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Evaluation of COI and ITS2 sequences ability to distinguish *Lysiphlebus fabarum* and *Lysiphlebus confusus* (Hymenoptera: Aphidiidae)

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Abstract

All wasp species in the genus *Lysiphlebus* Förster are solitary endoparasitoids of aphids and considered a taxonomically difficult group. In this study a part of COI and ITS2 was used to distinguish two closely related species *L. fabarum* (Marshall) and *L. confusus* Tremblay & Eady in Iran. The sequencing with a similarity about 99.5% showed COI's inability to separate the species *L. fabarum* and *L. confusus*. The ITS2 region of the rDNA partly separated the species with the similarity of 96.5-98.5%. The result is indicative of DNA barcoding deficiency in the identification of the species, as the two gene regions fail to efficiently separate the species.

Keywords: COI, DNA barcoding, ITS2, mitochondrial DNA, ribosomal DNA, *Lysiphlebus confusus*, *Lysiphlebus fabarum*

چکیده

زنبورهای پارازیتوئید جنس *Lysiphlebus* Förster پارازیتوئید انفرادی- داخلی شته‌ها هستند و از لحاظ تاکسونومیکی یکی از گروه‌های بسیار مشکل و ناشناخته محسوب می‌شوند. هدف اصلی این مطالعه استفاده از توالی قسمتی از ناحیه‌ی ژنی COI و ITS2 برای تفکیک دو گونه‌ی نزدیک به هم *L. fabarum* (Marshall) و *L. confusus* Tremblay & Eady جمع‌آوری شده از ایران است. داده‌های مولکولی به‌دست‌آمده از توالی‌یابی ناحیه‌ی COI نشان داد، این ژن قادر به تفکیک دو گونه‌ی فوق نیست و این گونه‌ها دارای شباهت بسیار زیاد (۹۹/۵٪) در توالی COI هستند. داده‌های مولکولی به‌دست‌آمده از توالی‌یابی ناحیه‌ی ریبوزومی ITS2 تنوع بیشتری را در بین این دو گونه نشان داد. تشابه موجود در این ناحیه‌ی ژنی ۹۸/۵-۹۶/۵٪ بود. علی‌رغم اهمیت DNA بارکدینگ، نواحی ژنی مورد استفاده در این مطالعه توانایی کافی در شناسایی و تفکیک دو گونه‌ی نزدیک به هم را نداشتند.

واژگان کلیدی: COI، DNA بارکدینگ، ITS2، DNA میتوکندریایی، DNA ریبوزومی، *Lysiphlebus confusus*، *Lysiphlebus fabarum*

Introduction

The genus *Lysiphlebus* Förster includes 30 described species that are solitary endoparasitoids of aphids. The species *L. fabarum* (Marshall) is an abundant generalist parasitic wasp in Guilan province with a very broad host range. The sympatric species *L. confusus* Tremblay & Eady together with the former species have a significant role in the control of aphid populations (Matin *et al*., 2009). The most important morphological character for separating *L. fabarum* from *L. confusus* is the lower marginal setae of forewings in females. In *L. confusus* lower marginal setae of forewing are longer than those on the surface while in *L. fabarum* these setae are as long as setae on the surface (Rakhshani *et al*., 2005). Based on the host plants, *L. confusus* can be divided into two groups: real *L. confusus* and *L.*

confusus-group. The pattern and the size of the lower marginal setae of forewing differs between the two groups, which make them difficult to identify, when are compared to *L. fabarum* (P. Stary, personal communication).

