic estimation, we used the program jModelTest 0.1.1 [39] to select which of the 88 models of DNA substitution better fits the data. The Bayesian analysis consisted of four chains with random starting trees and the GTR+G model of nucleotide substitution. The four chains, were run for 3×10^6 generations and trees were sampled every 100th generation. After plotting likelihood values for the four analyses we ascertained that the Markov chains reached stationarity approximately after 100,000 generations. We discarded the first 7,500 trees as burn-in and the remaining trees were used to construct a 50% majority rule consensus tree on which posterior probability scores were indicated.

3. Results and Discussion

ITS regions from 32 *C. wightii* individuals were sequenced and analyzed. The absence of other variable regions in the nuclear DNA of plants that could provide useful markers at both intra-family [13] and intra-genomic level differentiation [21], makes ITS ostensibly the best molecule for phylogenetic studies. The present study focuses on the insights of inter-individual variability to address questions related to diversity.

The amplified region of ITS1-5.8S rDNA-ITS2 in *C. wightii* varied between 711 to 727 bp. Similarity search of the sequences at the NCBI database showed ~95% similarity with other *Commiphora* spp., the sequences were deposited in the GenBank Database (Accession numbers-EU419958 to EU419989). The average GC content of the sequences was 64.6%, the substitution probabilities are given in Table 2 and the overall transition/transversion bias (*R*) was 1.319. Multiple sequence alignment of all the 32 ITS sequences resulted in a matrix of 757 characters (Supplementary data 2) out of which 70 were parsimony informative.

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution. Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row need to be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

	A	T	C	G
A	-	*4.84*	*8.22*	**22.43**
T	*3.94*	-	**10.13**	*8.57*
C	*3.94*	**5.96**	-	*8.57*
G	**10.33**	*4.84*	*4.22*	-

The overall estimate for nucleotide polymorphism is presented in Table 3. A five-fold level of polymorphism was observed between the populations, with the highest in Galta Hills (π = 0.05493 ± 0.01707, θ_w = 0.05307 ± 0.03221) and the lowest in Hirnoda (π = 0.01107 ± 0.00374, θ_w = 0.01107 ± 0.00709). Variability within NRTU families usually depends upon number of gene copies, rates of mutation, concerted evolution, number and chromosomal location of NRTU clusters, and proportion of sexual and asexual reproduction [40]. Polymorphism may arise when concerted evolution is not fast enough to homogenise repeats in face of high rates of mutation [41] or by loss of sexual recombination [19]. Also, concerted evolution is retarded in agamospermous plants [18]. As *C. wightii* is reported to be apomictic, lack of sexual reproduction could be an important contributor to polymorphism. Also, concerted evolution is retarded in agamospermous plants. In a simulation model, Adolfsson and Bengtsson [42] concluded that the spread of apomixis in a population is not necessarily associated with a substantial decrease in genetic variability and most of it is retained. These cumulative factors should increase the polymorphism among *C. wightii* populations, but the converse was observed. Our earlier studies revealed that sexuality in *C. wightii* is not uncommon [27], and the plant does revert to sexual mode of reproduction during its life cycle. Sexual reproduction may lower ITS sequence polymorphism by accelerating concerted evolution and homogenizing the ITS repeats.

Table 3. Nucleotide diversity of the ITS1- 5.8S- ITS2 fragment for the seven populations. π and θ_w refer to nucleotide diversity according to Nei and Li [43] and Watterson's parameter [32] respectively. Figures in parenthesis denote standard deviation.

Population	π	θ_w
Mangliawas	0.03473 (0.00908)	0.03446 (0.01590)
Ajmer	0.03078 (0.01080)	0.03148 (0.01742)
Kishangarh	0.03650 (0.00843)	0.03424 (0.01890)
Jobner	0.04445 (0.00550)	0.04966 (0.02180)
Bobas	0.03661 (0.01121)	0.03614 (0.02210)
Hirnoda	0.01107 (0.00374)	0.01107 (0.00709)
Galta Hills	0.05493 (0.01707)	0.05307 (0.03221)
All Samples	0.03905 (0.00307)	0.05418 (0.01685)

