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MOLECULAR SIGNATURE OF *nrDNA-ITS* MARKER IN *ISODON RUGOSUS* (LAMIACEAE)

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Abstract

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Molecular features of *nrDNA-ITS* sequences in medicinal plant *Isodon rugosus* were assessed with universal *ITS-1* and *ITS-2* primers. *ITS* sequences of 46 *Isodon* species were defined for their respective *ITS-1*, *5.8S* and *ITS-2* regions through *in-silico* datamining and analysis. Only in 32 species, sequence of all the three defined regions was complete, while in the rest of 14 species was partial. *Isodon rugosus* revealed five genic regions, namely, *18S rRNA* gene (partial sequence, > 1…..38), *ITS-1* (complete sequence, > 39…..236), *5.8S rRNA* gene (complete sequence, > 237…..400), *ITS-2* (complete sequence, > 401…..607) and large subunit *26S rRNA* gene (partial sequence, > 608…..672). The sequence regions were recorded with variable sites (VS), singleton (ST) and species-specific parsimonious-informative (PI) sites. The information was also collected in a phylogenetic tree obtained using the maximum likelihood (ML) method.

Keywords: *Isodon rugosus*, Lamiaceae, *nrDNA-ITS* marker.

INTRODUCTION

The genus name *Isodon* (Schrad. ex Benth.) Spach comes from the Greek word '*isos*' means equal and '*odous*' (or *odontos*) means tooth (Bentham, 1832; Spach, 1840). It consists of more than 150 species (Liu et al., 2017), distributed chiefly in tropical to temperate East Asia and tropical Africa (Mabberley, 2008; Chen et al., 2017). In India, the genus is represented with nearly 16 species (including one variety), mainly from the Himalayas, Western Ghats, and Nilgiri regions (Sharma, 2018). *Isodon rugosus* (Wall. ex Benth.) Codd. is an essential indigenous member of Indian Lamiaceae. Species is usually considered quite variable floristically due to obscure molecular identity, taxonomic relationship at inter and intracontinental level, and thus, studied under different names: *Isodon plectranthoides*, *Lumnitzera densiflora*, *Ocimum densiflorum*, *Plectranthus rugosus* and *Rabdosia rugose*.

DNA based markers such as *nrDNA-ITS*, *rbcL*, *matK*, etc. are reliable, reproducible and unaffected by the physical form and environment. They provide a convenient tool for identifying medicinal plants, crude drugs and even commercial products (Srivastava & Saggoo, 2014; Zhou et al., 2014). Among the different DNA markers, *nrDNA-ITS* has been supported as a remarkable marker where plastid markers fail to discriminate the plants at the species level (Hollingsworth, 2011). Therefore, *nrDNA-ITS* was chosen for study in the plant *Isodon rugosus*. This research is also in addition to our previous work in the plant *I. rugosus*, where the molecular autograph of plastid marker/gene (*matK*) was studied (Srivastava et al., 2020). The present study was undertaken to provide alternative DNA marker information in the taxon *I. rugosus* that could be employed along with other marker/s for its molecular identification and barcoding.

MATERIALS AND METHODS

Plant leaf material was used from the same specimen of *I. rugosus* (IRBSA-01), which has been studied previously by Srivastava et al. (2020) in the field population of Baru-Sahib valley of Himachal Pradesh (India). Except for primer pairs *18S* (forward) *ITS-U1*: 5'-GGAAGKARAAGTCGTAACAAGG-3' and *26S* (reverse) *ITS-U4*: 5'-RGTTTCTTTTCCTCCGCTTA-3' (Cheng et al., 2015), genomic DNA isolation (Chase & Hills, 1991), software applications DNAMAN version 7.0 (Lynnon Biosoft Corporation, USA), MEGA version 7.0 (Kumar et al., 2016) and *ITS* sequence analysis methodology were similar to that followed in previous of *mat-k* sequence study (Srivastava et al., 2020). Phylogeny with maximum parsimony (MP) criterion was avoided due to inconsistency with low scores. Sequence boundaries of different regions (i.e. *ITS-1*, *5.8S* and *ITS-2*) were determined by comparing the aligned sequences with the sequences of other related species of *Isodon* registered in NCBI GenBank (Table 1). The complete sequence of the *ITS* region was deposited at NCBI GenBank (accession No. MH931390).

RESULTS AND DISCUSSION

The sequence amplified by the primers *18S* (forward) *ITS-U1* and *26S* (reverse) *ITS-U4* showed the overall length of amplified *nrDNA-ITS* regions with 569 nucleotides (excluding partial sequences of *18S* and *26S rRNA* gene). The *ITS* sequences of 46 different *Isodon* spp. from the BLASTn result were retrieved from the NCBI GenBank (Table 1) and analysed with a sequence of *I. rugosus* (MH931390).

