Abstract leaves of salacia species contains active

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AbstractDiversityand phylogenetic relation of four Salaciaspecies collected from the western Ghats of Karnataka was assessed and comparedusing RAPD, ISSR markers and ITS sequence. For RAPD and ISSR marker 21 sampleswere analyzed using ten primers each. Nineteen ITS sequences along with anoutgroup was used to generated phylogenetic tree and diversity assessment. RAPDand ISSR primers generated 76 and 68 loci of which 70 and 61 loci respectively werepolymorphic.

In ITS analysis 18 sequences alignment generated overall length of739bp of which 137 bp were found to be polymorphic. Maximum likelihood analysisof the ITS sequences revealed three clades. UPGMA analyses of RAPD and ISSRbanding variation revealed two and four major clusters respectively. Similarclustering pattern was observed in PCoA. The level of polymorphism revealed byRAPD was 41. 45%±10%, ISSR is 33.

58%±6. 52% and ITS was 25. 50%± 17. 25%. AMOVArevealed significant variance within and among the Salacia species. Tajima’s D neutrality test were negative for allspecies indicating presences of rare alleles and population expansion. Keywords: Salacia species, Genetic Diversity, RAPD, ISSR, ITS, PCoA. AbbreviationsMI MarkerIndexRP ResolvingpowerPCoAPrinciple coordinate analysisPICPolymorphism information contentRAPD Randomamplified polymorphic DNAISSR Intersimple sequence repeatITS Inter transcribedspacersAMOVAAnalysis of molecular varianceUPGMAunweighted pair group method for arithmetic mean IntroductionSalacia is a liana which belongs to family Celastraceae subfamily Salacioideae.

In India, Salacia is distributed in Karnataka, Kerala, Tamil Nadu. Species of Salacia areused in ayurveda for treatment of diabetes and obesity as polyherbalpreparation or churnas such as Madhujeevan Churna (Salunkhe andWachasundar 2009) and Diajith (Rajalakshmy et al. 2014). Root, bark and leaves of Salacia species contains activeingredients which are anti-diabetic (Yoshikawa et al. 2001), anticancer (Yoshimi et al. 2001), antiviral agent effective against HIV, Herpes simplex(Guha et al.

1996; Zhengand Lu 1990). Salacia species containsactive compounds salacinol and kotalanol which have ?-glucosidase inhibitoryactivity (Xie et al. 2011). Speciesidentification in the genus Salaciais difficult when based solely on morphological characteristics. Although mostof the vegetative characters of the species within the genus are same differencesare observed in floral and some fruit characteristics (Udayan et al. 2012; Udayan et al. 2013). Therefore, accurate methods of validation andauthentication is indispensable to ensure safe use and efficacy of extractedraw drugs.

RAPDand ISSR simple and quick techniques which does not require any prior knowledgeDNA sequence of the target organism. RAPD detects nucleotide sequencepolymorphisms, using a single primer of arbitrary nucleotide sequence where asISSR detects polymorphisms in identicalinter-microsatellite loci oriented in opposite direction, using primers whichare di, tri, tetra or penta nucleotide simple sequence repeats (Zietkiewicz et al. 1994). Inter transcribed spacer (ITS) having universal set of primers is apopular choice for phylogenetic analyses (Alvarez and Wendel2003).

In the present study two DNA markersRAPD and ISSR, and a DNA barcoding region ITS was used to evaluate geneticdiversity within and among four Salaciaspecies-S. chinensis, S. macrosperma, S. fruticosa, S. oblonga sampled from Western Ghats of Karnataka. Materials and methodsSampling Twenty-onesamples were collected from various parts of western Ghats (Table 1 for details).

The 21 samples are grouped in four population of Salacia chinensis L., Salaciamacrosperma Wight., Salacia fruticosaLawson., Salacia oblonga Wall.

exWight & Arn. DNAisolation, RAPD and ISSR reaction GenomicDNA was isolated according to Stange et al. (1998) protocol. DNA was quantified using NanoDrop 2000Spectrophotometer (Thermo Fisher Scientific) and diluted to 25 ng for use in polymerasechain reaction (PCR). Reaction mixture contained 100 uM of each dNTPs (Merckbiosciences), 5 uMole of primer (Sigma, USA), 0. 5 Unit of Taq DNA polymerase(Merck biosciences) and 1x Taq buffer (Merck biosciences) in a total volume of20 ul. ISSR-PCR amplification was carried out for 40 cycles, with initialdenaturation for 5 minutes at 94°C, followed by cyclic process of denaturationfor 1-minute at 94oC, annealing at temperature standardized for eachprimer (Table 2) for 1 minutes and extension at 72°C for 1 minutes, and finalextension at 72 °C for 5 minutes in Applied Biosystems Veriti Thermal Cycler.