Molecular diagnostic tools provide a means for the rapid, accurate and inexpensive identification when morphological characters are not sufficient (Armstrong & Ball, 2005). In contrast to morphological characters, molecular data are expression of changes at the gene level and being less influenced by environment. Also molecular data are easily interpreted by producing more characters than visual characters. Genomic approaches use diversity among DNA sequences in order to identify organisms (Wilson, 1995). Selection of a suitable DNA region for phylogenetic analysis among species and genera, even between families, is always a challenge (Brower & DeSalle, 1994). The two most often targeted regions for insect systematic and population genetic studies are mitochondrial DNA and nuclear ribosomal DNA genes. In mtDNA, the most commonly used genes are 16S and 12S subunits, cytochrome oxidase I (COI) (Machado *et al*., 2001) and cytochrome oxidase II (COII) (Despres *et al*., 2002) and in ribosomal DNA, 18S, 28S subunits of rRNA, and the first and second internal transcribed spacer regions (ITS1 and ITS2) are the most common (Gariepy *et al*., 2007).

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's mitochondrial DNA for a quick identification of species (Ladoukakis & Zouros, 2001). A mitochondrial gene (COI) has been received more attention and leads the use of 500-650 bp from the first half of the COI gene in DNA barcoding.

Shufran *et al*. (2004) in a phylogenetic analysis, using COI and 16S gene region sequences, found that *Lysiphlebus testaceipes* (Cresson) population from Florida was set in basal of Great Plains Texas isolates with *L. fabarum* population. Sequence of COI gene region has been used for identification of many insect species such as *Torymus* spp. (Hym.: Torymidae) (Yara, 2004), *Encarsia* spp. (Hym.: Aphelinidae) (Monti *et al*., 2005), *Calliphora vicina* Robineau-Desvoidy and *C. vomitoria* (L.) (Dip.: Calliphoridae) (Ames *et al*., 2006), *Elachista* spp. (Lep.: Elachistidae) (Kaila & Stahls, 2006), mealybug species (Hem.: Pseudococcidae) (Saccaggi *et al*., 2008) and *Baetis vernus* Curtis and *B. macani* Kimmins (Ephem.: Baetidae) (Stahls & Savolainen, 2008).

In rDNA, ITS2 is a suitable choice for the identification of closely related species or sympatric populations and used in species barcoding and DNA array technologies (e.g., Engelmann *et al*., 2009; Landis & Gargas, 2007). It is typically 200-400 bp in length (in

Drosophila Fallén) and lies between the 5.8S and 28S genes, easily amplified by PCR from even miniscule amount of DNA, and is easy to sequence (Young & Coleman, 2004). Yu-Cheng & Greenstone (1999) by using ITS2 sequence, were able to distinguish *Aphelinus albipodus* Hayat & Fatima and *A. varipes* (Förster) and two strains of *A. asychis* Walker (Hym.: Aphelinidae) as endoparasitoids of *Diuraphis noxia* (Mordvilko) (Hem: Aphididae). Ciociola *et al*. (2001) identified the two closely related species *Trichogramma rojasi* Nagaraja & Nagarkatti and *T. lasalli* Pinto (Hym.: Trichogrammatidae) by ITS2 sequences. ITS1 and ITS2 of the rDNA could differentiate the bark beetle forest pest *Tomicus destruens* Wollaston from *T. piniperda* L. (Col.: Scolytidae), which has been mistakenly thought to be the same by some authors (Gallego & Galian, 2001). The ribosomal ITS2 sequences could also separate the two closely related populations of *Ageniaspis citricola* Logvinovskaya (Hym.: Encyrtidae) from Australia and Taiwan (Alvarez & Hoy, 2002). In a study by Stouthamer *et al*. (2004), ITS2 also was able to distinguish the two sibling species of *Trichogramma pretiosum* Riley and *T. deion* Pinto & Oatman. ITS2 sequence could also identify six species in the *Anopheles crucians* Wiedemann complex (Dip.: Culicidae) (Wilkerson *et al*., 2004).

Because of the limitations in appropriate diagnostic morphological characters, use of a reproducible and repeatable recognition tools like molecular makers will be more helpful in the identification of two closely related endoparasitic wasp species *L. fabarum* and *L. confusus* occurring in the same habitat. The purpose of this study was to evaluate the ability of regional gene COI and ITS2 as a barcoding tool for recognizing the two closely related wasp species *L. fabarum* and *L. confusus* in Guilan province, Iran.