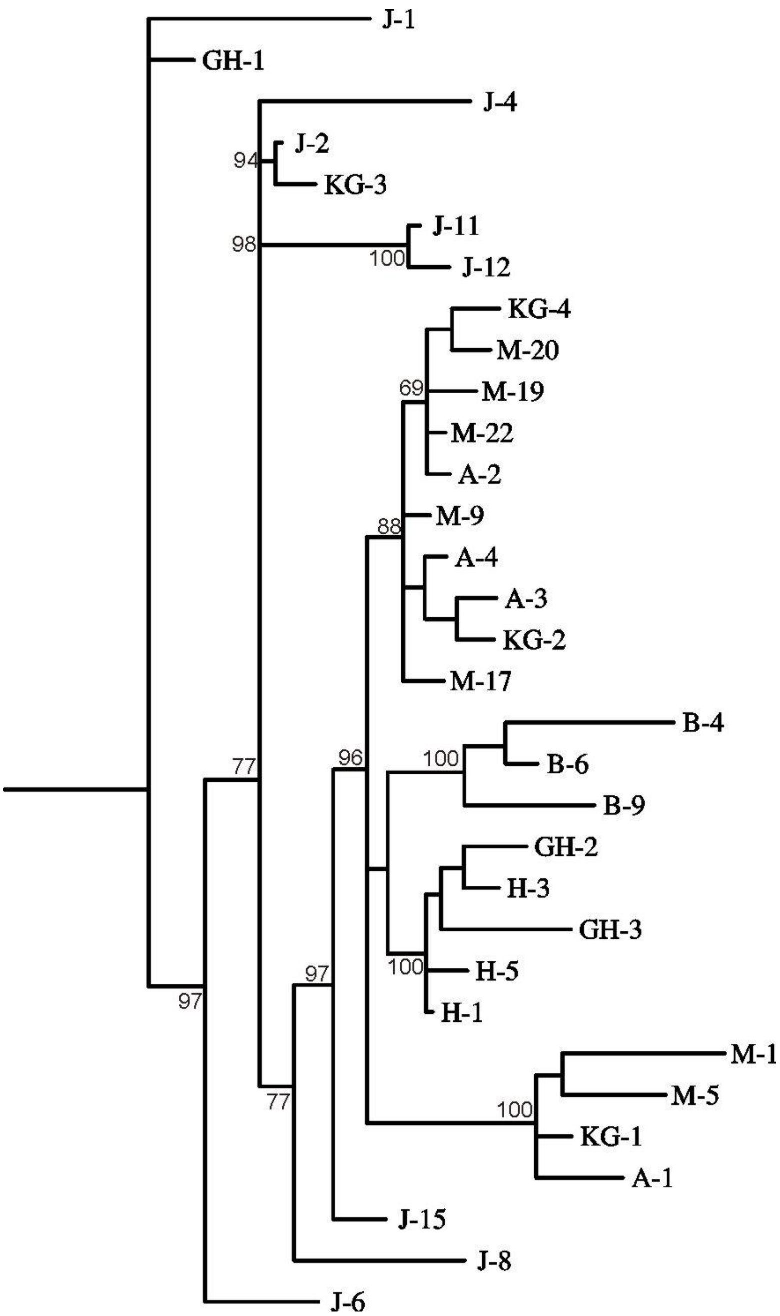
AMOVA was used to partition the genetic diversity and test whether there is any hierarchy of ITS sequence variation among individuals (Table 4). The genetic differentiation between the populations is high (Φ_{ST} = 0.206) (Nei [33] classified G_{ST} > 0.15 as high, Φ_{ST} and G_{ST} both denote fixation index and are comparable).

Table 4. Hierarchical analysis of molecular variance (AMOVA) within/among C. wightii populations. d.f.: degrees of freedom; SSD: sum of squared deviations; ΦST: fixation index; p-value: the probability of having a more extreme variance component than the observed values by chance alone.

