MOLECULAR IDENTIFICATION OF *ACHILLEA MILLIFOLIUM* L. FROM ITS MARKETED ADULTERANTS IN LAHORE, PAKISTAN

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ABSTRACT

Achillea millefolium L is an important medicinal plant (member of family Asteraceae) sold in herbal markets with local names of Biranjasif or Gomadar. Adulteration in precious medicinal plants is alarming issue in the herbal markets of Lahore. It is quite difficult to identify dried samples of medicinal plants sold in the herbal markets on their morphological basis. This research was conducted on marketed samples of Biranjasif or Gomadar (*Achillea millefolium*) with context to molecular identification. For this purpose four different DNA barcode sequences (matK, nrITS, rbcL and TrnH-PsbA) were selected. In this current study 11 market samples along with fresh taxonomically identified sample as positive control were considered by using four molecular markers. The results indicated that fresh sample of *A. millefolium* was original that was confirmed on the basis of three different barcodes while all market dried samples did not show any relationship with *A. millefolium* with any of the barcode sequence. The current study provides the information of authentication and significance of molecular markers (DNA barcodes) for identification of medicinal plants.

Key words: Biranjasif, DNA barcode, matK, nrITS, rbcL, TrnH-PsbA

INRODUCTION

Achillea millefolium L. is an important medicinal plant belonging to the family Asteraceae. It is locally known as Baranjasaf, Kangijari or Kala Chahu. It is found around the world as in Afghanistan, China, India and North America. However, in Pakistan, it is distributed in Skardu, Swat, Chitral, Kashmir, Kaghan, Hazara, Peshawar, Rawalpindi, Quetta, D.I. Khan, Ziarat, Karachi and Lahore. It is one of the extensively used herbal medicine to cure multiple diseases like gastrointestinal ailment, laxative, burn wounds, genital herpes, high blood pressure, gynocological disorders and anti-inflamatory (Ali and Cibas, 2017). Its flower is known to be effective in treatment of allergy and eczema. Its oil is used to treat flu, cold and swollen joints (Bremness, 2002). Leaf decoction is very useful to treat kidney stone and kidney infection. During trading, *A. millifolium* is usually adulterated with the *Adhatodavasica* Nees. The term adulteration means mixing or replacement of original herbal drugs with its resembled, inferior plant which has different chemical or therapeutic properties (Srirama *et al*., 2010; Kumar et al., 2015). Both plants although not have any morphological resemblance but they are adulterated either due to the lack of elementary knowledge about the authentic plant or confusion in vernacular names. Sometime Herbalist intentionally produce high degree of adulteration which cannot be determined easily (Sunita, 1992, Uniyal and Joshi, 1993, Sarin, 1996). Adulteration in the case of *A. millifolium* cause harmful impact on health of human beings such as diarrhoea, vomiting, miscarriages and reduced blood sugar level in diabetic persons (Kumar *et al*., 2010). Since morphological structures of traded herbal plants are not intact in crude herbal drugs form. Hence it is needed to select sensitive techniques. i.e. DNA barcoding for authentication of traded raw herbal drug.

To determine the adulteration in traded medicinal plant species, DNA barcoding is an important molecular technique to determine the adulteration which is a burning issue and impose various health

hazards. DNA barcodes; short sequences of DNA, are becoming important protocol for the recognition and identification of plant species (Kress *et al*., 2005). It is based on the various conserved regions of divergent species to develop large scale reference genomic library and useful for genetic diversity, phylogenetic analysis and discrimination of different species (Wattoo *et al*., 2016). DNA barcoding technique are free from subjective errors does not required any expertise as required in taxonomic parameters (Hebert and Gregory, 2005). One Nuclear (nrITS regions) and four chloroplast primers (mat, rbcL, trnH, trnH-psbA) were selected for the following research. Among them trnH-psbA was used in combination of trnH while remaining three were used singly. (Nithaniyal *et al*., 2016) worked on 112 traded raw drugs that were suffering the adulteration issue and created reference DNA barcoding library. They employed DNA barcoding markers i.e. matK, rbcL, to reveled adulteration persisted in 20 % of raw herbal drugs. In India, similar work on DNA barcoding was done by (Kumar *et al*., 2018). They emphasized on 30 important medicinal plants collected from 34 localities of India and showed about 12% market samples were adulterated among them. In current study we have determined the adulteration in the herbal drug i.e.*A. millifolium* L. by DNA barcoding techniques.

