

Interspecific relationships of *Piper* species in Sri Lanka as revealed by DNA barcode ITS

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Abstract

The genus *Piper* which is the largest genus in the family Piperaceae is economically important as it comprises with valuable crop species as well as several wild species. Although ten species of genus *Piper* are recorded in Sri Lanka, their interspecific relationships were not well studied. Therefore, the aim of this study was to determine interspecific relationships of *Piper* species in Sri Lanka using internal transcribed spacers from nuclear ribosomal DNA (ITS).

Ten varieties of *Piper nigrum* L., two varieties of each *P. betle* L. and *P. longum* L., *P. chuvya* (Miq.) C. DC., *P. siriboa* L., *P. sylvestre* Lam., *P. walkeri* Miq. and *P. zeylanicum* Miq. were used for the study. The genomic DNA was extracted from tender leaf samples using CTAB method, PCR amplified using ITS primer pair and subjected to DNA sequencing. The DNA sequence alignment analysis was carried out and a phenogram was constructed using the multiple sequence alignment programme MUSCLE.

According to the phenogram clear formation of two major clusters can be seen and ten *P. nigrum* varieties were clustered together however, variety Kuching has shown a separation from the main cluster. *P. longum*, *P. walkeri*, *P. sylvestre*, *P. siriboa*, *P. betle*, *P. chuvya* and *P. zeylanicum* formed the other cluster in which *P. siriboa* has shared close similarities with *P. betle*, while *P. sylvestre* and *P. walkeri* displayed close relationships and clustered together with *P. longum* varieties. Interspecific relationships between wild species and cultivated species (*P. nigrum*, *P. betle* and *P. longum*) indicated the potential of using the

wild species in breeding programs for crop improvement. However, further studies to reveal genetic and phenetic relatedness of those species are needed to deepen the knowledge on interspecific relationships of *Piper* species.

Keywords: *Piper*, DNA barcode, ITS, interspecific relationships

Introduction

Family Piperaceae is mostly found in the wet regions of the tropics and centered in the submontane and montane zones. The genus *Piper* which is the largest genus in the family contains aromatic, softly woody or herbaceous plants: erect, creeping or climbing by adventitious roots. (Huber, 1987; Parthasarathy, 2006). People throughout the tropics use *Piper* for many purposes, such as ornamentals, spices, perfumes, oils, insecticides, and many medicinal preparations. Despite its commercial and medicinal importance,

Piper has received very limited scientific attention (Wadt *et al.*, 2004).

The genus *Piper* is represented by ten species in Sri Lanka. The main cultivated crops in the genus include black pepper (*P. nigrum* L.) and betel (*P. betle* L.). *P. zeylanicum* Miq., *P. trineuron* Miq. and *P. walkeri* Miq. are endemic to the country, whereas five species are considered as introduced. *P. walkeri* Miq. and *P. trineuron* Miq. are found to be recorded in few localities of the country and *P. hymenophyllum* Miq. is considered as an extremely rare species. *P. sylvestre* Lam. is the most widespread *Piper* species in Sri Lanka. *P. siriboa* L., *P. longum* L. and *P. chuyva* (Miq.) C. DC. are commonly used in traditional medicines (Huber, 1987; Edirisinghe, 2009; Senaratna, 2001; Liyanage & Senanayake, 2010).

Most of the crop plants are susceptible to various diseases which in turn causes economic losses to the country. Bacterial leaf blight is one of the major diseases which damages the

betel cultivation, caused by a bacteria called *Xanthomonas campestrisbetlicola*. No control measure has been identified other than destruction of seriously diseased plants. (Mahesha *et al.*, 2009). Pepper cultivations are also affected by serious diseases such as *Phytophthora* foot rot and virus diseases. (Ravindran *et al.*, 2000). The recommended control measures cannot be considered for prevention or eradication of the diseases of black pepper effectively (International Pepper Community, 2016).