In-silico datamining showed only 32 *Isodon* species with complete *nrDNA-ITS* sequences, out of which nine species (accessions No. KF032259.1,

Table 1. *Isodon* species from the BLASTn result and their respective GenBank accession numbers

No.	Species (abbreviations)	Gene Bank No.	No.	Species (abbreviations)	Gene Bank No.
1.	*Isodon rugosus* (I–1)*	MH931390	25.	*I. loxothyrsus* (I–25)	FJ593381.1
2.	*I. adenanthus* (I–2)	FJ593355.1	26.	*I. lungshengensis* (I–26)	FJ593382.1
3.	*I. adenolomus* (I–3)	FJ593356.1	27.	*I. megathyrsus* (I–27)#	MH117581.1
4.	*I. amethystoides* (I–4)#	KF032259.1	28.	*I. melissoides* (I–28)	FJ593385.1
5.	*I. anisochilus* (I–5)	KF855420.1	29.	*I. nervosus* (I–29)#	KF032257.1
6.	*I. brachythyrsus* (I–6)	KF855427.1	30.	*I. oresbius* (I–30)	KF855434.1
7.	*I. bulleyanus* (I–7)	FJ593358.1	31.	*I. parvifolius* (I–31)	KF855425.1
8.	*I. coetsa* (I–8)#	KM877351.1	32.	*I. pharicus* (I–32)	KF855430.1
9.	*I. dawoensis* (I–9)	KF855429.1	33.	*I. phyllostachys* (I–33)	FJ593388.1
10.	*I. effuses* (I–10)	KF855444.1	34.	*I. pleiophyllus* (I–34)	FJ593389.1
11.	*I. enanderianus* (I–11)#	JQ389514.1	35.	*I. rosthornii* (I–35)	FJ593390.1
12.	*I. eriocalyx* (I–12)	FJ593364.1	36.	*I. rubescens* (I–36)#	KF032249.1
13.	*I. flabelliformis* (I–13)	FJ593365.1	37.	*I. rugosiformis* (I–37)	FJ593391.1
14.	*I. flavidus* (I–14)	FJ593366.1	38.	*I. scoparius* (I–38)	FJ593392.1
15.	*I. flexicaulis* (I–15)	FJ593367.1	39.	*I. sculponeatus* (I–39)	FJ593393.1
16.	*I. forrestii* (I–16)	FJ593368.1	40.	*I. serra* (I–40)#	KF032262.1
17.	*I. gesneroides* (I–17)	FJ593369.1	41.	*I. setschwanensis* (I–41)	KF855412.1
18.	*I. glutinosus* (I–18)	FJ593370.1	42.	*I. shikokianus* (I–42)	KF855449.1
19.	*I. grandifolius var. atuntzeensis* (I–19)	FJ593371.1	43.	*I. shikokianus var. occidentalis* (I–43)	KF855450.1
20.	*I. inflexus* (I–20)#	KF032266.1	44.	*I. taliensis*(I–44)	FJ593394.1
21.	*I. irroratus* (I–21)	KF855439.1	45.	*I. wikstroemioides* (I–45)	FJ593396.1
22.	*I. japonicas* (I–22)#	KF032254.1	46.	*I. xerophilus* (I–46)	FJ593397.1
23.	*I. leucophyllus* (I–23)	KF855421.1	47.	*Rabdosia setschwanen-sis var. yungshengensis* (I–47)**	KF855436.1
24.	*I. longitubus* (I–24)	KF855445.1	48.	*Mentha suaveolens****; *M. canadensis****	AF369161; KC473228

*Query taxon, ***Rabdosia setschwanensis* var. *yungshengensis* – Syn. – *Isodon setschwanensis*, ***out group species, # sequences in nine taxa already defined for their respective regions in NCBI databases.

KM877351.1, JQ389514.1, KF032266.1, KF032254.1, MH117581.1, KF032257.1, KF032249.1 and KF032262.1) were observed with defined regions (e.g. *ITS-1*, *5.8S, ITS-2*), which were originally provided in the NCBI database (Table 1). Remaining all the species that were not defined by their respective regions hitherto is now defined and presented in Table 2. The information regarding accession numbers, length, sequence span and GC content [calculated as G + C% = {(G + C) / (A + T + G + C)} × 100] of the different *ITS* regions (i.e. *ITS-1*, *5.8S* and *ITS-2*) of various *Isodon* species are provided in Table 2.

Amplified sequence in *nrDNA-ITS* sequence in *I. rugosus* was reported with five regions: *18s* ribosomal RNA gene (partial sequence, > 1…..38), *ITS-1* (complete sequence, > 39…..236), *5.8S rRNA* gene (complete sequence, > 237…..400), *ITS-2* (complete sequence, > 401…..607) and large sub unit (*26S rRNA* gene partial sequence, > 608…..672).

ITS-1 region

The sequence of *ITS-1* in *I. rugosus* was observed with a length of 198 nt bases. Excluding the sites with missing (or ambiguous) data and gaps produced significant alignment (SA) in the *ITS-1* region at 198 nt sites (> 39…..236). From the aligned positions of the *ITS-1* region of 47 taxa, in *I. rugosus*, 49 sites were variable (VS), 30 sites were singleton (ST) and 19 sites of which were observed as parsimonious-informative (PI). Distribution of informative sites with their respective nucleotide bases (e.g. A, T, G or C) was observed to be positioned at 60 (C), 67 (A), 77 (C), 82 (T), 84 (C), 94 (A), 100 (C), 106 (C), 114 (G), 116 (G), 147 (A), 166 (A), 167 (T), 180 (C), 183 (C), 205 (G), 213 (C), 221 (G) and 229 (C) (Table 3). The GC content in the *ITS-1* region of *I. rugosus* was 66.16%. The spanning length of the *ITS-1* region varied from 194 nt to 198 nt, with a more frequent (in 44 species) occurrence of 196 nt (Table 2). Length variation was observed in three taxa, namely, *I. rugosus* (> 39.....236; 198 nt); *I. brachythyrsus* (> 01…..194; 194 nt) and *I. coesta* (> 33…..227; 195 nt).