MATERIALS AND METHODS

Samples Collection

Raw herbal sample of *A. millefolium* L were purchased from different herbal shops located in herbal market (Akbari mandi) of Lahore. For making DNA comparison with genuine plant source, fresh sample of *A.millefolium* was collected from Jinnah garden Lahore. Identification of field sample was done by comparing its morphological characters with already available preserved samples in herbariums of Government College University Lahore (GCU) and Lahore College for Women University Lahore (LCWU). For DNA isolation leaves of fresh sample were washed, shade dried and frozen in plastic zipper bag.

Extraction of plant DNA

Fresh sample and marketed samples were grinded in fine powder form to make the extraction easy. DNA was extracted from samples by following the method of Doyle and Doyle, 1990. Quality of extracted DNA was tested by running on 1% agarose gel and quantified by using nanodrop spectrophotometer.

PCR amplification

Four barcoding markers matK, rbcL, nrITS and trnH-psbA were selected for the amplification of plant DNA. List of primer sequence used for DNA amplification given in the Table 1.

Table 1. List of primers used to amplify the chloroplast and nuclear DNA barcodes.

Barcode name	Primer Name	Primer sequence	Reference
matK	matK F	5′-TAATTTACGATCAATTCATTC-3′	(Ford et al., 2009)
	matK R	5′-CTTCCTCTGTAAAGAATTC-3′	
rbcL	rbcL F	5′-ATGTCACCACAAACAGAAAC-3′	(Asmussen and Chase, 2001)
	rbcL R	5′-TCG CAT GTA CCY GCA GTT GC-3′	
nrITS	nrITS F	5′-CCTTATCATTTAGAGGAAGGAG-3′	(Stanford et al., 2000)
	nrITS R	5′-GGAAGTAAAAGTCGTAACAAG-3′	
trnH-psbA	trnH-psbA F	5′-GTTATGCATGAACGTAATGCTC-3′	(Sang et al., 1997)
	trnH-psbA R	5′-CGCGCATGGTGGATTCACAAATC-3′	

Chloroplast and nuclear DNA barcodes and their primer names and sequences used in this study PCR was conceded with reaction volume of 25µL, in which template DNA was 4 µL (10ng/µL), each of forward and reverse primer were 2.5 µLand 12.5µL of blue Master mix wizpure (Wizbiosolutions) with concentration of 200 µm and sterile distilled water was 3.5 µL. PCR amplification was done In thermo cycler (Qiagen, Netherland) with thermal profile: 94°C for 5min followed by the 40 cycles of 94°C for 1min annealing at 52°C for 1min and 72°C also for 1 min and final extension at 72°C for 10 min and hold it at 4°C. Annealing temperature was variable for all the four selected primers. i.enrITS primer (52°C), matK (50°C), rbcL (54°C) and trnH-psbA (58°C). PCR amplification was verified by 1% agarose gel.

Data analysis

The amplified product by matK, nrITS, rbcL and TrnH-PsbA were commercially sequenced (macrogen), and sequenced results of each primer were analysed by using MEGA 7.0. The phylogenetic trees were constructed by using neighbour joining metheod in MEGA 7.0. Bootstrap testing of 1000 replicates was performed to estimate the confidence level of the topology of the consensus tree.

RESULTS AND DISCUSSION

PCR based amplification was done of conserved regions using four pairs of universal primers i.e.matK, nrITS, rbcL and TrnH-PsbA. Good results of PCR amplification from fresh sample and marketed samples of *A.millifolium* L. were obtained as all four pairs of primers resulted in efficient amplification (Figure 1). Amplicons (matK (910 BP), nrITS (679 BP), rbcL (709 BP) and TrnH-PsbA (548) with specific size were obtained from all the primers used in this study. DNA star applications (Lasergen Inc. USA) were used for construction contigs and DNA sequences cleaning (Ilham *et al*., 2022).