Material and methods

Insect sampling and identification

In spring 2008, samples of *L. fabarum* and *L. confusus* were collected in the Guilan province cities: Rasht (University of Guilan, 37º 11' 38.32" N, 49º 38' 18.82" E), Fouman (Ghaleh-Roud-Khan, 37º 05' 43.71" N, 49º 15' 36.98" E) and Shaft (Emamzadeh_Ebrahim, 37º 00' 10.94" N, 49º 14' 22.60" E). A few specimens of *L. confusus*-group were collected from the south-western province of Khouzestan on *Malva* sp. The species were identified by Dr. P. Stary (Biology Centre, AS CR, Institute of Entomology).

In order to rear parasitoid wasps, the parasitized mummified aphids were collected on different host plants (table 1) and put in 14 × 12 × 5 cm plastic cages provided with a ventilation hole covered by cheese cloth under laboratory conditions (22 ± 1 ºC, 70 ± 5% RH

and 16L: 8D) until the emergence of adult parasitoids. The adult wasps were later preserved in absolute ethanol and identified, using the identification key by Rakhshani *et al*. (2005), and stored at -20 ºC for molecular analysis.

DNA extraction, amplification and sequencing

DNA was extracted from *L. fabarum* and *L. confusus* specimens, using the CTAB method of Juen & Traugott (2005) with the adaptation of 600 µl extraction buffer[1]. The DNA pellet was suspended in 50µl of TE[2] and stored at -20˚C.

The primers C1-J-1718 and C1-N-2191 (Simon *et al*., 1994) were used to amplify COI gene from the *L. fabarum* and *L. confusus*. PCR amplifications, carried out in 50 µl total volume containing 33.8 µl ddH_2O, 5 µl reaction buffer (10X PCR buffer), 2 µl $MgCl_2$ (50 mM), 2 µl each of primers, 1 µl dNTPs (dNTP mix 10 mM), 0.2 µl of *Taq* DNA polymerase (5 U/µl) and 4 µl of DNA template (150-200 µg/ml). The reaction mix was put into a 0.2 ml tube and amplification was performed in a MJ mini™ (BIORAD) thermocycler with an initial denaturation at 95 ºC for 2 min, followed by 35 cycles at 94 ºC for 1 min, 56 ºC for 1 min, and 72 ºC for 1 min. A final extension period at 72 ºC for 5 min followed the 35 cycles.

The primer set 5′-TGTGAACTGCAGGACACATGAA-3′ and 5′-ATGCTTAAATTTA GGGGGTAGTC-3′ were used as forward and reverse respectively (Cornel *et al*., 1996) for amplification of ITS2 in *Lysiphlebus* wasps. PCR amplifications were carried out using Hot Start PCR in 50 µl total volume of 33.4 µl ddH_2O, 5 µl reaction buffer (10X PCR buffer), 1 µl $MgCl_2$ (50 mM), 4 µl each of primers, 1 µl dNTPs (dNTP mix 10 mM), 0.6 µl of *Taq* DNA polymerase (5 U/µl) and 1 µl of DNA template (150-200 µg/ml). The reaction mix without *Taq* DNA polymerase was put into a 0.2 ml PCR tube and amplification was performed in a MJ mini™ (BIORAD) thermocycler with an initial denaturation at 92 ºC for 3 min, then thermocycler was paused and *Taq* DNA polymerase added to PCR tube and process followed by 39 cycles at 92 ºC for 45 sec, 54 ºC for 45 sec, and 72 ºC for 45 sec. A final extension period at 72 ºC for 3 min followed the 39 cycles. PCR products were separated by electrophoresis at 80 V for 45-60 min on a 1.2% agarose gel contained 0.5 µg/ml ethidium bromide for staining. PCR products were visualized and photographed by GelDoc (Bio-Rad, Italy). A negative control was included in all experiments.