Source of variation	d.f.	SSD	Estimated variance	Total variance (%)	Φ_{ST}	p-value
Among population	6	301.118	6.046	21		
Within population	25	508.796	23.359	79	0.206	0.01
Total	31	885.094	29.405			

The constructed maximum parsimony tree separated the samples into five major clusters (data not shown). The majority rule consensus tree prepared by Bayesian analyses of the ITS sequence data also showed that the populations were grouped according to their geographic location (Figure 2). Apart from Bobas, samples from a particular population did not separate into a single cluster and were found to be mixed. Samples collected from Ajmer, Kishangarh and Mangliawas were distributed in two clusters, so were the Hirnoda, Jobner and Galta Hills samples. It seems that genetic exchange might be taking place between samples from nearby areas (i.e., districts), while long distance transfer is being restricted; this may be responsible for high population structure and differentiation.

Figure 2. Relationship between the taxa studied, inferred using the maximum likelihood method (MrBayes). Numbers at the nodes are Bayesian posterior probabilities.



4. Conclusions

It appears that *C. wightii* plants have evolved under reproductive isolation, probably due to population fragmentation and habitat loss. The reproductive isolation, coupled with the reported apomictic behaviour may have a detrimental effect on genetic variation. Mantel's test showed an average correlation (r^2 = 0.3647) between genetic and geographic distance that was statistically significant (p = 0.023). These samples appeared to be the remnants of a previously large population that was present in the area. Since population differentiation is high, the population continuum has been disrupted, possibly due to over-exploitation, unsustainable utilization and other anthropogenic activities. Educating the villagers and promoting scientific gum-resin tapping practices seem to be the best conservation strategies, along with habitat restoration. Besides, initiative should be taken on surveying and mapping the distribution of the plant for future germplasm collection and maintenance.