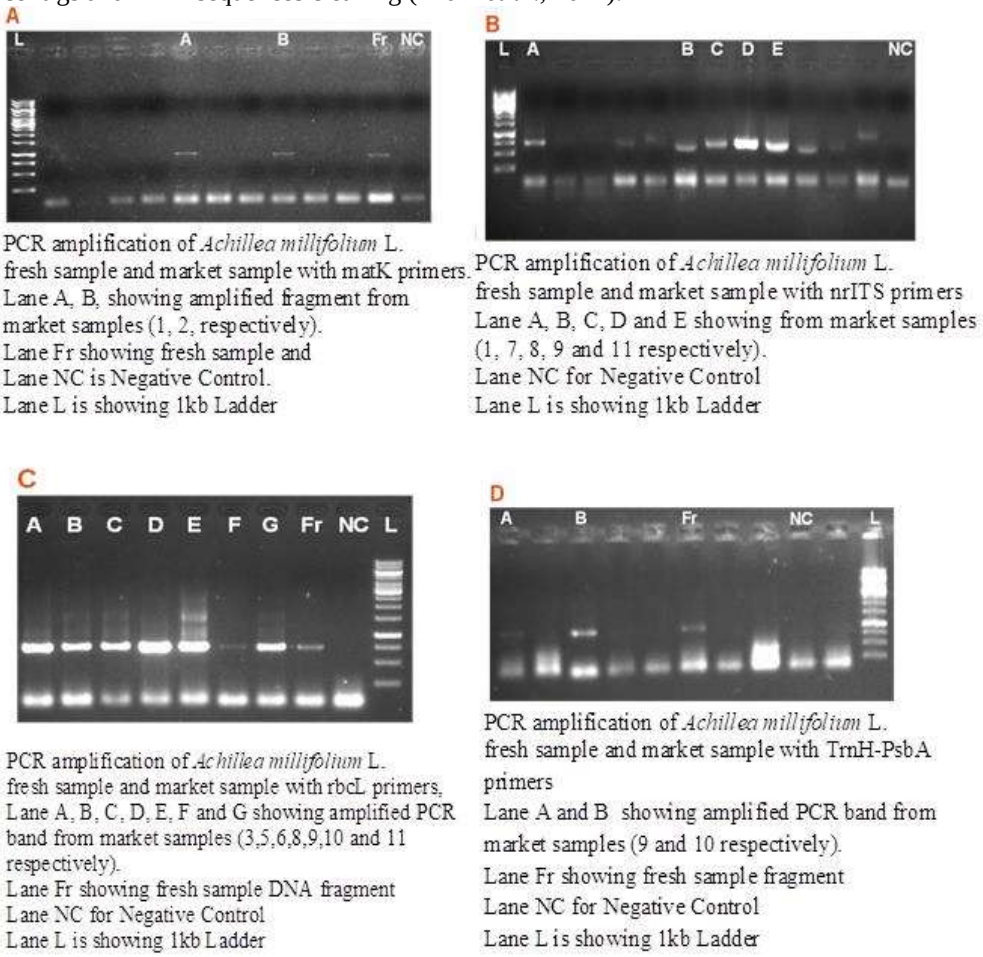

Fig. 1. PCR amplification of *Achillea millifolium* L. fresh sample and market sample with matK primers (A), nrITS primers (B), rbcL primers (C) and TrnH-PsbA primers (D).

In the present study 11 different samples were purchased from different shops of local market Lahore Pakistan which were being marketed with the local name of Biranjasif or Gomadar (Yarrow; *A.millefolium*) but marketed medicinal plants don't have any authentic way to identify them. Phylogenetic results showing matK, nrITS, rbcL and TrnH-PsbA sequences of fresh and market samples of *A. millefolium* L. with their closely related groups have been mentioned in table 2 and 3. All the samples (11 market and used as a positive control fresh sample) were analysed with barcode sequences to assess the close relationship with *A. millefolium* (Franzen, 1988; Asahina *et al*., 2010; Estevez *et al*., 2015). It was found that no samples showed close relationship with *A. millefolium* out of 11 market samples. Fresh sample of *A. millefolium* analysed with 3 different barcode sequences and it proved as *A. millefolium* with all three barcode sequences. Dried marketed samples of medicinal plants can't be identified on morphology basis (Ganie *et al*., 2015).