PCR products were purified via column-based purification kit (Millipore, USA) using

1. 12.5 mg CTAB, 360 µl ddH_2O, 62.5 µl 1M Tris, 175 µl 5M NaCl, 25 µl 0.5M EDTA and 1.25 µl β-mercaptoethanol.
2. 10 mM Tris-Hcl pH 8.0, 1 Mm EDTA.

vacuum filtering and then were sequenced using Sanger sequencing method by ABI3730XL sequenced analyzer (Applied Biosystems, USA) via Macrogen Company (South Korea) in forward and reverse directions. Samples were sequenced twice.

Sequence alignment

Sequencing results edited by the CHROMAS program version 2.01 (http://www.technelysium.com.au). The basic local Alignment Search Tool (BLAST) was used to compare the similarity of obtained sequences with GenBank database (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignment was performed using ClustalW ver. 1.82 (http://www.ebi.ac.uk/clustalw/).

Results and discussion

Multiple sequence alignment of mtDNA COI gene region for *L. fabarum* and *L. confusus* with a difference only at one or two nucleotides in their sequences (fig. 1) and lack of variation in amino acids (not presented here) show a high similarity among them (99.5%). This result suggests that an identification system based on COI gene region is not applicable and COI divergences appear too low to distinguish *L. fabarum* from *L. confusus*. The most interesting point among these results is that *L. confusus*-group collected on *Malva* sp. from south-western Khuzestan province has a similar COI sequence to *L. fabarum* collected on *Rubus idaeos* from Guilan province in the north of Iran.

Table 1. Sequencing results of COI and ITS2 regions for *L. fabarum* and *L. confusus*.

Species	Accession number	Host plant	Sampling area	Gene	Length (bp)	% of nucleotides			
						A	T	C	G
L. fabarum	FJ799353	*Rubus idaeos*	Fuman*	COI	464	28.23	45.69	10.99	15.09
L. fabarum	FJ870105	*Faba vulgaris*	Rasht (Pirbazar)	ITS2	681	40.70	41.56	8.20	9.54
L. fabarum	FJ870106	*F. vulgaris*	Rasht (Pirbazar)	ITS2	675	40.44	41.50	8.44	9.62
L. fabarum	GU566736	*R. idaeos*	Rasht*	ITS2	679	40.50	41.38	8.25	9.72
L. confusus	FJ799354	*Salix aegyptiaca*	Shaft*	COI	470	28.10	45.94	11.28	14.68
L. confusus	FJ799355	*S. aegyptiaca*	Rasht*	COI	462	28.57	45.67	11.04	14.72
L. confusus	GQ359416	*S. aegyptiaca*	Rasht*	COI	476	27.94	45.59	11.55	14.92
L. confusus -group	GQ359415	*Malva* sp.	Khuzestan	COI	472	28.18	45.76	11.44	14.62
L. confusus	GQ359414	*S. aegyptiaca*	Rasht*	ITS2	479	43.26	37.22	9.26	10.26

* See "Insect sampling and identification" for more information. A = adenine, T = thymine, C = cytosine, G = guanine, bp = base pairs.