5.8S region

The spanning length of the *5.8S* region of *nrDNA* in *I. rugosus* was 164 nt bases (> 237…..400). In *I. rugosus*, out of 164 aligned positions, four sites were variable, three sites were singleton, and one (1) site was parsimonious informative. The parsimonious informative site in the aligned sequences of the *5.8S* region was observed (Table 4) at position 237 (A). The GC content (G + C%) in the region was 54.88%. In 45 species, the length of the *5.8S* region was observed with a similar value of 164 nt bases, while GC content was identical in 40 species (Table 2). One species, i.e. *I. phyllostachys* (I–33; > 197…..359), was reported with single nucleotide deletion, making its spanning length of 163 nt bases (Table 2). The GC content differed in six species, namely, *I. coesta* (I–08, G + C%: 55.49), *I. inflexus* (I–20, G + C%: 54.27), *I. lungshengensis* (I–26, GC%: 55.49), *I. parvifolius* (I–31, G + C%: 54.27), *I. phyllostachys* (I–33, G + C%: 55.21) and *I. shikokianus var. occidentalis* (I–43, G + C%: 55.49).

ITS-2 region

Alignment in the *ITS-2* region of *I. rugosus* was significant at 207 nt positions (> 401…..607). There were 38 nt sites, which were variable, 26 sites were singleton, and 12 sites were parsimonious informative. Parsimonious informative sites (Table 4) were observed at positions 418 (A), 419 (C), 424 (G), 425 (C), 429 (C), 433 (G), 450 (T), 502 (C), 551 (C), 558 (T), 565(T) and 568 (C). The GC richness was reported as 68.12% (G + C%). Among the 47 *Isodon* spp. (including *I. rugosus*), the spanning length of *ITS-2* regions was partial in 15 species (193 nt in 3 sp., 194 nt in 7 sp., 195 nt in 1 sp., 197 nt in 2 sp., 199 nt in 1 sp., 205 nt in 1 sp.) and complete in 32, excluding *I. rugosus*, species (207 nt in 4 spp., 208 nt in 20 spp., 209 nt in 4 spp., 210 nt in 1 sp., 211 nt in 2 spp. and 212 nt in 1 sp.) (Table 2).

It was found that only nine species of *Isodon*: *I. amethystoides* (I-4, KF032259.1), *I. coetsa* (I–8, KM877351.1), *I. enanderianus* (I–11, JQ389514.1), *I. inflexus* (I-20, KF032266.1), *I. japonicas* (I–22, KF032254.1), *I. megathyrsus* (I–27, MH117581.1), *I. nervosus* (I–29, KF032257.1), *I. rubescens* (I–36, KF032249.1) and *I. serra* (I–40, KF032262.1) were defined previously as complete for all the three regions of *nrDNA-ITS* (i.e. *ITS-1* + *5.8S* + *ITS-2*) by their respective authors in NCBI database (Table 1). By taking reference to the similar definition or defined regions of these nine taxa, only 32 species (I–01, I–03, I–04, I–07, I–08, I–11, I–12, I–13,

Table 2. Defined length (L), spanning region (>) and nucleotide composition (G + C%) of the *ITS-1*, *5.8S*, *ITS-2* regions of *Isodon rugosus* and related species