Table 02. Phylogenetic results showing matK, nrITS, rbcL and TrnH-PsbA sequences of fresh and market samples of *A. millefolium* L with their closely related closely groups.

Barcode sequences	Sample	Closely grouped with
matK sequences of fresh sample *A. millefolium* L.	fresh sample *A. millefolium* L.	*A. millefolium* already available in the databases (Accession # EU385315),
	market sample 1	Prunus species
	market sample 2	*Justicia adhatoda* (Accession # KY828464)
	market sample 5	*Deinbollia kilimandscharica* (Accession # JN191116)
	market sample 8	Different species of Morus genis.
nrITS primer sequence	market sample 1 and 9	*Vigna radiata* (Accession # LC193790),
	sample 7	*Foeniculum vulgare* cultivar (Accession # HQ377210),
	Sample 8	*Morus alba* (Accession # HQ144172)
	Sample 11	*Foeniculum vulgare* (Accession # FJ980395)
rbcL fresh sample	fresh sample	*Achillea millefolium* (Accession # KM360610)
	samples 3	Monechma (Accession # AB586154),
	samples 5	*Sphaeranthus indicus* voucher (Accession # JQ933489),
	sample 6	*Sorbaria sorbifolia* (Accession # KY419928),
	samples 8	*Morus australis* (Accession # KY420004),
	samples 9	*Vigna radiata* (Accession # AP014692),
	samples 10	*Justicia adhatoda* voucher (Accession # JQ231000)
	samples 11	*Sorbaria sorbifolia* (Accession # KY419928)
TrnH-PsbA	fresh sample	*Achillea millefolium* (Accession # MF348813)
	market sample 9	*Vigna radiata* cultivar (Accession # KT224678)
	market sample 10	*Justicia candicans* voucher (Accession # KT161342)