Domesticated plants have been moved into and adapted to new environments and they have been reshaped according to human needs therefore they have been fundamentally altered from wild relatives. Modern crops are the result of thousand years of evolutionary process. Crop evolution includes two fundamental processes namely the creation of diversity and selection. For agriculture, the continuation of this evolutionary process is necessary. Therefore, an essential criterion of crop evolution is the availability of genetic diversity (Brush, 2000). Genetic diversity is usually considered as the amount of genetic variability among individuals of a variety, or population of a species. It results from the many genetic differences between individuals and it may be visible in different ways such as differences in biochemical characteristics (e.g. in protein structure or isoenzyme properties), in physiological properties (e.g. abiotic stress resistance or growth rate) or in morphological characters such as flower colour or plant form (Brown, 1983).

The information on genetic diversity and relationship within and among crop species is essential for the efficient utilization of plant genetic resources (Irwin *et al.*, 1998). Genetic resources assist plant breeders to create plants with novel gene combinations and select crop varieties more suited to the needs of diverse agricultural systems (Glaszmann *et al.*, 2010).

Researchers have found out that, though Crop Wild Relatives (CWR) are beneficial, the attention given to the utility and conservation of crop wild relatives is very limited. Plant breeders face many obstacles in using CWR effectively and there is insufficient recognition that CWR bring to farmers by facilitating the gene flow from wild populations to crop genetic diversity in cultivations. Because of negligence of conservation, many are being threatened and are at risk of disappearing (Jarvis *et al.*, 2008). CWR are often well adapted to marginal level environments and they can thrive well in biotic and abiotic stresses than modern crop varieties (Maxted *et al.*, 2012 a).

DNA barcoding provides an insight into species-level taxonomy in groups which has simple morphologies and those that have received inadequate taxonomic attention to characterize properly the diversity they contain (Hollingsworth *et al.*, 2011). It also assists in the process of identifying and authentication of unknown specimens. DNA barcode uses a standard short genomic region that is universally present in target lineages which has sufficient sequence variation to discriminating among species that uses for taxonomic identification (Kress *et al.*, 2005).

ITS is one of the most widely used DNA fragment as its high resolution of inter and intraspecific relationships. DNA barcoding in tropical plants may be challenging as compared to temperate plants and have limited attention yet. DNA extraction makes difficulties at some instances due to the presence of high level of secondary metabolites (Coley and Barone 1996). Tripathi *et al.* 2013 have tested different plant barcode loci, (*rbcL*, *matK*, ITS, *trnH-psbA*, and the recently proposed ITS2) for their efficacy using 300 accessions of tropical tree species. They have tested these loci for PCR, sequencing success and species discrimination. According to them *rbcL* was best as far as PCR and sequencing success were concerned, but it was not successful for the species discriminating ability of tropical species. According to them ITS and *trnH-psbA* were the second best loci in PCR and sequencing success, respectively. They have suggested that

both ITS and *trnH-psbA* would be helpful as barcode markers for tropical species than *matK* and *rbcL*.

The present study aims to determine interspecific relationships of *Piper* species in Sri Lanka using internal transcribed spacers from nuclear ribosomal DNA (ITS) with a view to provide molecular data or barcoding.

Methodology

Collection of plant material

The following plant species were selected for the analysis (Table 1). Samples were collected from Intercropping and betel research station-Narammala, natural habitats and from the growers. Varieties were used for the three cultivated species; *P. nigrum*, *P. betle* and *P. longum*.

Table1: List of collected plant species and varieties

Species	Variety
<i>P. nigrum</i>	MB 12, MW21, IW 05, MW 18, Panniyur, Kuching, KW 30, KW 31, KW 33, GK 49
<i>P. betle</i>	Ratadalu, Nagawalli
<i>P. longum</i>	Thippili, Gaja Thippili
<i>P. siriboa</i>	
<i>P. sylvestre</i>	
<i>P. chuyva</i>	
<i>P. walkeri</i>	
<i>P. zeylanicum</i>	