Accession No.	*ITS-1*		*5.8S*		*ITS-2*	
	L > region	G + C%	L > region	G + C%	L > region	G + C%
MH931390 (I–1)	198 > 39…..236	66.16	164 > 237…..400	54.88	207 > 401…..607	68.12
FJ593355.1 (I–2)	196 > 01…..196	63.78	164 > 197…..360	54.88	205 > 361…..565	65.85
FJ593356.1 (I–3)	196 > 01…..196	65.82	164 > 197…..360	54.88	208 > 361…..568	67.79
KF032259.1 (I–4)	196 > 15…..210	65.82	164 > 211…..374	54.88	207* > 375…..567	67.15
KF855420.1 (I–5)	196 > 01…..196	64.80	164 > 197…..360	54.88	197 > 361…..557	69.04
KF855427.1 (I–6)	194 > 01…..194	65.46	164 > 195…..358	54.88	194*** > 359…..552	68.04
FJ593358.1 (I–7)	196 > 01…..196	64.29	164 > 197…..360	54.88	211 > 361…..571	68.72
KM877351.1 (I–8)	195 > 33…..227	64.62	164 > 228…..391	55.49	208 > 392…..599	66.35
KF855429.1 (I–9)	196 > 01…..196	65.31	164 > 197…..360	54.88	194 > 361…..554	68.56
KF855444.1 (I–10)	196* > 01…..196	64.79	164 > 197…..360	54.88	194* > 361…..554	67.53
JQ389514.1 (I–11)	196 > 06…..201	65.82	164 > 202…..365	54.88	208 > 366…..573	66.35
FJ593364.1 (I–12)	196** > 01…..196	64.29	164 > 197…..360	54.88	208* > 361…..568	67.31
FJ593365.1 (I–13)	196 > 01…..196	64.80	164 > 197…..360	54.88	212 > 361…..572	66.03
FJ593366.1 (I–14)	196 > 01…..196	65.82	164 > 197…..360	54.88	208 > 361…..568	66.83
FJ593367.1 (I–15)	196 > 01…..196	65.82	164 > 197…..360	54.88	208 > 361…..568	67.79
FJ593368.1 (I–16)	196* > 01…..196	65.82	164 > 197…..360	54.88	208 > 361…..568	68.27
FJ593369.1 (I–17)	196 > 01…..196	65.31	164 > 197…..360	54.88	209 > 361…..569	67.94
FJ593370.1 (I–18)	196* > 01…..196	65.31	164 > 197…..360	54.88	209 > 361…..569	67.94
FJ593371.1 (I–19)	196 > 01…..196	64.80	164 > 197…..360	54.88	208 > 361…..568	67.79
KF032266.1 (I–20)	196 > 15…..210	64.80	164 > 211…..374	54.27	211 > 375…..585	67.77
KF855439.1 (I–21)	196*** > 01…..196	63.78	164 > 197…..360	54.88	194* > 361…..554	68.56
KF032254.1 (I–22)	196 > 15…..210	65.31	164 > 211…..374	54.88	208 > 375…..582	67.31
KF855421.1 (I–23)	196 > 01…..196	66.33	164 > 197…..360	54.88	195 > 361…..555	69.74
KF855445.1 (I–24)	196 > 01…..196	65.31	164 > 197…..360	54.88	193 > 361…..553	67.36
FJ593381.1 (I–25)	196* > 01…..196	65.82	164 > 197…..360	54.88	208 > 361…..568	67.79
FJ593382.1 (I–26)	196 > 01…..196	65.82	164 > 197…..360	55.49	208 > 361…..568	67.79
MH117581.1 (I–27)	196 > 33…..228	64.80	164 > 229…..392	54.88	208 > 393…..600	67.79
FJ593385.1 (I–28)	196* > 01…..196	65.31	164 > 197…..360	54.88	208 > 361…..568	67.79
KF032257.1 (I–29)	196 > 17…..212	66.33	164 > 213…..376	54.88	209 > 377…..585	67.94
KF855434.1 (I–30)	196 > 01…..196	65.82	164 > 197…..360	54.88	199**** > 361…..559	67.34
KF855425.1 (I–31)	196 > 01…..196	66.33	164 > 197…..360	54.27	197* > 361…..557	69.04
KF855430.1 (I–32)	196*** > 01…..196	63.78	164 > 197…..360	54.88	194* > 361…..554	68.56
FJ593388.1 (I–33)	196 > 01…..196	64.29	163^{d} > 197…..359	55.21	208 > 360…..567	67.79
FJ593389.1 (I–34)	196*** > 01…..196	62.76	164 > 197…..360	54.88	208*** > 361…..568	66.35
FJ593390.1 (I–35)	196 > 01…..196	65.31	164 > 197…..360	54.88	208* > 361…..568	67.31
KF032249.1 (I–36)	196 > 17…..212	64.80	164 > 213…..376	54.88	208 > 377…..584	67.79
FJ593391.1 (I–37)	196 > 01…..196	65.82	164 > 197…..360	54.88	208 > 361…..568	66.83
FJ593392.1 (I–38)	196 > 01…..196	65.31	164 > 197…..360	54.88	208 > 361…..568	67.79
FJ593393.1 (I–39)	196 > 01…..196	64.80	164 > 197…….360	54.88	207 > 361…..567	66.67
KF032262.1 (I–40)	196 > 17…..212	65.81	164 > 213…..376	54.88	210 > 377…..586	68.10
KF855412.1 (I–41)	196* > 01…..196	65.31	164 > 197…..360	54.88	194 > 361…..554	69.07
KF855449.1 (I–42)	196 > 01…..196	64.80	164 > 197…..360	54.88	193 > 361…..553	68.39
KF855450.1 (I–43)	196 > 01…..196	64.80	164 > 197…..360	55.49	193* > 361…..553	66.84
FJ593394.1 (I–44)	196 > 01…..196	65.31	164 > 197…..360	54.88	209* > 361…..569	66.99
FJ593396.1 (I–45)	196 > 01…..196	64.80	164 > 197…..360	54.88	208 > 361…..568	67.79
FJ593397.1 (I–46)	196 > 01…..196	66.33	164 > 197…..360	54.88	207 > 361…..567	67.15
KF855436.1 (I–47)	196 > 01…..196	65.82	164 > 197…..360	54.88	194** > 361…..554	68.56

L – length in nucleotide bases, N –any nucleotide base, * – N (1), ** – N (2); *** – N (3), ; ****N (4), d – deletion 1.