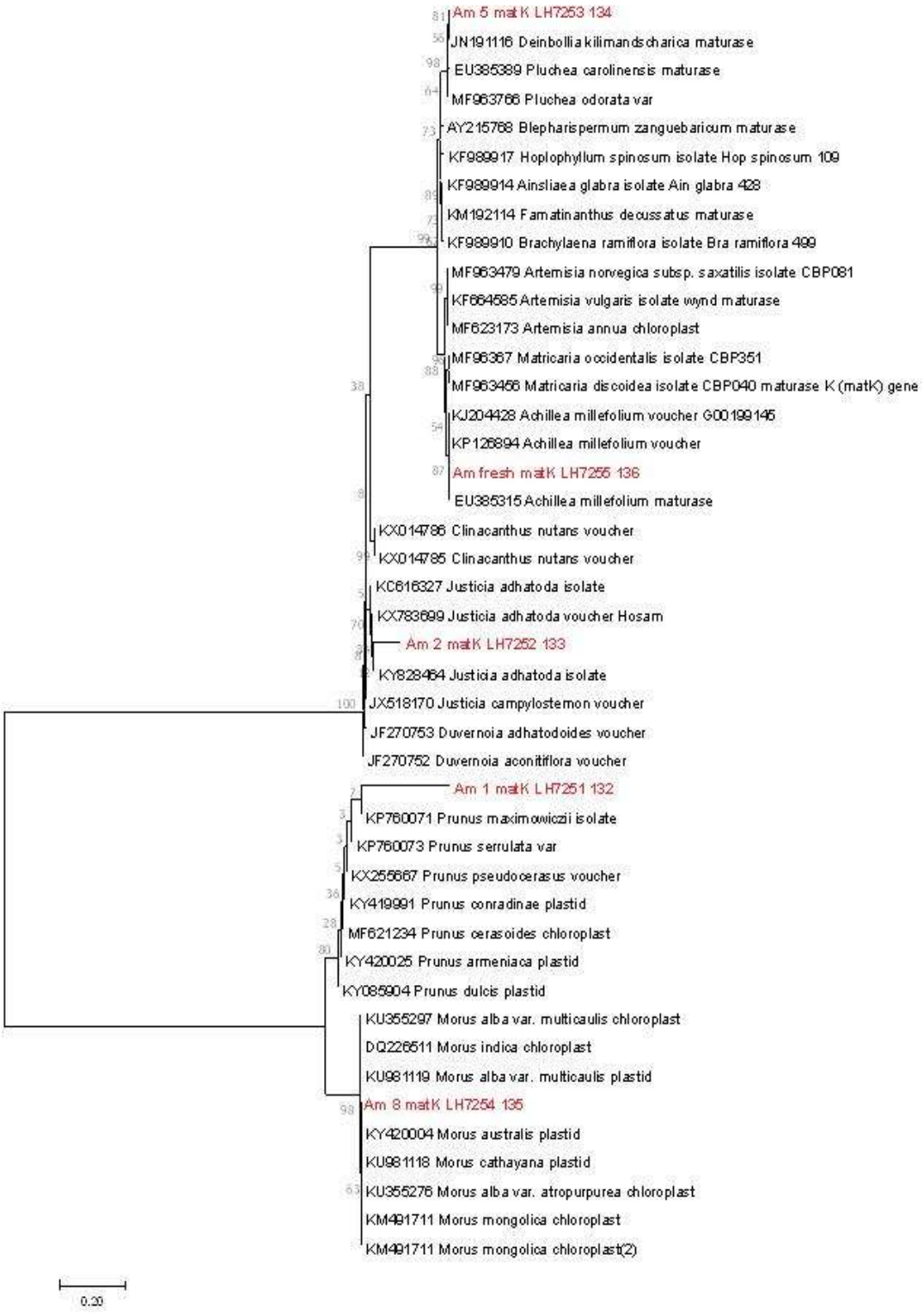

Fig. 2. Phylogenetic analysis of matK sequences of fresh and marketed samples (shown in red font colors) of *Acillea millefolium*. Other sequences were retrieved from the databases based on BLAST analysis. Neighbour joining algorthim was used for phylogenetic tree construction in Mega 6 sofware tool.