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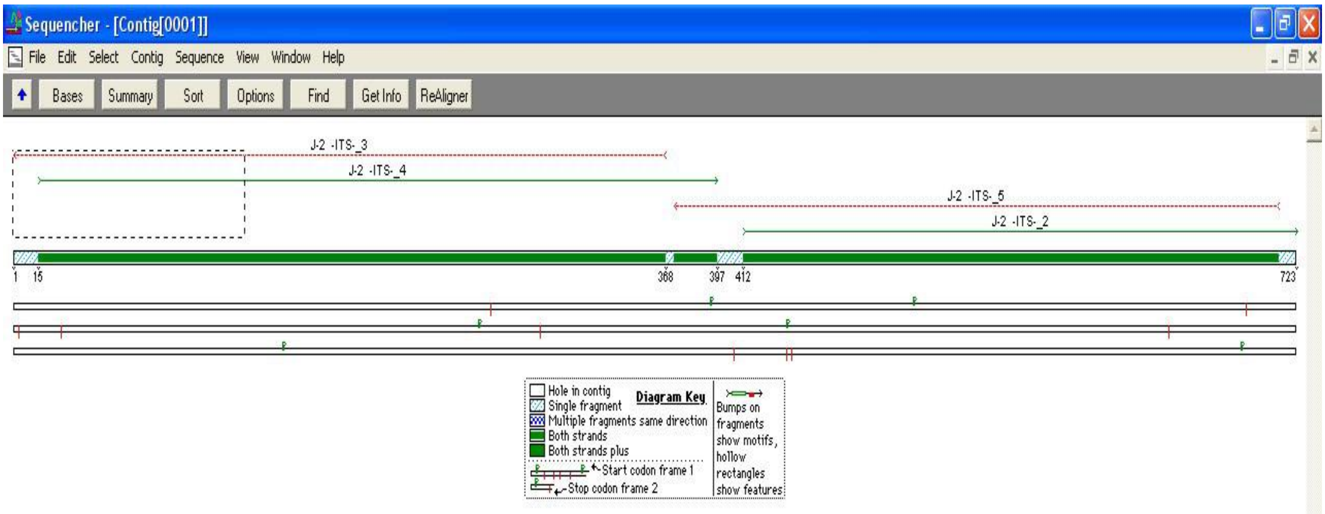
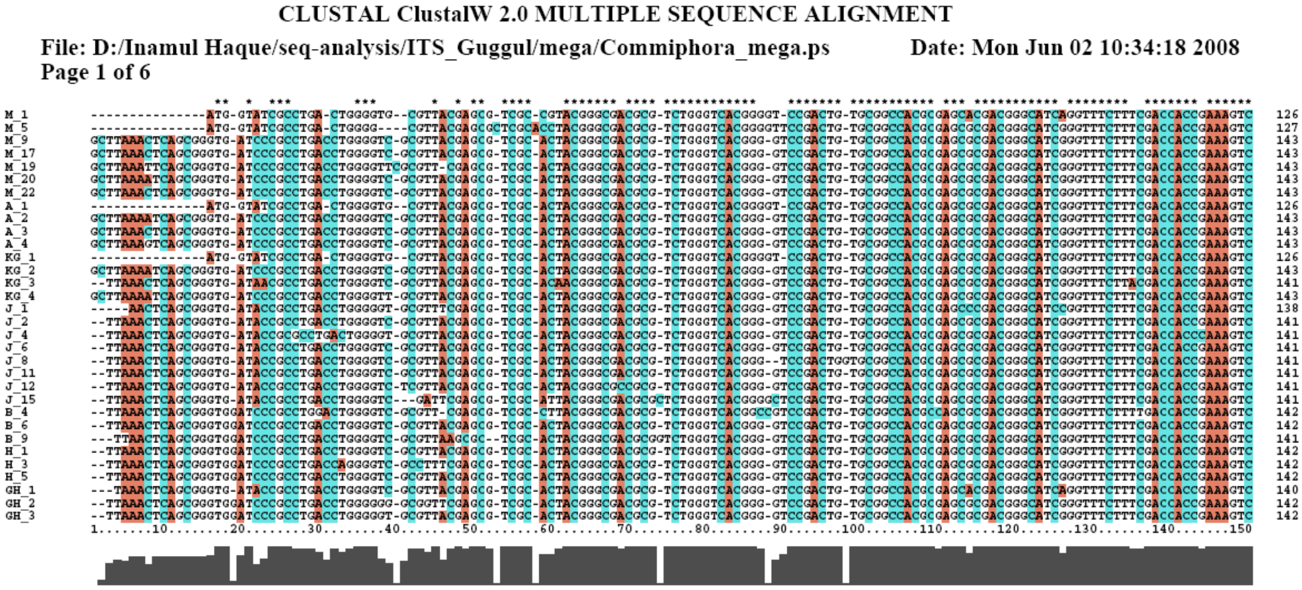
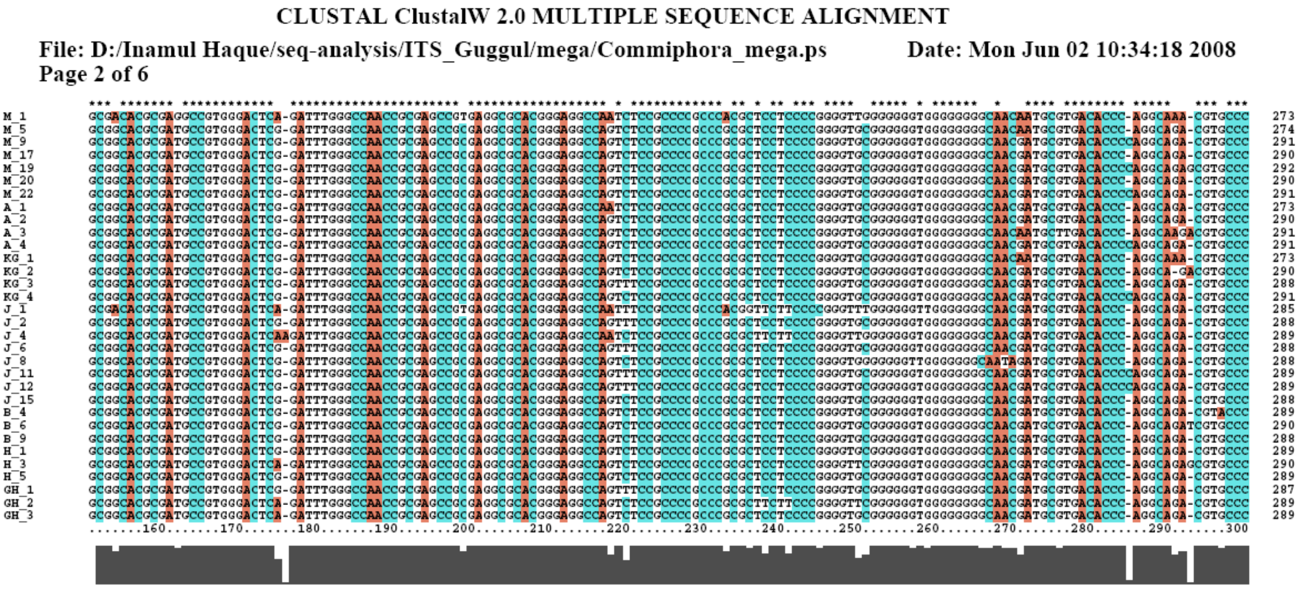