Extraction of DNA from the plant leaves

DNA of all the taxa were extracted using CTAB method. Tender leaves were taken as samples. Extraction buffer (500 µl) was added into a capped eppendorf tube and kept for about 10-15 minutes for pre warming. Tender leaves (0.1 g) were placed in the motor and finely ground. To the pre warmed extraction buffer 0.5 µl of β-mercaptoethanol was added. The tube was capped and mixed gently. It was incubated for 30 minutes at 60 °C in the water bath. The tube was mixed gently every 10 minutes by inverting the tube. One volume (500 µl) of chloroform-isoamyl alcohol (24:1) was added to the tube and mixed for 10 minutes. The tube was centrifuged for 10 minutes at 12000 rpm at room temperature. The upper aqueous phase was transferred into a tube and the volume was measured. Then 0.6 volume of ice- cold iso propanol was added to the tube. The tube was closed and mixed gently but thoroughly by inverting the tube several times. After that the tube was kept in a -20 °C freezer for about 20 minutes and centrifuged at 4 °C for 10 minutes at 12000 rpm. The supernatant was discarded and 750 µl of washing solution was added. The pellet was washed by gently suspending and then the tube was centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatant was removed and 750 µl of washing solution was added again. The pellet was agitated gently for few minutes and centrifuged at 12000 rpm for 10 minutes at 4 °C. Then the supernatant was carefully removed and the tube was kept inverted on tissue paper for about 1 hour to dry. After drying appropriate volume of TE buffer was added. The pellet was kept to dissolve at 4 °C without agitation. Extracted DNA from all the plant species were subjected to polymerase chain reaction (PCR) using universal primers for the internal transcribed spacer (ITS) region. The primer sequences were as follows:

ITS5af (5'- CCTTATCATTTAGAGGAAGGAG-3')

ITS4r (5'- TCCTCCGCTTATTGATATCG-3')

The 15 µL of PCR mixture was prepared using 7.5 µL of GoTaq green master mix, 5.5 µL of Nuclease free water, 0.5 µL of Primer (Forward), 0.5 µL of Primer (Reverse) and

1.0 μ L of DNA sample.

The PCR conditions for ITS regions were 3 minutes at 94 °C for initial denaturation, followed by 30 cycles of 30 seconds at 94 °C for denaturing, 30 seconds at 52 °C for annealing and 1 minute at 72 °C for extension. Amplified ITS regions were purified using a GeneClean kit and subjected to sequencing using an ABI 3500 genetic analyser (Applied Biosystems®).

Data analysis

The DNA sequence alignment analysis was carried out and a phenogram was constructed using the multiple sequence alignment programme MUSCLE (Multiple Sequence Comparison by Log-Expectation).

Results and Discussion

Amplified ITS regions were approximately 800 bp in size (Plate 1).