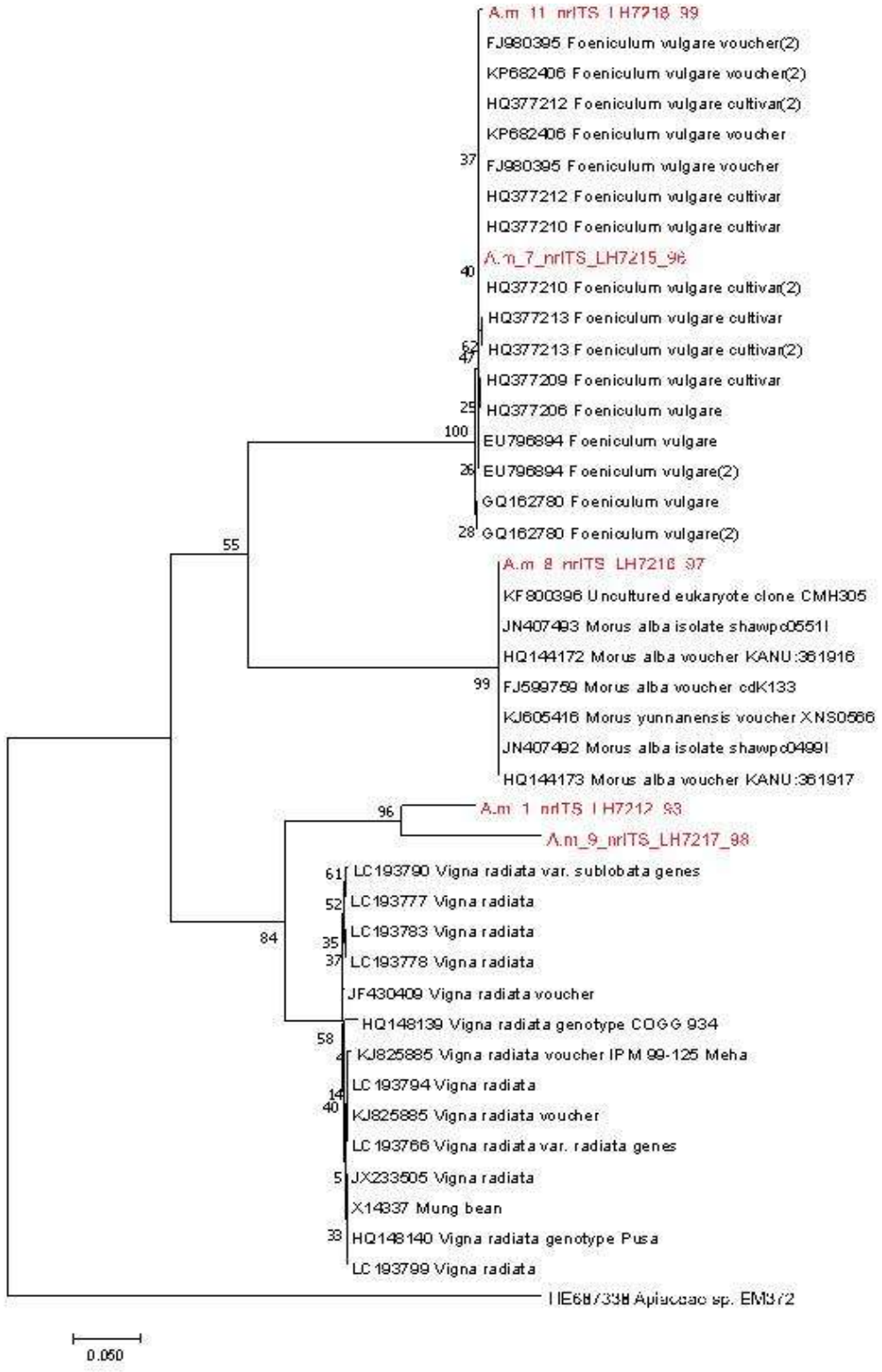

Fig. 3. Phylogenetic analysis of nrITS sequences of fresh and marketed samples (shown in red font colors) of *Acillea millefolium*. Other sequences were retrieved from the databases based on BLAST analysis. Neighbour joining algorthim was used for phylogenetic tree construction in Mega 6 sofware tool.