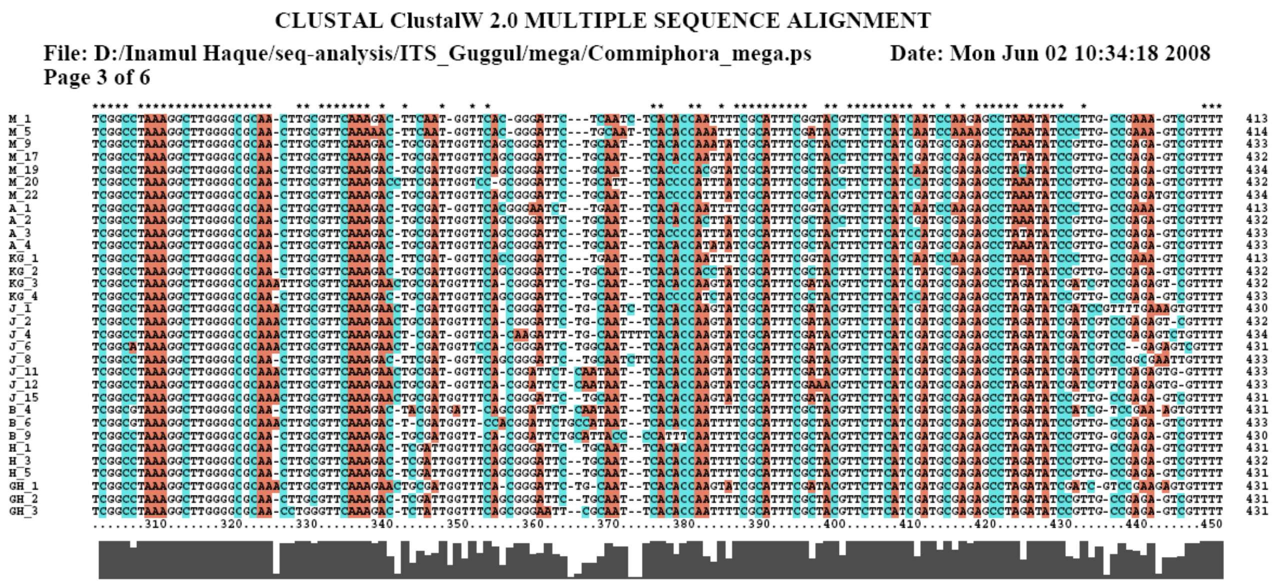
Supplementary Data 1. Screenshot showing complete sequence assembly of ITS region obtained with four primers.



