Systematic position and habitat distribution modelling for reintroduction of critically endangered medicinal plant *Pittosporum eriocarpum* Royle (Pittosporaceae)

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Abstract

Pittosporum eriocarpum Royle (Pittosporaceae) is an endemic and endangered species of Indian Western Himalaya which is found in the restricted pockets of Uttarakhand and Himachal Pradesh only. The species is facing critical threats on its natural population mainly due to habitat loss, climate change and other anthropogenic activities. In order to improve the conservation status of species, potential suitable habitats for reintroduction, were predicted using Maxent. The species shows high variability in its morphological characteristics due to which sometimes it is being confused with other allied species *P. napaulense* and to avoid future confusion, systematic position of the species has been ascertained using nrDNA Internal Transcribed Spacer sequence data.

Key words: – nrDNA ITS, ML, ENM, Endangered

INTRODUCTION

The genus *Pittosporum* Banks ex Gaertn. (Pittosporaceae) is represented by ca.240 species occurring in different tropical regions of the world (Carolin & Bittrich 2018; Chandler *et al.* 2007), many of which are introduced for horticultural purposes. In India, the genus is represented by 11 species distributed in the Himalayas and in Western Ghats. Of these, only two species viz. *P. eriocarpum* Royle and *P. napaulense* (DC.) Rehder & E.H. Wilson are reported to occur naturally in North-West Himalaya (Nayar & Giri 1993). *Pittosporum eriocarpum* is endemic to Indian Western Himalaya and so far reported from the states of Himachal Pradesh and Uttarakhand. The species was first described by J.F. Royle from the upper hills of Sahastradhara and Mussoorie (Royle 1834). The species was considered endemic to Uttarakhand Himalaya from the range between Jumna (=Yamuna) to Sarda valley (Hooker & Thomson 1872; Brandis 1874; Gamble 1902; Brandis 1906). Parker (1918) reported it from, then called Suket state, Kangra district (now in Mandi district) of Himachal Pradesh. Somehow it remained unnoticed and the species was considered as endemic to Uttarakhand only (Hajra 1983; Nayar & Sastry 1987; Nayar 1996; Gaur 1999; Pullaiah 2006; Singh *et al.* 2015). The species has been reported from a few localities of Uttarakhand viz. Mussoorie, JhariPani, HathiPaon, Kempty Range, Nagni, Dhaulagiri, Sahastradhara, Nainital etc. (Kanjilal 1928; Hajra 1983; Gaur 1999; Pundir 2014).

The species shows high variability in its morphology due to which sometimes it is being confused with other allied species *P. napaense* hence to avoid future confusion systematic position of the species has been ascertained using nrDNA ITS phylogeny.

The habitat area of *Pittosporum eriocarpum* has been depleted mostly due to habitat fragmentation, over-exploitation, increasing human population and demand of land for cultivation and other economic development activities (Pragya *et al.* 2018). It is, therefore, necessary to get detailed information on potential suitable habitats for the reintroduction as measure for conservation of this medicinally important plant. Habitat distribution modelling helps to identify the suitable areas for reintroduction and in developing effective species conservation strategies.

MATERIAL AND METHODS

Morphology : Different floristic explorations during 2012 to 2019 to Western Himalayas several specimens of *P. eriocarpum* were collected for *ex-situ* conservation, mass propagation and further *in-situ* conservation. The study of the vegetative as well as flowering stages of the collected plants revealed its close resemblance to *P. napaense* and is critically endangered due to its overharvesting for medicinal uses.

Sampling : Four accessions of *P. eriocarpum* were collected from different localities in Uttarakhand (Table 1). Young leaves preserved in silica gel was used for molecular work. The Sanger sequences generated were added to a dataset comprising of 62 species of *Pittosporum* downloaded from GenBank including outgroups. The sequence alignment is available from the authors on request.