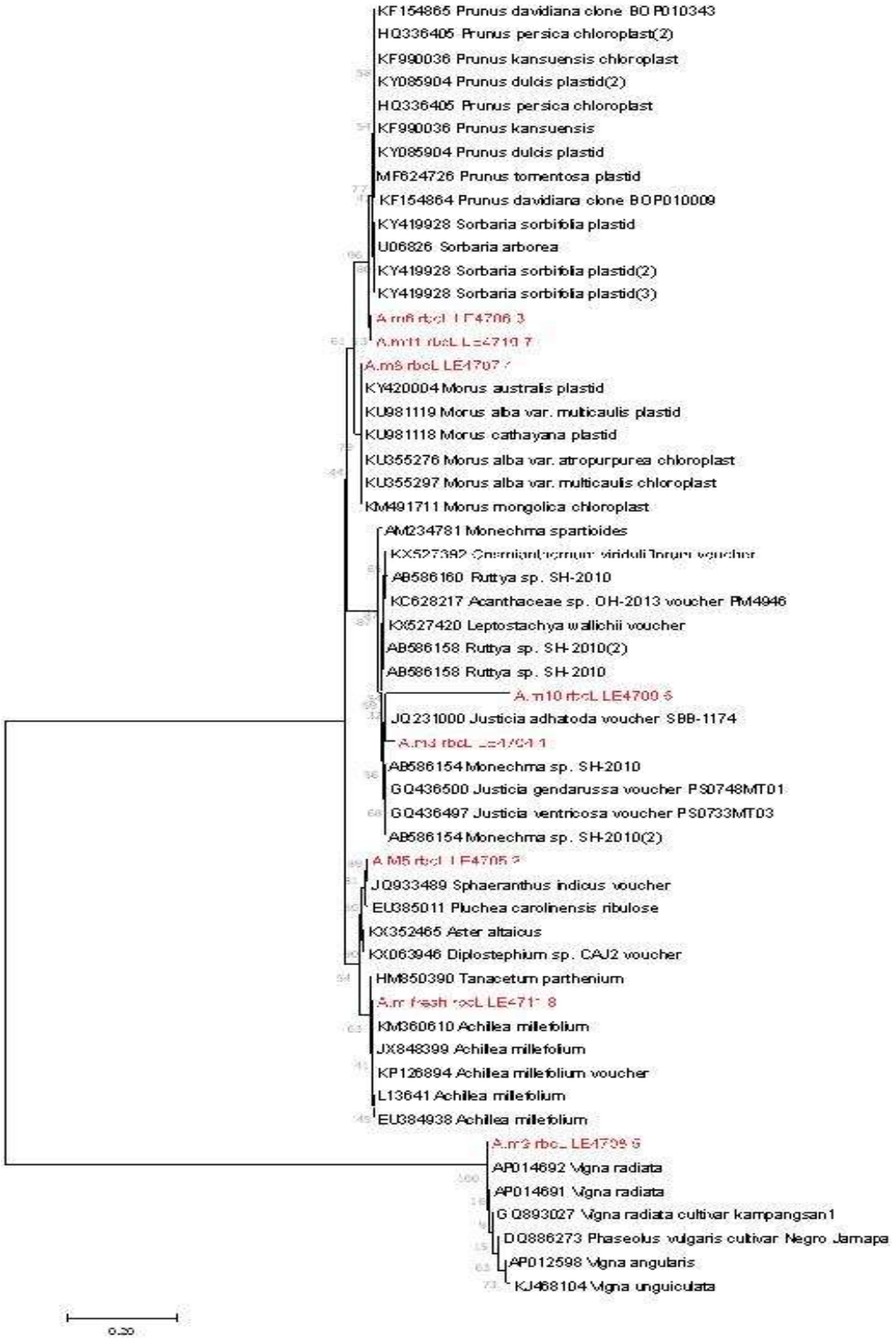

Fig. 4. Phylogenetic analysis of rbcL sequences of fresh and marketed samples (shown in red font colors) of *Acillea millefolium*. Other sequences were retrieved from the databases based on BLAST analysis. Neighbour joining algorthim was used for phylogenetic tree construction in Mega 6 sofware tool.