Table 3. Distribution of informative (PI) sites and nucleotide base positions in the aligned *ITS-1* region of *Isodon rugosus*

Species*	*ITS-1* region (> 39…..236)																		
	60	67	77	82	84	94	100	106	114	116	147	166	167	180	183	205	213	221	229
I–01	C	A	C	T	C	A	C	C	G	G	A	A	T	C	C	G	C	G	C
I–02	–	–	–	–	–	–	–	–	A	T	C	–	–	–	–	–	–	A	–
I–03	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–
I–04	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	A
I–05	–	–	–	–	–	–	T	–	–	–	C	–	–	–	–	T	–	–	–
I–06	–	–	–	–	–	–	**	–	–	T	C	–	–	–	–	–	–	–	–
I–07	T	–	–	–	–	–	–	–	–	–	C	–	–	–	–	T	–	–	T
I–08	T	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–09	–	–	–	–	–	–	–	–	–	T	T	–	–	–	–	–	–	–	–
I–10	–	–	–	A	–	–	–	–	–	–	C	–	–	–	A	–	–	–	A
I–11	–	–	–	–	–	G	–	–	–	A	C	–	–	–	–	–	–	A	–
I–12	–	–	–	–	–	G	–	–	–	T	–	–	–	**	–	–	–	–	–
I–13	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–
I–14	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–
I–15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
I–16	–	–	–	–	–	–	–	–	–	**	C	–	–	–	–	–	–	–	–
I–17	–	–	–	–	–	–	–	–	–	–	C	–	–	T	–	–	–	–	A
I–18	–	–	–	–	–	–	–	–	–	**	C	–	–	–	–	–	–	–	–
I–19	–	–	–	–	–	–	–	–	T	T	–	–	–	–	–	–	–	–	–
I–20	–	–	–	–	T	–	–	–	–	–	C	T	–	–	A	–	–	–	A
I–21	–	–	A	–	–	–	–	–	–	T	C	–	–	–	–	-	-	-	**
I–22	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	A
I–23	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	-
I–24	–	–	–	–	T	–	–	–	–	–	C	T	–	–	–	A	–	–	–
I–25	–	T	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	**	-
I–26	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–
I–27	–	–	–	–	–	–	–	A	–	–	C	–	–	–	–	–	–	–	–
I–28	–	T	–	A	–	–	–	–	–	**	C	–	–	–	–	–	–	–	–
I–29	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–30	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–
I–31	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–32	–	–	T	–	–	–	–	–	T	T	**	–	–	–	–	-	-	–	–
I–33	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–
I–34	–	–	–	–	–	–	Y	–	–	–	C	–	–	–	–	–	T	–	–
I–35	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	T	–	A
I–36	–	–	–	–	–	–	–	–	–	A	C	–	–	–	–	–	T	–	A
I–37	–	T	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–38	–	–	–	–	–	–	–	A	–	–	C	–	–	T	–	–	–	–	–
I–39	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–
I–40	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–41	–	–	A	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–42	–	–	–	–	–	–	–	–	–	–	–	T	A	T	–	–	–	–	–
I–43	–	–	–	–	T	–	T	–	–	–	C	T	A	–	–	A	–	–	–
I–44	–	–	–	–	–	–	T	–	–	–	C	–	–	–	–	–	–	A	–
I–45	–	–	–	–	–	–	–	–	T	T	C	–	–	–	–	–	–	–	–
I–46	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–
I–47	–	–	–	–	–	–	–	–	–	T	C	–	–	–	–	–	–	–	–

*Abbreviation of species see in Table 1, '–' – bars represents the identical nucleotides as that of *I. rugosus* in their respectively aligned sequence sites, **N – any nucleotide base.

Table 4. Distribution of informative (PI) sites and nucleotide base positions in the aligned *5.8S nrDNA* and *ITS-2* region of *Isodon rugosus*

Species*	*5.8S* region (> 237…..400)	*ITS-2* region (> 401…..607)										
	237	418	419	424	425	429	433	450	502	551	558	565	568
I–01	A	A	C	G	C	C	G	T	C	C	T	T	C
I–02	–	–	–	–	T	–	–	–	–	gp	C	–	T
I–03	–	C	A	T	–	–	–	–	–	–	C	–	T
I–04	–	C	A	T	–	–	–	–	–	–	C	–	T
I–05	–	C	A	C	–	–	–	–	–	–	C	–	–
I–06	–	C	A	**	–	–	–	–	–	–	C	–	–
I–07	–	C	A	C	–	–	–	–	–	–	C	–	–
I–08	G	–	–	–	–	–	–	–	–	–	–	–	T
I–09	–	C	A	T	–	–	–	–	–	–	C	–	–
I–10	–	C	A	T	–	**	–	–	–	–	C	–	T
I–11	–	C	A	T	–	–	–	–	T	T	C	–	T
I–12	–	C	A	T	–	–	–	–	–	–	C	–	T
I–13	–	C	–	C	–	–	–	–	–	T	C	–	T
I–14	–	C	A	T	T	–	–	–	–	–	C	C	T
I–15	–	C	A	T	–	–	–	–	–	–	C	–	T
I–16	–	C	A	T	–	–	–	–	–	–	C	C	T
I–17	–	C	–	T	–	–	–	–	–	–	C	–	T
I–18	–	C	A	T	–	–	–	C	–	–	C	–	T
I–19	–	C	A	T	–	–	–	–	–	–	C	–	T
I–20	–	–	–	T	–	A	–	–	–	–	C	–	T
I–21	–	C	A	T	–	–	–	–	–	–	C	–	T
I–22	–	C	A	T	–	–	–	–	–	–	C	–	T
I–23	–	C	–	T	–	–	–	–	–	–	C	C	–
I–24	–	–	A	–	T	–	–	–	T	–	C	–	T
I–25	–	C	A	T	–	–	–	–	–	–	C	–	T
I–26	G	C	A	T	–	–	–	–	–	–	C	C	T
I–27	–	C	A	T	–	–	A	–	–	–	C	C	T
I–28	–	C	A	T	–	–	–	–	–	–	C	–	T
I–29	–	C	A	T	–	–	–	–	–	–	C	–	T
I–30	–	C	A	C	–	–	–	–	–	–	C	–	T
I–31	–	C	A	C	**	–	–	–	–	–	C	C	T
I–32	–	C	A	T	–	–	–	–	–	–	C	**	T
I–33	–	C	A	T	–	–	–	–	–	–	C	C	T
I–34	–	C	A	–	–	–	–	–	–	**	C	–	T
I–35	–	C	A	T	–	–	–	C	–	T	C	–	T
I–36	–	C	A	T	–	–	–	–	–	–	C	–	T
I–37	–	C	A	T	–	A	–	–	–	–	C	–	T
I–38	–	C	A	T	–	–	A	–	–	–	C	C	T
I–39	–	C	A	A	–	–	–	–	–	T	C	–	T
I–40	–	–	A	T	–	–	–	–	–	–	C	–	T
I–41	–	C	A	C	–	–	–	–	–	T	C	–	–
I–42	–	–	–	–	T	–	–	–	T	–	C	–	T
I–43	–	–	–	–	T	–	–	–	T	–	C	–	T
I–44	–	–	gp	T	–	–	–	–	–	**	C	–	T
I–45	–	C	A	T	–	–	–	–	–	–	–	C	T
I–46	–	–	–	–	T	–	–	–	T	–	C	–	T
I–47	–	C	A	T	–	–	–	–	–	–	C	**	**