Table 1. Species, source and GenBank accession number for the DNA accessions used in the present study. Taxa sequenced for ITS region of Nuclear DNA

Taxon	Locality	GenBank accession number
<i>Auranticarpa edentata</i> L.W. Cayzer, Crisp & I. Telford	Australia	AY829013
<i>A. licifolia</i> L.W.Cayzer, Crisp & I.R.H.Telford	Australia	AY829014
<i>Billardiera lehmanniana</i> F.Muell.	Australia	AY829020
<i>B. longiflora</i> Labill.	Australia	AY829021
<i>Bursaria incana</i> Lindl.	Australia	AY829023
<i>B. spinose</i> Cav.	Australia	AY829024
<i>Griselinia jodiniifolia</i> (Griseb.) Taub.	Chile: Valdivia prov.	AJ536580
<i>G. racemosa</i> (Phil.) Taub.	Chile: Chiloe prov.	AJ536583
<i>G. scandens</i> (Ruiz & Pav.) Taub.	Chile	AJ536586
<i>Hymenosporum flavum</i> F.Muell.	Australia	AY829026
<i>Marianthus bicolor</i> (Putt.) F. Muell.	Australia	AY829028
<i>M. ringens</i> (J. Drumm. ex Harv.) F. Muell.	Australia	AY829027
<i>Rhytidosporum alpinum</i> McGill.	Australia	AY829045
<i>P. angustifolium</i> Lodd.	Australia	AY829029
<i>P. angustifolium</i>	Australia	HM116993
<i>P. arborescens</i>	Cook Island, Rarotonga	AF302025
<i>P. arborescens</i> Rich ex. A. Gray	Kingdom of Tonga, Vava'u	AF302026
<i>P. balfouri</i> Cufod.	Mauritius	AF302015

Taxon	Locality	GenBank accession number
<i>P. bracteolatum</i>	Australia	
<i>P. coccineum</i> Beauvis.	New Caledonia, Poya	AF302033
<i>P. cornifolium</i> A. Cunn.	New Zealand, cultivated	AF302030
<i>P. crassifolium</i> Banks & Sol. ex A. Cunn.	New Zealand	EF635471
<i>P. dallii</i> Cheeseman	Kahurangi National Park, New Zealand	U63196
<i>P. eriocarpum</i> Royle	Nainital, Uttarakhand, India	GB Acc. No. awaited
<i>P. eriocarpum</i>	Gangolihat, Uttarakhand, India	GB Acc. No. awaited
<i>P. eriocarpum</i>	Sahastradhara, Uttarakhand, India	GB Acc. No. awaited
<i>P. eriocarpum</i>	Mussoorie, Uttarakhand, India	GB Acc. No. awaited
<i>P. fairchildii</i> Cheeseman	New Zealand: Three Kings Islands	EF635472
<i>P. ferrugineum</i> W.T. Aiton	Australia	AY829032
<i>P. gatopense</i> Guillaumin	New Caledonia, Poya	AF302034
<i>P. glabratum</i> Lindl.	China: Dinghushan National Nature Reserve	KP092543
<i>P. glabratum</i> Lindl.	China: Dinghushan National Nature Reserve	KP092542
<i>P. illicioides</i> Makino	China: Hualong Mountain in Daba Mountains Range	MH710957
<i>P. illicioides</i> Makino	China	JX106283
<i>P. kogbiense</i> Guillaumin	New Caledonia, Mt. Dmuzac	AF302035
<i>P. lancifolium</i> (F.M. Bailey) L.W. Cayzer, Crisp & I.Telford	Australia	AY829033
<i>P. phillyraeoides</i> DC. (= <i>P. ligustrifolium</i>)	Australia	AY829034
<i>P. mannii</i> Hook.f.	Kenya	MK696153
<i>P. moluccanum</i> Miq.	Australia	AY829035
<i>P. multiflorum</i> (A.Cunn. ex Loudon) L.W. Cayzer, Crisp & I. Telford	Australia	AY829036
<i>P. oreilhanum</i> C.T. White	Australia	AY829037
<i>P. podocarpum</i> Gagnep.	China: Hualong Mountain in Daba Mountains Range	MH710978
<i>P. ralphii</i> Kirk	New Zealand	EF635473
<i>P. ramiflorum</i> Zoll. ex Miq.	Papua New Guinea	DQ499124
<i>P. resiniferum</i> Hemsl.	Philippines and Malaysia	DQ007353
<i>P. revolutum</i> Aiton	Illinois, USA	AY829038
<i>P. rubiginosum</i> A. Cunn.	Australia	AY829039
<i>P. spinescens</i> (F.Muell.) L.W.Cayzer, Crisp & I.Telford	Australia	AY829040
<i>P. taitense</i> Putterl.	Society Island, Bora Bora	AF302027
<i>P. tenuifolium</i> subsp <i>colensoi</i>	New Zealand	DQ499125
<i>P. truncatum</i> E. Pritz.	China: Qinling Mountains Range	MH710783