Hebert *et al*. (2003) considered the mitochondrial DNA as one of the best choices for DNA barcoding in animals. Although mtDNA genes have been used as useful markers in identification of species, some aspects make their application difficult. The present study showed that DNA barcoding based on this part of COI gene sequence is not suitable for the identification of the two closely related species *L. fabarum* and *L. confusus* but we cannot completely reject the use of DNA barcoding because of our insufficiently collected samples. Other researchers such as Norrbon *et al*. (1999) obtained similar results as a partial COI gene sequence failed to recognize the cryptic individual in *Anastrepha fraterculus* Wiedemann complex (Dip.: Tephritidae). Morrow *et al*. (2000) in a similar work showed COI gene sequence is unable to identify cryptic individual in *Bactrocera tryoni* Froggatt and *B. dorsalis* Hendel (Dip.: Tephritidae) complex. Whitworth *et al*. (2007) unsuccessfully tried to separate the species of the genus *Protocalliphora* Hough (Dip.: Calliphoridae) by barcoding of the COI gene sequence. Two closely related species, *Ochlerotatus portonovoensis* Tiwari & Hiriyan and *O. wardi* Reinert (Dip.: Culicidae) could not be identified as separated species based on DNA barcode approach (Kumar *et al*., 2007). Mallet & Willmot (2003) in their study showed that the identification systems based on mtDNA markers would fail because of the prevalence of horizontal transfers of mitochondria between divergent lineages and the regularly shared mitochondrial polymorphisms among closely allied species over the millions of years. However, the advantages of COI gene as a useful tool to identify species cannot be disregarded. North American bird individuals have been identified to species level with a rate ranging from 98 to 100% based on mtDNA markers (Hebert *et al*., 2004). Australian fish (Ward *et al*., 2005), tropical Lepidoptera (Hajibabaei *et al*., 2006) and morphologically indistinguishable parasitoid flies (Tachinidae) were identified by mtDNA markers (Smith *et al*., 2006).

Inability of COI gene region in separating of *L. fabarum* and *L. confusus*, demonstrated that a different genetic region should be considered for the identification of these species. Ribosomal DNA (rDNA) unlike mtDNA, that has maternal inheritance, comes from both parents. Among rDNA gene regions, ITS2 as a non-coding region has highly repetitive and relatively divergent sequence among closely related populations, and have proven useful for comparison of closely related insect species, subspecies, or populations (Yu-Cheng *et al*., 2004). Many studies showed usefulness of ITS2 region in the identification of closely related species (e.g. Young & Coleman, 2004). Results of multiple sequence alignment for rDNA ITS2 region indicated that *L. confusus* and *L. fabarum* have differences in eight nucleotide

positions (fig. 2). This level of variation was enough to separate these two species. However, in a study by Stouthamer *et al*. (2000) ITS2 sequences did not separate superficially similar species *Trichogramma miutum* Riley and *T. platneri* Nagarkatti (Hym.: Trichogrammatidae).

Infection by a microorganism called *"Wolbachia"* proved that it potentially influences mtDNA variation at the intra or inter-specific level. Numerous studies have demonstrated that selection acting on *Wolbachia* has indirectly reduced mtDNA polymorphism in the infected population or species (e.g. Jiggins, 2003; see review in Hurst & Jiggins, 2005). In insects, at least three cases were described where *Wolbachia* infection caused mitochondrial introgression between closely related species: (1) between several members of *Drosophila melanogaster* (Meigen) subgroup (Ballard, 2000), (2) two sister species of sub-Saharan butterflies *Acraea encedon* (L.) and *A. encedana* Pierre (Lep.: Nymphalidae) (Jiggins, 2003), and (3) between the yellow and the brown type of *Eurema hecabe* (L.), two yet unnamed sibling species of Japanese butterflies (Narita *et al*., 2006). In addition, *Wolbachia*-induced thelytokous populations were confirmed in three species of the genus *Lysiphlebus* (*L. cardui* (Marshall), *L. confusus* and *L. fabarum*) in the West Palaearctic subregion (Stary, 1999). An infection by *Wolbachia* could be the cause of overall similarity of COI sequences gene region between *L. fabarum* and *L. confuses* that needs to be tested for its possible existence among the *Lysiphlebus* populations of Guilan province.