ForRAPD-PCR, the protocol was similar to ISSR except for the annealing temperaturewhich was 36°C for all the primers. For the PCR amplification of the ITSsequence, primers ITS4-TCCTCCGCTTATTGATATGC and ITS5-GGAAGTAAAAGTCGTAACAAGGdesigned by WHITE (1990) were used. ITS

amplification was carried for 28cycles with initial denaturation at 95°C for 1 minute 30 seconds, cyclicprocess of denaturation at 95°C for 30 seconds, annealing at 42°C for 1 minute, extension at72°C for 1 minute and final extension 72°C for 3 minutes. Amplifiedproducts were separated in 1. 8% agarose gel containing ethidium bromide using1x TBE buffer. DNA fragments were visualized under UV light.

The band patternswere photographed using Gel Doc™ XR (Bio-Rad). Phylogenetic analysis of ITS sequence Theamplified products were sent to Chromous biotech, Bangalore for sequencing. Thesequence generated were submitted to NCBI database. For phylogeneticanalysis of ITS sequence MEGA 5 (Tamura et al.

2011) software was used. Nineteen samples from currentstudy and an outgroup sequence was used for sequence analysis. The multiplesequence alignment was performed using CLUSTAL W, version 1. 6 (Thompson et al. 2002). Using MEGA 5 best-fit Model-test was performed andmodel with the lowest Bayesian Information Criterion (BIC) score was selectedfor further analysis. The Maximum Likelihood tree was constructed using thebest fit model with least BIC score. Datacollection and Analysis Thebanding patterns obtained from RAPD and ISSR were scored as present (1) orabsent (0) and binary matrix was created for RAPD and ISSR primers.

Thepolymorphic information content (PIC) proposed by Roldàn-Ruiz et al. (2000), marker index (MI) described by Varshney et al. (2007) and resolving power (RP) by Prevost and Wilkinson(1999) of each marker was calculated

and multiplex ratio was calculates as product of total number monomorphic and polymorphic loci/number of assays. POPGENE(Yeh et al.

1999)was used to calculate various paraments such aspercentage of polymorphic band, observed number of alleles (na), effectivenumber of alleles (ne), Shannon's information index (I) and Nei's genediversity (H) total heterozygosity (Ht), average heterozygosity (Hs) and geneflow (Nm) between the populations and among the individuals within eachpopulation. The similarity matrix was subjected to cluster analysis byunweighted pair group method for arithmetic mean (UPGMA) and a dendrogram wasgenerated. GenAlEx6(Peakall and Smouse2006) was also used to calculate Principal Coordinates Analysis (PCoA) thatplots the relationship between distance matrix elements based on their firsttwo principal coordinates.

The product-moment correlation (r) based on Mantel Zvalue was computed to measure the degree of relationship between similarityindex matrices produced by any two-marker systems. The RAPD, ISSR and ITS datawere subjected to analysis of molecular variance (AMOVA), as described by . ResultsRAPD andISSR analysis details In present study, initially 40RAPD primers that is 2 set of Operon primer kits OPG and OPR (20 primer fromeach kits) were used to detect genetic polymorphism of S. oblonga, S.

fruticosa, S. chinensis and S. macrosperma. Out of the 40 RAPD primers, 10 primers i. e. OPG-02, 14, -16, -17, -18, -19 and OPR-02, -03, -07, -08 showed reproducible amplifiedDNA polymorphism. All the chosen primers amplified fragments across the 21samples, with the number of amplified fragments

ranging from 4 to 12. Minimum numberof loci were seen in the primer OPG18 (4 bands) and maximum bands were observedin primer OPG17-12 bands.

From the ten primers, a total of 76 loci weregenerated of which 70 were polymorphic, making polymorphism generated by RAPDmakers to be 92. 11%. Multiplex ratio of RAPD analysis was calculated to be 7.

6. Cumulative resolving power of 10 RAPD primer was 54. 67. While in ISSR analysis 10 primersproduced 67 loci of which 61 bands were polymorphic, accounting for 91. 04% ofpolymorphism. Number of loci varied from minimum of four in primer ISSR 5 tomaximum of nine in ISSR 10. Multiplex ratio of ISSR analysis is calculated tobe 6. 8.