Taxon	Locality	GenBank accession number
<i>P. truncatum</i> E. Pritz.	China: Qinling Mountains Range	MH711550
<i>P. truncatum</i> E. Pritz.	China: Qinling Mountains Range	MH710692
<i>P. undulatum</i> Guill.	Australia	AF302014
<i>P. undulatum</i> Guill.	Australia	HM116994
<i>P. venulosum</i> F. Muell.	Australia	AY829043

Molecular analyses: Genomic DNA was extracted from silica dried materials using a Dneasy Plant Mini Kit (Qiagen, Amsterdam, Netherlands). DNA amplification and sequencing of the entire ITS region (ITS1, 5.8S and ITS2) were performed using the primers ITS1 and ITS4 (White *et al.* 1990). The polymerase chain reaction (PCR) was performed with standard methods using RedDye Master mix (12.5 µl) and PCR amplification was performed with 35 cycles (Denaturation for 1 min at 94°C, annealing for 1 min at 49°C, and 1 min of extension at 72°C followed by a last cycle of final extension for 5 min at 72°C). PCR products were checked for the presence of appropriate bands on a 0.8% agarose gel, purified, and sequenced at 1Base Labs., South Korea. Forward and reverse sequences were edited and assembled using the computer program and DNA Baser vers. 4 (2013, Heracle Biosoft). All sequences have been deposited in GenBank.

Phylogenetic Analyses: Maximum Likelihood (ML) performed after determining the models of evolution selected in jModelTest v.2.1.5 (Darriba *et al.* 2012) by Akaike test (option AIC), ML analyses was performed at CIPRESS Science gateway (Miller *et al.* 2010). Maximum Likelihood analyses were performed using RAxML-HP2 on XSEDE ver. 8.2.12 (Stamatakis 2014) on the gateway. RAxML was run under the model selected from the jModel test with 20 heuristic searches from distinct random stepwise addition sequence parsimony starting trees, followed by selection of best scoring tree. RAxML bootstrap (BS) values were calculated using 1000 replicates. The outgroups were selected from previous studies (Chandler *et al.* 2007).

Species distribution modelling and future prediction : For the analysis 60 primary distributional records of the species were used which were obtained through field surveys. The coordinates of all the occurrence points were recorded to an accuracy of 3 – 10 m using GPS (Garmin). These coordinates were then converted to decimal degrees for use in modelling the distribution of habitats of the species. Bioclimatic variables (Hijmans *et al.* 2005) with 30 seconds spatial resolution, downloaded from World Clim dataset (www.worldclim.org) were used in the present study. These World Clim data (period 1950 – 2000) are derived from measurements of altitude, temperature and rainfall from weather stations across the globe (Khanum *et al.* 2013) and are frequently used in modelling species distribution (Kumar & Stoghlgren 2009; Sanchez *et al.* 2011; Khanum *et al.* 2013; Adhikari *et al.* 2015).