*Abbreviation of species see in Table 1, '–' – bars represent the identical nucleotides as that of *Isodon rugosus* in their respectively aligned sequence sites, ** N – any nucleotide base, gp – gap.

I–14, I–15, I–16, I–17, I–18, I–19, I–20, I–22, I–25, I–26, I–27, I–28, I–29, I–33, I–34, I–35, I–36, I–37, I–38, I–39, I–40, I–44, I–45 and I–46) were found complete in all the three regions of *nrDNA-ITS* (*ITS-1*, *5.8S* and *ITS-2*). From the above 32 species, for *ITS-2* region, 19 species: *I. adenolomus* (I–3), *I. coetsa* (I-8), *I. enanderianus* (I–11), *I. eriocalyx* (I–12), *I. flavidus* (I–14), *I. flexicaulis* (I–15), *I. forrestii* (I–16), *I. grandifolius* var. *atuntzeensis* (I–19), *I. japonicas* (I-22), *I. loxothyrsus* (I-25), *I. lungshengensis* (I–26), *I. megathyrsus* (I–27), *I. melissoides* (I–28), *I. phyllostachys* (I–33), *I. pleiophyllus* (I–34), *I. rosthornii* (I–35), *I. rubescens* (I–36), *I. rugosiformis* (I–37), *I. taliensis* (I–44) possessed similar spanning length of 208 nt (Table 2). Alignment in these taxa reflected a maximum length span of 212 nt