Cumulative resolving power of 10 ISSR primer was 58. 48. The markerindex for RAPD and ISSR was 6. 54 and 5. 45 respectively. Observed number of alleles, effective number of alleles, Nei’s genetic diversity, Shannon’s informationindex, for 21 samples of Salaciaspecies analyzed using ten each of RAPDand ISSR primer were found to be 1. 9211, 1. 4537, 0.

2785, 0. 4294 and 1. 9104, 1. 5108, 0.

2988, 0. 4509 respectively. Total genotype diversity among population (Ht) was estimated to be 0.

2713 while within populationdiversity (Hs) was estimated to be 0. 1514 for RAPD and for ISSR Ht was 0. 3055and Hs was 0. 1222. Mean coefficient of gene differentiation (Gst) value forRAPD was 0. 4418 and ISSR was 0. 5999.

Suggesting that 55. 8% and 40. 1 % of thegenetic diversity resided within the population as per RAPD and ISSR markers. Estimates of gene flow in the population for RAPD and ISSR were 0. 6318 and0. 3334 respectively. (Table 3).

Dendrogram and PCoA of RAPD and ISSRIn RAPD dendrogram, 21samples of Salacia grouped into two clusters(Cluster 1 and 2). Cluster 1 contained S. chinensis SC1 to SC5 and cluster 2 was further divided into twosub-clusters (sub-cluster 1 & 2). In cluster 2, sub-cluster 1 contained allsamples of S.

macrosperma along withtwo samples of S. fruticosa SF1 (Fig 1) and sub-cluster 2 contained three remaining samples of S. fruticosa along with S.

oblonga samples. The cumulative totalvariation of three principle components accounted for 65. 68 % of variation. Dendrogram of ISSR datashowed that the samples clearly grouped into four clusters (I, II III and IV)of its respective species S. chinensis, S. macrosperma, S. fruticosa, S.

oblonga. For ISSR analysis cumulativetotal variation of three principle components accounted for 74. 05% of thevariation. The results of RAPD and ISSR PCoA analysis were comparable to thecluster analysis (Fig 2). ITS analysis For ITS analysis, 19 samples ofcurrent study and an outgroup Pristimerapreussii belonging to sub-family Hippocrateoideae was used to constructphylogenetic tree.

Two samples SM1 and SM14 produced faint bands and could notbe sequenced. Sequence alignment of 20 samples resulted in overall sequencelength of 752 bp, of which 221 bp (29. 38%) were conserved, 503

bp (66. 88%) werevariable sites and 103 bp (13. 69%) were parsimony informative sites. Threemajor clades were observed from ML tree. Clade 1 contained all the samples of S.

macrosperma along with samples of S. oblonga which were nested with-in theclade. Clade 2 and 3 contained S.

chinensis and S. fruticosasamples respectively (Fig 3). Comparative analysis of population Values of observed number ofalleles, effective number of alleles, Nei’s genetic diversity, Shannon’sinformation index of each population were compared to observed diversity anddegree of polymorphism with-in the population (Table 3 and 4). In comparison, the RAPD values were marginally higher than the ISSR except in the S. fruticosa population. Significantdifferences were observed in all the parameters. Highest percentage ofpolymorphism and highest polymorphic loci were seen in S. fruticosa population in RAPD analysis.

In RAPD, ISSR and ITS analysishigh degree of polymorphism was seen in S. macrosperma and S. fruticosa populationfollowed by S. chinensis population. Althoughonly two samples are in S.

oblongapopulation, RAPD, ISSR and ITS analysis detected polymorphism of 15. 79%, 16. 42%, 23. 36%respectively.

Also, in parameters such as Ht, Hs, Gst and Nm significantdifferences in value were observed (Table 4). The level of polymorphismrevealed by RAPD was (41. 45%±10%) which was higher than ISSR (33. 58%±6. 52%) andITS (25.

50%±17. 25%). The polymorphism of each population of S. chinensis, S. macrosperma, S. fruticosa and S. oblonga from RAPD was35. 53%, 55.

26%, 59. 21%, 15. 79%and ISSR was32. 84%, 47. 76%, 37. 31%, 16.

42% respectively. For comparative analysis of ITSwith the RAPD and ISSR, sequences data of ITS was analyzed in GenAlEx. Beforeexporting the data, the outgroup sequence and sequence SF5 were removed. Onlypolymorphic nucleotide positions were converted to numeric codes (A= 1, C= 2, G= 3, T= 4, hypen/colon= 5) and 137 sites showed the polymorphism which were usedfor the further analysis. The polymorphism of each population of S. chinensis, S.

fruticosa, S. macrospermaand S. oblonga from ITS analysis was6.