Supplementary Data 2. Complete alignment matrix of the ITS sequences from *C. wightii*.







Supplementary Data 2. *Cont.*

CLUSTAL ClustalW 2.0 MULTIPLE SEQUENCE ALIGNMENT

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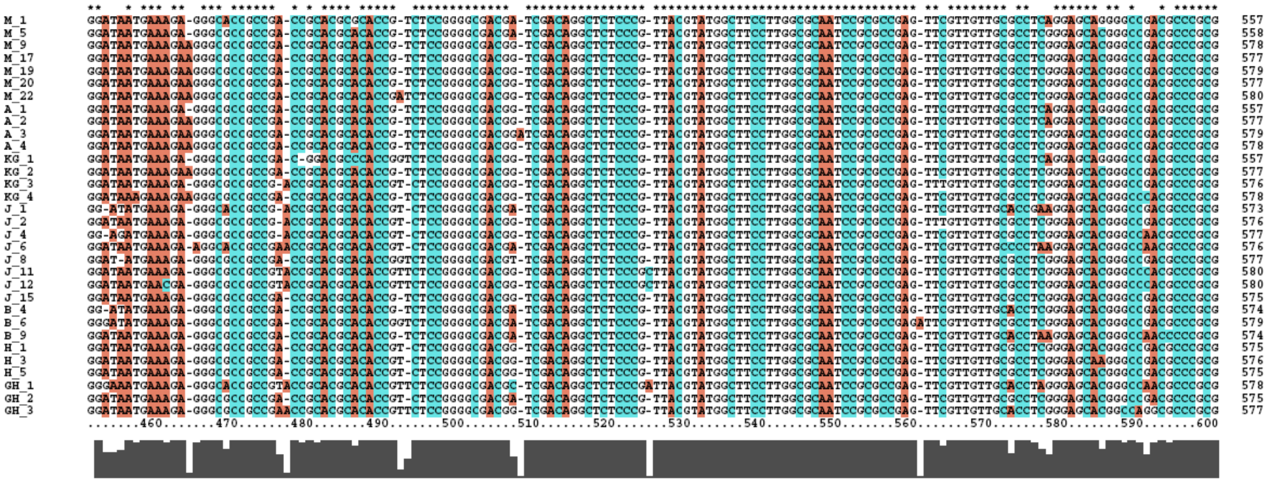

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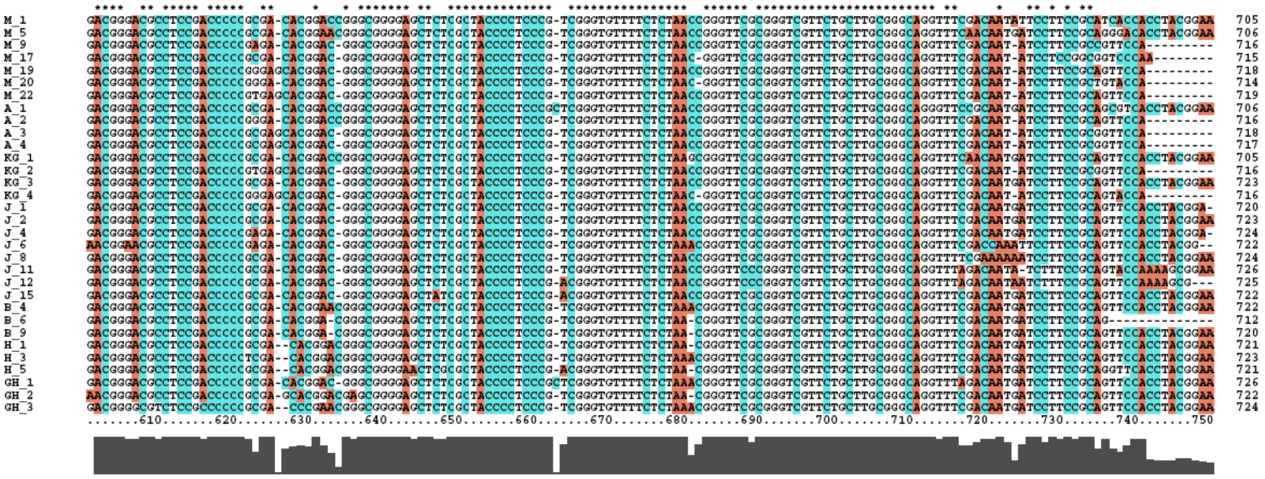

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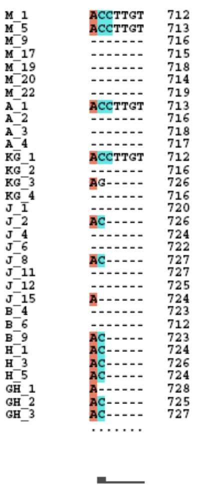

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