For habitat modelling, the model was developed using Maximum entropy distribution software (Maxent version 3.3.3; Phillips *et al.* 2006; <http://www.cs.princeton.edu/wschapire>), which generates an estimate of probability of the presence of species that varies from 0 to 1, where 0 is the lowest and 1 is the highest probability. Of the 60 records, seventy five percent were used for model training and twenty five percent for testing. To validate the model robustness, 10 replicated models runs for the species with a threshold rule of 10 percentile training presence was executed. In the replicated runs, cross validation technique was employed, where samples were divided into replicate folds and each fold was used for test data. Other parameters were

set to default as the program is already calibrated on a wide range of species datasets (Phillips & Dudík 2008). From the replicated runs average, maximum, minimum, median and standard deviation were generated. Jackknife procedure and percent variable contributions were used to estimate the relative influence of different predictor variables. Receiver operating characteristics (ROC) analyses the performance of a model at all possible threshold by a single number called, the area under the curve (AUC).. Higher AUC values correspond to better model quality and accuracy. The Area under the ROC curve (AUC) was used to evaluate model performance. AUC is a measure of model performance and varies from 0 to 1 (Fielding & Bell 1997). An AUC value of 0.50 indicates that model did not perform better than random whereas a value of 1.0 indicates perfect discrimination (Swets 1988). Future climate scenario data for 2050 (A2a emission scenario) were obtained from Consultative Group on International Agricultural Research (CGIAR). (<http://ccafsclimate.org>). These future climate projections are based on IPCC 4th assessment data and were calibrated and statistically downscaled using the data for 'current' conditions.

RESULTS

Sequence analyses

For the study a total of 62 sequences were analyzed of which four accessions (GenBank accession number [MT677940, MT677941, MT677942, MT677943, submitted]) have been newly generated from *P. eriocarpum* collected from different localities of Uttarakhand [Western Himalaya]. This is the first study to have analyzed any Indian species of *Pittosporum*.

Multiple sequence alignment

The alignments of the ITS regions (ITS1, 5.8s ITS2) consists of 715 characters; 8.5 % of which are gaps; GC content is 61.6 %; 298 identical sites represent 41.7 %; pairwise percentage identity is 89.4 %; parsimonious informative sites are 257. Final alignment has been submitted to TreeBASE also can be obtained from corresponding author on request.

Evolutionary model estimation

The best fit model according to BIC criterion is TNe+G4 as evaluated in jModeltest (Dariba *et al.* 2012). Model selected implemented as -f parameter for subsequent phylogenetic analyses. The rate parameters were - A-C: 1.000; A-G: 3.1690; A-T: 1.000; C-G: 1.000; C-T: 6.8834; and G-T: 1.000.

ML phylogenetic analyses

The tree topology retrieved (Figure 1) has similar topology to that from the previous studies (see Chandler *et al.* 2007). The log-likelihood of the tree is -5605.384. The total tree length i.e. sum of all branch length is 1.5651. Sum of internal branch length is 0.6297. All the species of *Pittosporum* after originating from the last common ancestor grouping in single clade. Four accessions of Indian species of *P. eriocarpum* (BP 100) is placed along *P. balfauri* and *P. mannii* (BP. 75).

Taxonomic description

Shrubs or small trees, 4 – 8m tall. Bark grayish brown, somewhat rough, young shoots and branchlets densely tomentose, glabrous later. Leaves simple, exstipulate, alternate or falsely whorled usually clustered towards the tips of branchlets; lamina obovate to oblanceolate to elliptic-oblong, 4 – 12 x 2 – 5cm, entire, acuminate or acute, sometimes mucronate, base cuneate, membranous, lateral veins about 12-16 on each side, midrib impressed above and slightly prominent beneath, transversely horizontal; petiole 15 – 20 mm long, tomentose. Inflorescence terminal or pseudoterminal, paniculately corymbose, glabrous; peduncles 2 – 4 cm long; pedicels