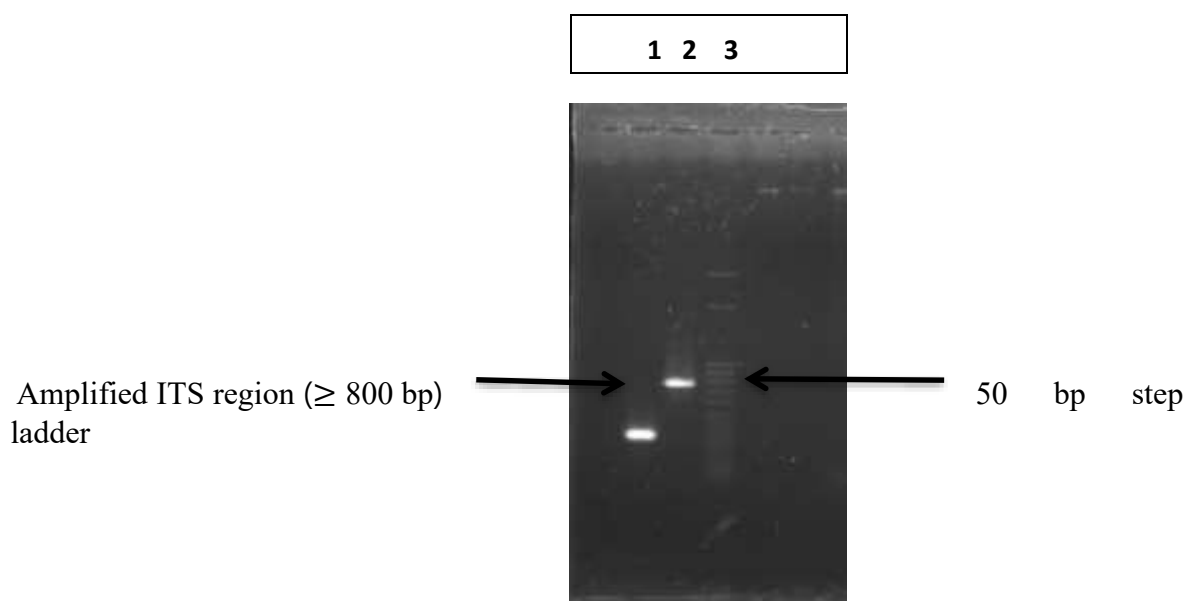


Plate 1: Gel electrophoresis image of PCR amplified products of *P. longum* (Lane 1: Amplified *trnH-psbA* regions, Lane 2: Amplified ITS regions, Lane 3: 50 bp step ladder)

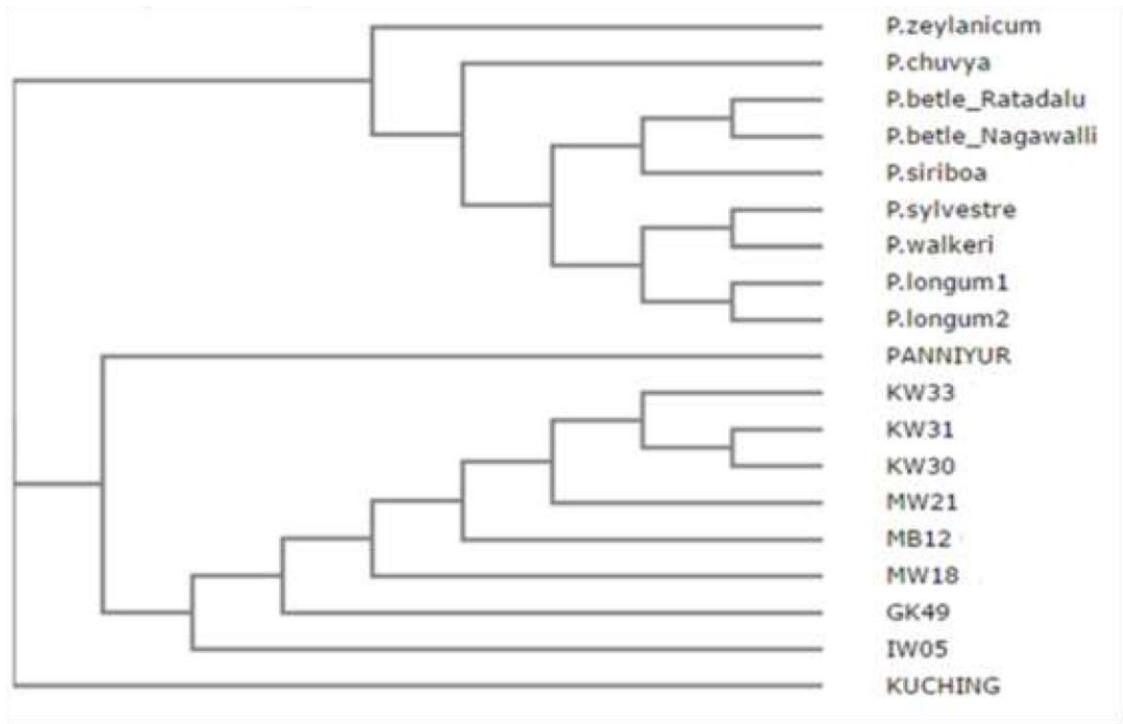


Figure 1: Phenogram constructed using the multiple sequence alignment programme MUSCLE

Table 2: List of species denoted in percent identity matrix

Number	Species name
1	<i>P. zeylanicum</i>
2	Panniyur
3	Kuching
4	<i>P. chuvya</i>
5	<i>P. betle</i> (Ratadalu)
6	<i>P. siriboa</i>
7	<i>P. betle</i> (Nagawalli)
8	<i>P. sylvestre</i>
9	<i>P. walker</i>
10	<i>P. longum</i> (Thippili)
11	<i>P. longum</i> (Gaja thippili)
12	KW33
13	IW05
14	KW31
15	KW30
16	MW21
17	MB 12
18	MW18