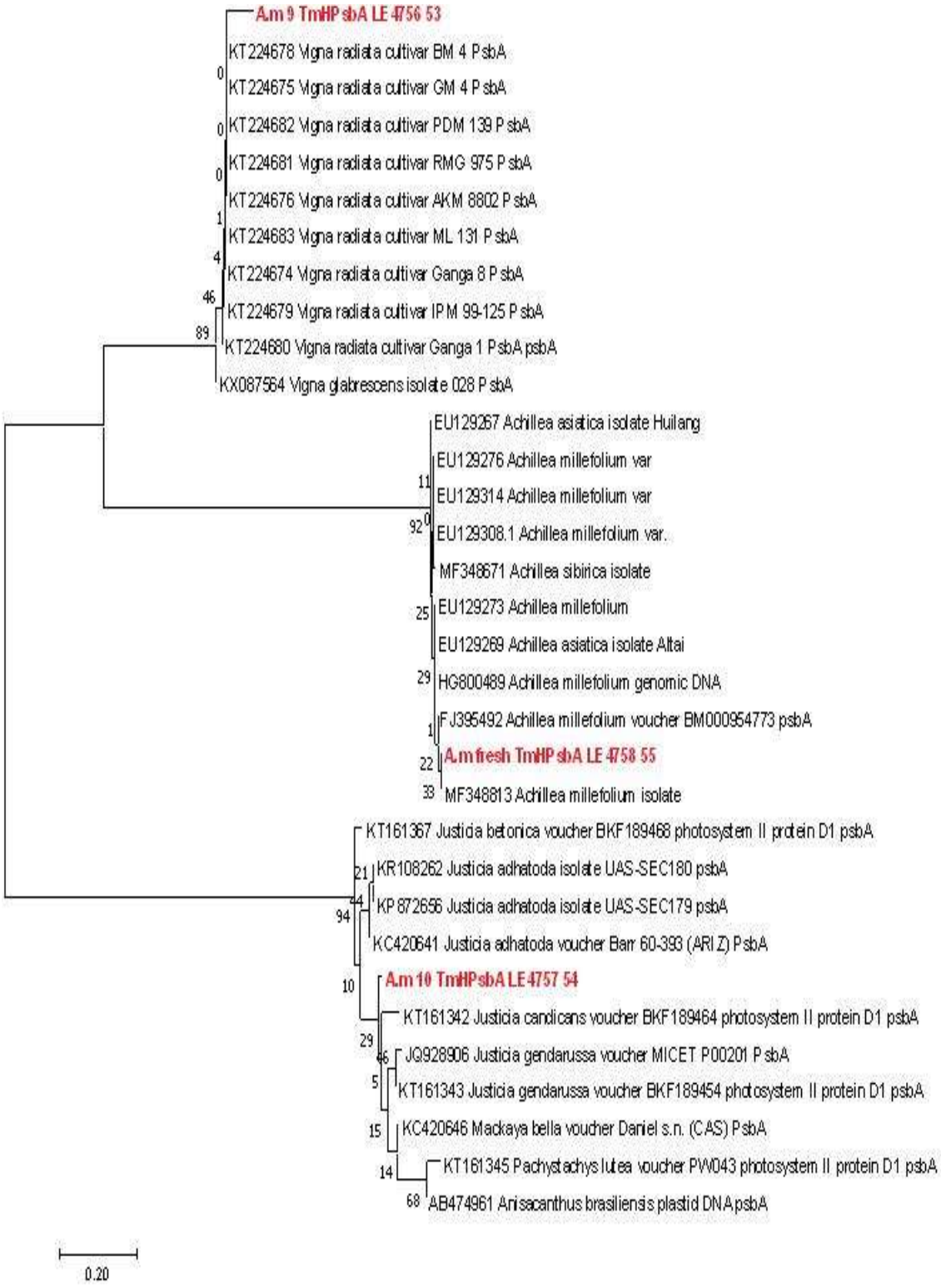

Fig. 5. Phylogenetic analysis of TrnH-PsbA sequences of fresh and marketed samples (shown in red font colors) of *Acillea millefolium*. Other sequences were retrieved from the databases based on BLAST analysis. Neighbour joining algorthim was used for phylogenetic tree construction in Mega 6 sofware tool.

Table 3. Phylogenetic results of fresh sample with matK primer, rbcL primers and trnH-psbA primers and their closely relation with *A. millefolium*.

samples	Amplification with	Closely related with
market sample 1	matK	different species of genus Prunus
	nrITS	*Vigna radiata*
market sample 2	matK primers	*Justicia adhatoda*
market sample 3	rbcL primers	Monechma
sample 5	matK primers	*Deinbollia kilimandscharica*
	rbcL primers	*Sphaeranthus indicus* voucher
Market sample 6	rbcL primers	*Sorbaria sorbifolia*
Sample 7	nrITS primers	*Foeniculum vulgare* cultivar
Market sample 8	matK	different species of *Morus australis*
	nrITS	
	rbcL	
Sample 9	nrITS	*Vigna radiata.*
	rbcL	
	TrnH-PsbA	
Market sample 10	rbcL	*Vigna radiata*
	TrnH-PsbA	
Sample 11	nrITS	*Foeniculum vulgare*
	rbcL	*Sorbaria sorbifolia*

Molecular identification is the most authentic and free of human biasness (Ramzan et al., 2019). Using multiple barcodes make the results more reliable. These barcode method is helpful in genotyping of medicinal plants in the international trades. Based on the sequences obtained from fresh sample of *A. millefolium*, species specific primers can be designed on any of the DNA barcode. These species specific primers may facilitate the large scale screening of the medicinal plants with a simple diagnostic PCR reaction, without doing sequencing and sequence analysis (Gharibi *et al*., 2011; Kumar *et al*., 2021; Atiyha *et al*., 2022).

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CONCLUSION

The study would be very fruitful for providing the information about the identification and authentication of crop species on the basis of molecular markers. In current study 11 market and one fresh sample of *Achillea millefolium* L were used for determination the correlation and identification among the samples by using four DNA markers. The fresh samples of *Achillea millefolium* L showed significant result and found to be used as a genetic and morphological identification of the medicinal plants on large scale basis. This can be used as a positive control for the screening of medicinal plants on the basis of various characteristics. The study would be equally beneficial both for scientific and farmers community.

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