5 – 8mm long, Flowers bisexual, buds oblong, 6 - 9 mm across; sepals 5, subequal, lanceolate-ovate, 2 x 1 mm, margins minutely ciliate, subacute, minutely pilose; petals 5, usually ligulate, linear oblong, 9 - 10 x 2 - 2.5 mm, obtuse, pal yellow; -stamens 5, free, alternate to petals; filaments slender, glabrous, about 5 mm long; anthers sagittate, dorsifixed, oblong, 2 – 2.5 mm long; ovary superior, ovoid, densely brown-tomentose, about 2 mm across. Capsules sub-globose, transversely ribbed, 12 - 15 mm across, pericarp hard woody, densely pilose-tomentose, dehiscing with 2 – 5 valves; seeds many, oblong-ovoid, irregularly compressed, embedded with glutinous, resinous pulp, reddish-black.

Distribution

Endemic to Indian Western Himalaya. Reported only from Uttarakhand and Himachal Pradesh from an altitudinal range of 900–2000 m.

Morphological variations

The species shows different types of variations in both vegetative and flowering characteristics with response to changes in environmental and habitat conditions.

a. Growth habit: variations have been observed in its growth pattern in different habitats. While growing on rocky slopes plants are shorter and bushier, whereas when growing on gentle slopes or planted in plain land it become straight and attains good height

b. Shape of lamina: In vegetative state, the shape of lamina varies from ovate or obovate to lanceolate even on a single branch. In some populations, the leaves are broader becoming slightly ovate whereas it is lanceolate in other populations.

c. Flowering and Fruiting: The flowers of this species are heterostylous having variations in size and functionality of stamens and stigma. Some of the plants bears functional female flowers having fertile stigma but sterile stamens whereas some trees produce only functionally male flowers having well developed and fertile stamens but underdeveloped stigma. Some plants also behave as sexually labile and behaves as either male or female in different years. However, sometimes fruit set is also seen in functionally male flowers due to leaky dioecism (Das *et al.* 2017).

Variations are also observed in shape and size of fruits in some habitats. The fruits collected from Sahastradhara were somewhat angular. Also, the thickness of fruit valves (pericarp) varied in fruits collected from planted trees in GBPNIHESD, Kullu with that of other populations in Uttarakhand. The fruit valves of former plants were thin as compared to fruits of other localities which may be due to variable responses to climatic conditions prevailing in those localities.

In light of the above morphological variations sometimes the species shows overlapping characteristics with its allied species *P. nepaulense* thus leading to misidentification (Srivastava *et al.* 2017).

Molecular diagnosis

Accessions of *P. eriocarpum* differs at several positions from *P. balfourian* ITS alignment with its related species: position 81 G/A, 89 T/C, 100 & 101 CC/GT, 138-140 TTG/CTT, 185 C/T, 193 A/G, 203 T/C, 217 C/T, 227 A/G, 233 A/G, 382 T/C, 399 C/T, 421- 423 GTT/ACC

Potential suitable habitat for reintroduction

The maxent model for *P. eriocarpum* performed well with an average AUC value of 0.998. Most suitable habitat is predicted in Uttarakhand, India with an area of 9084 km² of high suitability and 6040 km² as very highly suitable (Figure 2a & 3a). Table 2 shows the relative contributions of the predictor variables in Maxent for distribution of *P. eriocarpum*. Precipitation of Driest

Figure 1. Best maximum likelihood phylogeny based on the nrDNA ITS data (724 base pairs). Likelihood bootstrap values >70 shown at the nodes *Pittosporum eriocarpum* highlighted in red, for GB-sequence accession number provided from GB (downloaded from GenBank.)