A search among other gene regions present in GeneBank database (encoding elongation factor 1 alpha and 18S rDNA) to separate *L. fabarum* and *L. confusus* showed none of above-mentioned genes could distinguish these two species except ATPase subunit 6 of mtDNA (unpublished data). Sanchis *et al*. (2000) investigated phylogeny of Aphidiinae subfamily by using 18S rDNA region and reported that *L. fabarum* and *L. confusus* with 86% bootstrap support value are monophyletic. Similarity of COI mtDNA gene region and somehow ITS2 rDNA gene sequences between *L. fabarum* and *L. confusus* suggest that both may have recently diverged from a common ancestor.

In this research, nucleotide similarities found in *L. fabarum* and *L. confusus*, challenge the separation of these two species based on available morphological characters. Hence, in order to justify similarities or differences between *L. fabarum* and *L. confusus*, other gene regions for these species need to be sequenced and compared, and also more specimens from the same collecting site should be examined to find out more and reliable morphological characters.

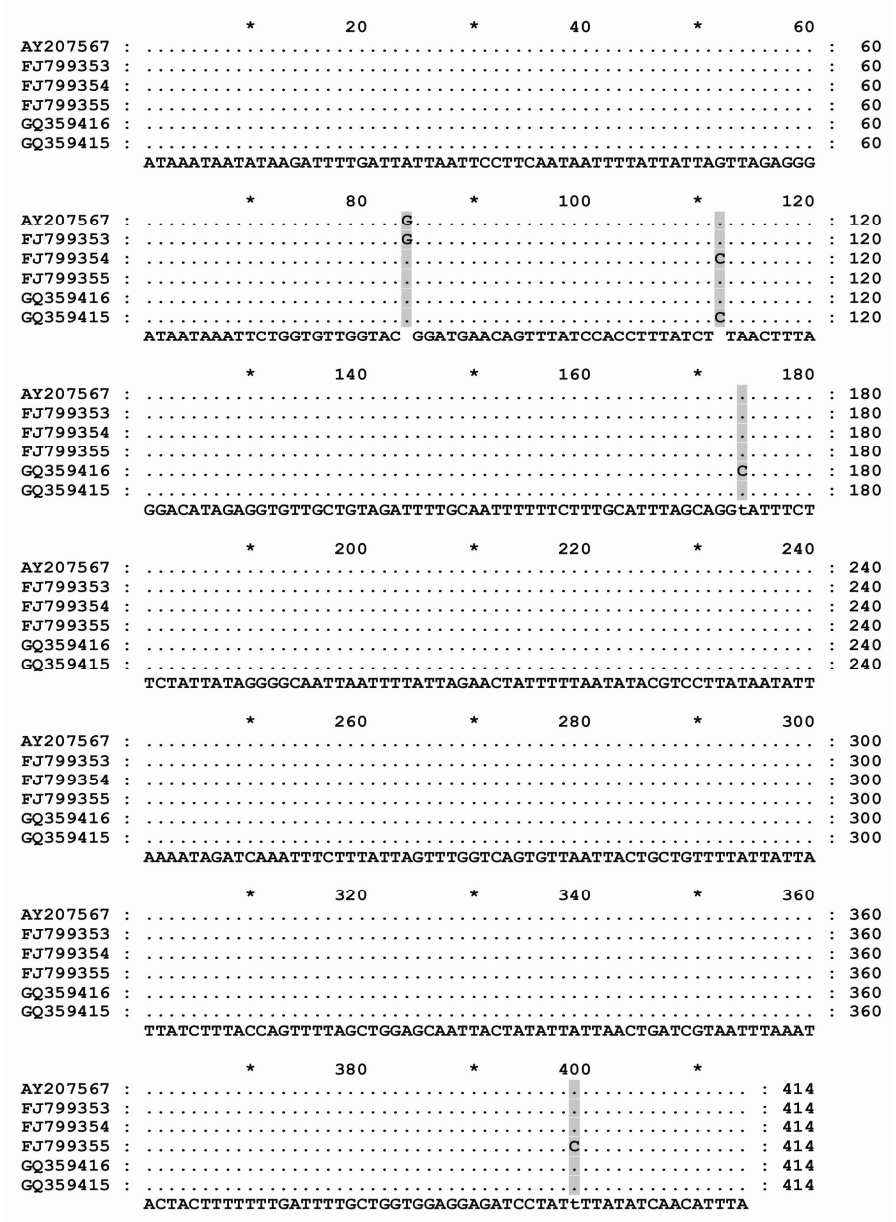

Figure 1. Multiple sequence alignment of mtDNA COI gene region for *L. fabarum* and *L. confusus* using ClustalW ver. 1.82. The same nucleotides are shown by dot (.). The highlighted positions indicate nucleotides change.

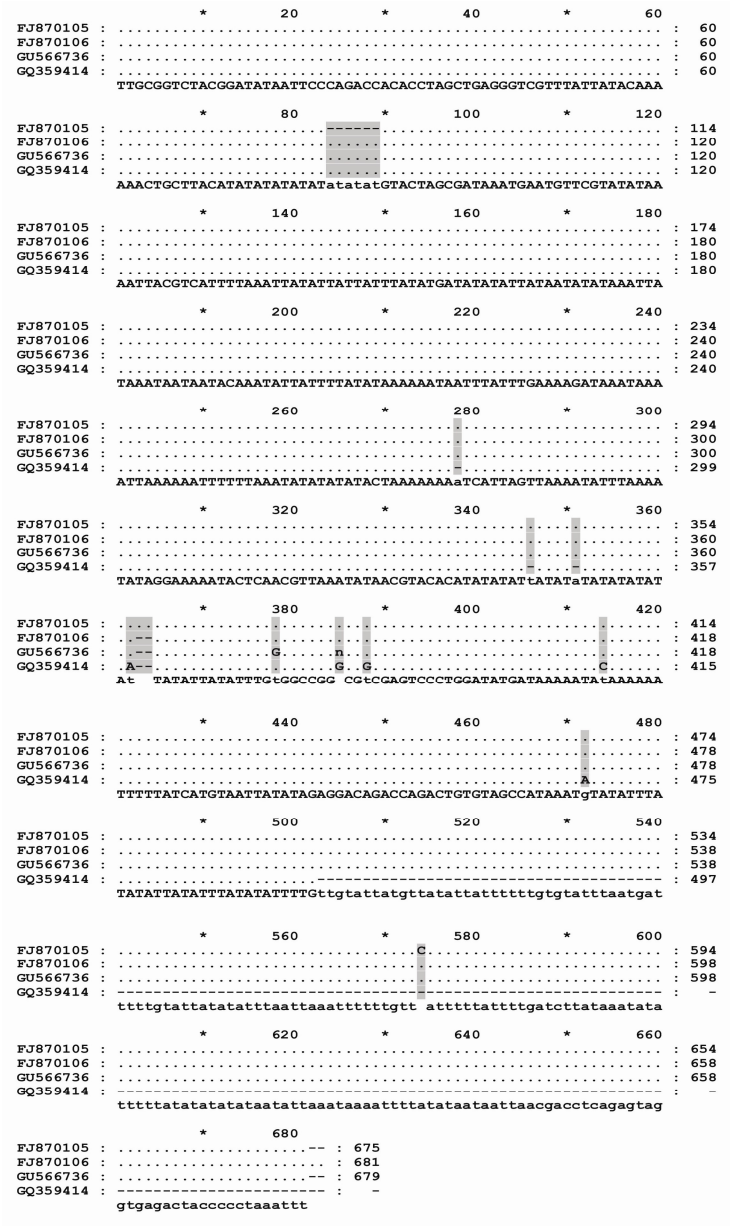

Figure 2. Multiple sequence alignment of ITS2 region for *L. fabarum* and *L. confusus* using ClustalW ver. 1.82. The same sequences are shown by dot (.) and gaps indicated with a (-). The highlighted positions indicate nucleotides change.

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