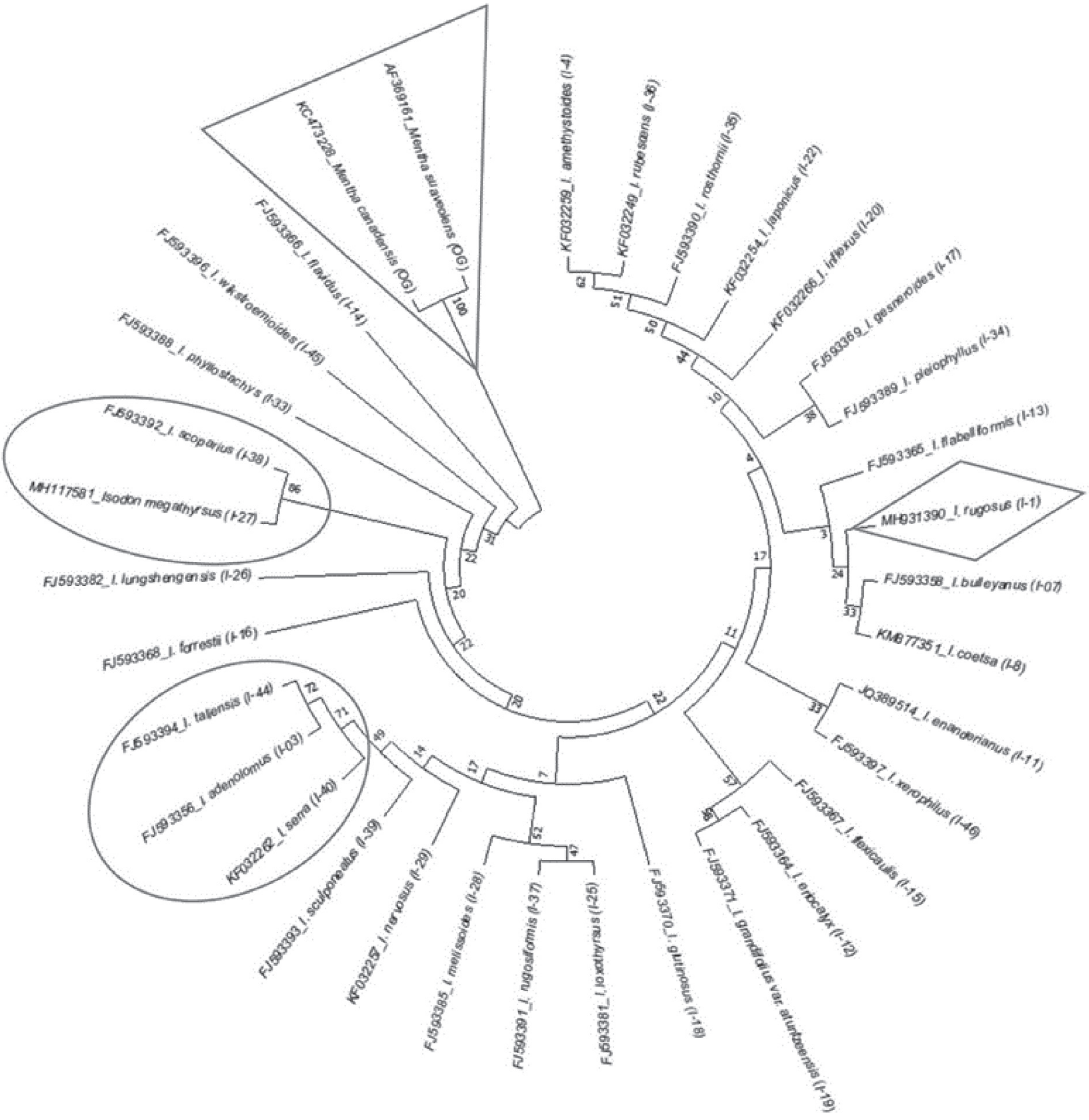

Fig. 1. Phylogenetic tree of *Isodon* species with two different out-group members (within a triangle), *Mentha suaveolens*-AF369161 and *M. canadensis*-KC473228, generated using *ITS* sequence according to the Maximum likelihood (ML) method. Numbers above lines are the bootstrap values in 1000 replicates, species within circles have bootstrap closeness above 70, query taxon *Isodon rugosus* within rhombus

in *I. flabelliformis* (I–13) and a minimum of 207 nt in four species, namely *I. rugosus* (I–01), *I. amethystoides* (I–04), *I. sculponeatus* (I–39) and *I. xerophilus* (I–46). Species *I. flabelliformis* (I–13) was reported with the lowest (66.03%) GC content. It was highest (68.72%) in *I. bulleyanus* (I–07), wherein 11 species, e.g. *I. adenolomus* (I–03), *I. flexicaulis* (I–15), *I. grandifolius var. atuntzeensis* (I–19), *I. loxothyrsus* (I–25), *I. lungshengensis* (I–26), *I. megathyrsus* (I–27), *I. melissoides* (I–28), *I. phyllostachys* (I–33), *I. rubescens* (I–36), *I. scoparius* (I–38) and *I. wikstroemioides* (I–45), were reported with same (67.79%) GC content.

The 32 species referred above with complete *nrDNA-ITS* regions (*ITS-1* + *5.8S* + *ITS-2*) were used to check the phylogenetic relationship of *I. rugosus*. Two different species of genus *Mentha* were used to confirm the out grouping, namely *M. suaveolens* (AF369161) and *M. canadensis* (KC473228). Nucleotide bases counts of *nrDNA-ITS* in *Isodon* species was observed with similar trends in their respective regions as C > G > A > T in *ITS-1*, G > C > A > T in *5.8S* and C > G > T > A in *ITS-2*. The estimation of average evolutionary divergence in overall sequence pairs (i.e. overall mean distance in 34 sequences) was d ± S.E. = 0.023 ± 0.002 by the p-distance method and d ± S.E. = 0.024 ± 0.002 by both Jukes-Cantor's (Jukes & Cantor, 1969) and Kimura 2 parameters (Kimura, 1980).

Phylogenetic tree generated by the maximum likelihood (ML) method (Fig. 1) placed the *I. rugosus* with a low bootstrap value near to the clade having species *I. coesta* (I–08) and *I. bulleyanus* (I–7). It indicated its divergence from the probable most recent common ancestor (*MRCA*) with missing linked members. The clades obtained above the bootstrap value 50 were informative. Two different clades (a clade having *I. scoparius* – *I. megathyrsus* and another having *I. adenolomus* – *I. taliensis*) show relatedness reasonably up to a good extend with their respective *MRCA* as in both clades bootstrap value was above 73.

The molecular signature of *ITS* marker in different medicinal plants has been studied by various researchers (Alice & Campbell, 1999; Doh et al., 2016; Liu et al., 2019; Srivastava & Saggoo, 2014). In genus *Isodon*, 46 species were studied with *ITS* marker, and they are included in the present study (Table 1). Similar to the above, species *I. rugosus* has been studied previously for *matK* marker by Shinwari et al. (2018) and Srivastava et al. (2020) and *psbA-trnH* intergenic spacers by Pekhna (2019).

Presently, taxon *I. rugosus* studied for its molecular (*nrDNA-ITS*) signature showed the presence of 32 informative (PI) sites together in the *nrDNA-ITS* regions (i.e. *ITS-1* + *5.8S* + *ITS-2* =19 + 1 + 12). This information could be used as additional taxonomic knowledge for the species to confirm its presence in the local flora.