Quarter (Bio 17), Precipitation Seasonality (Bio 15), Precipitation of Warmest Quarter (Bio 18) and Mean Temperature of Coldest Quarter (Bio 11) were the strongest predictors for the distribution of *P. eriocarpum* with 59.2%, 18.7%, 11.1% and 5.6% contributions, respectively. The probability of presence of *P. eriocarpum* increased with an increase in precipitation of driest quarter and precipitation seasonality (Figure 3 a, b). Relative importance of different environmental variables based on results of jackknife tests in Maxent are shown in Figure 4. Jackknife results also showed that Precipitation of Driest Quarter is the most important predictor for the distribution of *P. eriocarpum*.

Table 2: Selected environmental variables and their percent contribution in maxent model for *P. eriocarpum*

Environment Variables	Percent Contribution
Precipitation of Driest Quarter (Bio 17)	59.2
Precipitation Seasonality (Bio 15)	18.7
Precipitation of Warmest Quarter (Bio 18)	11.1
Mean Temperature of Coldest Quarter (Bio 11)	5.6
Precipitation of Wettest Quarter (Bio 16)	2.3
Precipitation of Coldest Quarter (Bio 19)	1.4
Minimum Temperature of Coldest Month (Bio 6)	0.9
Mean Temperature of Driest Quarter (Bio 9)	0.7

Figure 2. Predicted current and future potential suitable habitat of *P. eriocarpum* in India

Compared with the area of most suitable habitat (6040 km²) under current climate prediction, the future prediction model for 2050 (A2a emission scenario) showed loss of habitat (Figure 1b), with the most optimal geographic distribution of 3023 km² (Figure 2b). Although the location of future potential distribution of *P. eriocarpum* is very similar to the current potential distribution, the model results suggest that there is a significant loss of very highly suitable habitat of 50.04 % area.

Figure 3. Area under different suitability grades for current and future potential suitable habitat of *P. eriocarpum* in India. Figures at the top of each bar represent the area

Figure 4. Response curves showing relationships between probability of presence of a species and two top bioclimatic predictors of *P. eriocarpum* (a, b)

Figure 5. Relative predictive power of different bioclimatic variables based on the jackknife of regularized training gain in maxent model for *P. eriocarpum*

DISCUSSION AND CONCLUSION

The ML analyses showed the monophyletic nature of the genus *Pittosporum* Banks ex Gaertn. of Pittosporaceae. Species of *Pittosporum* are found to be grouping in well supported (BP 100 Figure 1) clade and supports the previous study by Chandler *et al.* (2007). *Pittosporum eriocarpum* collected from Uttarakhand (Western Himalaya) is placed within the monophyletic clade. Systematic position of Indian *P. eriocarpum* is very interesting due to its placement along with *P. balfourii* from Mauritius. The best explanation for the placement could be the Gondwanan origin of *P. balfourii* (Gemmill *et al.* 2002). The ITS sequence data has ascertained the systematic position of the species, morphological description provided in the present communication binds with the molecular data and will help taxonomists, biochemists and ecologists to identify, eradicate adulterants and the ENM data generated (Figure 2) will be helpful for conservation biologists. The ENM data reveals the most suitable habitat for *Pittosporum eriocarpum* is predicted in Western Himalaya. The model results showed that 7.03% of the total predicted area is highly potential. In regard to future distribution of *P. eriocarpum*, the modelling suggests that its geographic distribution would shrink. The restricted distribution of highly suitable habitats of *P. eriocarpum* in Western Himalaya suggests that the plant is endemic to Western Himalayan region of India, which has also been suggested through previous field surveys, herbarium records and literature (Padalia *et al.* 2010). The zone mapped in the study with higher model thresholds suggests that these areas have ideal habitat conditions for reintroduction of the *P. eriocarpum*. The current and predicted map of suitable habitat and identification of suitable bioclimatic variables can be of significant use for government and non-government organizations responsible for management and conservation of threatened medicinal plants (Malay *et al.* 2018).

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