19 GK49

Table 3: Percent identity matrix

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	95.99																
3	95.06	96.57															
4	94.21	94.70	94.98														
5	92.83	93.26	92.84	93.05													
6	93.26	93.69	92.45	94.34	97.69												
7	93.36	93.54	93.24	94.06	98.63	98.23											
8	90.95	90.70	90.29	90.66	92.03	91.64	92.43										
9	90.48	89.98	89.10	89.20	91.36	91.55	92.06	94.76									
10	88.03	87.98	87.52	88.03	89.00	88.48	89.12	88.37	86.71								
11	89.82	88.29	88.37	89.27	89.53	89.29	89.66	89.88	88.95	90.28							
12	75.31	75.97	75.80	74.06	73.54	73.06	74.17	70.95	66.07	69.76	70.60						
13	82.69	83.64	83.10	81.25	79.94	80.00	80.53	77.56	73.84	77.08	72.22	84.82					
14	70.48	71.16	71.47	69.31	68.61	68.56	68.79	65.96	62.14	65.30	66.15	85.67	82.32				
15	73.77	74.78	74.47	72.74	71.91	71.43	71.95	69.39	65.65	68.03	68.60	86.70	83.45	91.47			
16	78.69	79.97	79.40	78.27	79.89	76.81	77.21	74.18	71.15	72.83	73.12	86.88	86.93	87.45	90.70		
17	83.06	84.53	84.05	82.39	81.17	80.92	81.22	78.36	73.84	77.08	77.75	87.74	89.72	84.70	86.00	89.33	

18 83.38 84.33 84.08 82.52 81.04 80.95 81.49 78.49 75.31 76.89 77.87 86.83 90.34 84.44 85.98 90.20 91.57

19 84.04 85.36 84.63 83.19 82.10 81.59 82.01 78.98 75.62 77.93 78.64 85.43 89.69 83.85 85.45 90.74 92.22 93.37

The phenogram (Figure 1) showed separation of two major clusters where ten *P. nigrum* varieties were clustered together. However, variety Kuching has shown a separation from the main cluster. *P. longum*, *P. walkeri*, *P. sylvestre*, *P. siriboa*, *P. betle*, *P. chuvya* and *P. zeylanicum* formed the other cluster in which *P. siriboa* has shared close similarities with *P. betle*, while *P. sylvestre* and *P. walkeri* displayed close relationships and clustered together with *P. longum* varieties.

Black pepper varieties Kuching and Panniyur shared more similarities with *P. zeylanicum* (95.06% and 95.99% respectively) which is an endemic species to Sri Lanka (Table 3). Out of the other black pepper varieties GK 49, MB 12 and MW 18 have showed high relationships with *P. Zeylanicum* (84.04%, 83.06%, and 83.38% respectively). The other endemic species (*P. walkeri*) has showed close similarities with *P. sylvestre* (94.76%) and *P. betle* varieties (Ratadalu 91.36%, Nagawalli 92.06%) while *P. chuvya* has shared more similarities with variety Panniyur (94.70%).

The present study revealed the interspecific relationships with crop species of *Piper* with the wild species of the genus. Being in the wild environment make the crop wild relatives as more potent candidates for the crop improvement programmes by means of a source of strong genetic material. Among the wild species *P. zeylanicum* has displayed more similarities with black pepper varieties which would suggest the possibility of using its beneficial traits for the crop improvement programmes. Further, *P. walkeri* and *P. sylvestre* will also be rich with promising characters, which have displayed more similarities with crop species.

Conclusions

According to the phenogram (Figure 1) and the percent identity matrix (Table 3) constructed using the multiple sequence alignment programme MUSCLE, interspecific relationships between wild species and three cultivated species (*P. nigrum*, *P. betle* and *P.*

longum) were clearly indicated. These information have provided a valuable indication of the potential of using the wild species such as *P. zeylanicum*, *P. walkeri* and *P. sylvestre* in breeding programmes for crop quality improvement. However, further studies are

needed to reveal genetic relatedness of those species to deepen the knowledge on interspecific relationships of wild and crop species of genus *Piper*.

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