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REFERENCES

Alice L.A., Campbell C.S., 1999: Phylogeny of *Rubus* (rosaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. – American Journal of Botany, 86(1): 81–97.

Bentham G., 1832: Labiatarum: genera et species. James Ridgway and Sons. – London.

Chase M.W., Hills H.H., 1991: Silica Gel: an ideal material for field preservation of leaf samples for DNA studies. – Taxon, 40(2): 215–220.

Chen Y.P., Xiang C.L., Hu G.X., Peng H., 2017: Taxonomic notes on *Isodon* (Nepetoideae, Lamiaceae) in China I: Revision of four species. – Nordic Journal of Botany, 35(2): 162–175.

Cheng T., Xu C., Li L., LI C., Zhang Y., Zhou S., 2015: Barcoding the kingdom plantae: new PCR primers for *ITS* regions of plants with improved universality and specificity. – Molecular Ecology Resources, 16(1): 138–149.

Doh E.J., Paek S.H., Lee G., Lee M.Y., Oh S.E., 2016: Application of partial internal transcribed spacer sequences for the discrimination of *Artemisia capillaris* from other *Artemisia* species. – Evidence-Based Complementary and Alternative Medicine, 2016: 1–12. doi: 10.1155/2016/7043436.

Hollingsworth P.M., 2011: Refining the DNA barcode for land plants. – Proceedings of National Academy of Sciences, 108: 19451–19452.

Jukes T.H., Cantor C.R., 1969: Evolution of Protein

Molecules: 21–132. – New York.

Kimura M., 1980: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. – Journal of Molecular Evolution, 16(2): 111–120.

Kumar S., Stecher G., Tamura K., 2016: MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. – Molecular Biology and Evolution, 33(7): 1870–1874.

Liu M., Wang W.G., Sun H.D., Pu J.X., 2017: Diterpenoids from *Isodon* species: An update. – Natural Product Reports, 34(9): 1090–1140.

Liu Z., Gao Y., Zhou J., 2019: Molecular authentication of the medicinal species of *Ligusticum* (Ligustici Rhizoma et Radix, “Gao-ben”) by integrating non-coding internal transcribed spacer 2 (*ITS-2*) and its secondary structure. – Frontiers in Plant Science, 10(429): 1–8.

Mabberley D.J. (eds), 2008: Mabberley’s plantbook. A portable dictionary of the plants, their classification and uses. – Cambridge.

Pekhna B., 2019: In-silico datamining and study of molecular (DNA) barcode in medicinal taxon *Isodon rugosus* (Lamiaceae) from the flora of Baru Sahib, Himachal Pradesh (India). Master’s Thesis, Department of Botany, Eternal University. – India.

Sharma A., 2018: Cyto-morphology, anatomy, phytochemical screening and antimicrobial study in wild *Isodon rugosus* (syn. *Plectranthus rugosus*) from Baru-Sahib area of Himachal Pradesh. Master’s Thesis, Department of Botany, Eternal University. – India.

Shinwari Z.K., Jan S.A., Khalil A.T., Khan A., Ali M., Qaiser M., Zahra N.B., 2018: Identification and phylogenetic analysis of selected medicinal plant species from Pakistan: DNA barcoding approach. – Pakistan Journal of Botany, 50(2): 553–560.

Spach E., 1840: Histoire naturelle des végétaux IX. Librarie Encyclopédique de Roret. – Paris.

Srivastava D.K., Saggoo M.I.S., 2014: Molecular identification of *Verbascum thapsus* L. (Ban Tambaaku) and its *ITS* sequence comparison with other *Verbascum* L. species. –Medicinal Plants – International Journal of Phytomedicines and Related Industries, 6(1): 1–7.

Srivastava D.K., Bansal P., Singh P.K., Saggoo M.I.S., 2020: Molecular autograph of maturase-k gene in *Isodon rugosus* (Lamiaceae). – Botanica, 26(1): 95–100.

Zhou J., Wang W., Liu M., Liu Z., 2014: Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum* and its substitutes and adulterants by DNA barcoding technique. – Pharmacognosy Magazine, 10(40): 385–390.

MOLEKULINIS *ISODON RUGOSUS* (LAMIACEAE) *nrDNA-ITS* ŽYMENS PARAŠAS

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Santrauka

Vaistinio augalo *Isodon rugosus* molekulinės nrDNA-ITS sekos buvo įvertintos, naudojant universaliuosius ITS-1 ir ITS-2 pradmenis. Atlikus *in-silico* duomenų analizę buvo nustatyta, kad *Isodon* spp. 46 rūšių ITS sekos atitiko ITS-1, 5.8S ir ITS-2 regionus. Tačiau tik 32 rūšių sekos atitiko visus tris regionus, kitų rūšių sekos buvo dalinės. *Isodon rugosus* tyrimas atskleidė penkių genų regionus: 18S rRNR (dalinė seka, > 1… .38), ITS-1 (visa seka, > 39… .236), 5.8S rRNR (visa seka, > 237… ..400), ITS-2 (visa seka, > 401… ..607) ir subgeno 26S rRNR (dalinė seka, > 608… .. 672). Sekos regionai buvo nustatyti kintamose, singletoninėse ir rūšims būdingose informatyviose srityse. Informacija buvo pateikta filogenetiniame medyje, sudarytame naudojant maksimalios tikimybės metodą.