57%, 19. 71%, 24. 82%, 23. 36% and overall polymorphism was 18. 61%±4. 16%. For ITS coefficientof evolutionary differentiation was 0. 797which indicated that 20.

3% of thegenetic diversity resided within the population. Tajima’s D neutrality tests wereperformed to check whether genus Salaciapopulations followed a neutral model of evolution with constant population sizeover time. The observed values of Tajima’s D neutrality tests were -1. 089757for S. macroperma and S. oblonga population, -1. 105205 for S. fruticosa and -0.

174749 for S. chinensis and-1. 181277 for all the19 samples. After removing sample SF5 since it showed high divergence, neutrality test was performed for 18 sample of Salacia which gave observed value of 0. 606285. AMOVA,

which helps inpartitioning of the overall variations among groups and among populationswithin the group were performed for RAPD, ISSR and ITS data matrices. From RAPD, 39% of molecular variance was found among population while, within the populationthis value was found to be 61% indicating that there were more variationswithin the population. While in ISSR, 55% molecular variance was found amongpopulation and 45%within the population.

For ITS sequence analysis 80%variances was among the population and 20% variance was within population whichwas similar to coefficient of evolutionary differentiation. (Table5). Nei genetic pairwisedistance of Salacia species was foundto be > 0. 5 for RAPD, ISSR and ITS sequence. But in ITS sequence analysis, the pair-wise distance between the S.

oblongaand S. macrosperm was 0. 061suggesting that they are very closely related. In addition, the pair-wisedistance and identity of S. oblongaand S. fruticosa was 0. 915 and 0. 088indicating that they are highly dissimilar.

(Table 6). Statistical comparative analysis Mantel test was employed todetermine the coefficient of correlation between the genetic distance matricesgenerated by RAPD and ISSR markers. The coefficient of correlation between RAPDand ISSR marker was R2= 0. 3781, r= 0. 614 which is high. This value signifiesthat there was considerable correlation between RAPD and ISSR genetic distancesmatrices. Twenty-one samples grouped into two clusters in RAPD dendrogramwhereas in ISSR dendrogram four cluster were observed.

Comparing RAPDdendrogram with ISSR dendrograms we can notice that S. oblonga was an Operational Taxonomic Units (OTU). In allanalysis, results of cluster analysis were comparable to PCoA. Mantel test was also employed toanalyze the ‘ goodness of fit’ for each marker system. This was done bycomparing cophenetic similarity matrices of genetic distance with copheneticsimilarity matrices with the Nei’s Genetic Distance for each marker technique. It revealed values higher than 0. 80 for all the markers used RAPD (r = 0.

827, P = 0. 01), ISSR (r = 0. 816, P = 0. 01) thus confirming their authenticity andvery good fit of PCA clustering. Discussion DNA markers have been used toevaluate genetic diversity in various plant species. In general, RAPD isincreasingly being employed in genetic research owing to its speedy process andsimplicity. On the other hand, ISSR marker has high potential to reveal polymorphismat intra- and intergenomic level to determine diversity than compared RAPDs(Zietkiewicz et al.

, 1994). In current study, we have comparedthe applicability of ISSRs and RAPDs as genetic markers to characterize the Salacia species. The only reports ongenetic diversity on genus Salacia wascarried out by Priya et al. (2016) who used RAPD molecular markers to asses diversity ofsamples collected from Wayanad region in Kerala. In the present study, anattempt has been made to examine the level of genetic variation within Salacia species sampled in the WesternGhats of Karnataka. From numbers and values obtainedin the current study it was quite that obvious RAPD is a better marker than ISSRin evaluating diversity of Salaciaspecies.

However, on careful observation it can be observed that RAPD marker wasnot able to differentiate S. oblongasamples and it was grouped within S. fruticosa samples. This could be attributed to the fact that the putativelysimilar bands originating from RAPD analysis in different individuals may notnecessarily have to be homologous, although they may be of same size in basepairs which in-turn results the erroneous calculation of genetic relationships (Fernandez et al. 2002). This also explains the fact that Nei’s geneticdistance and identity between S. oblonga andS.

fruticosa were considerably highwhich was contrary to observation seen in dendrogram and PCA. Resolving powerof ISSR was marginally higher than RAPD. Also, the differences in clusteringpattern in RAPD and ISSR markers may also be attributed to differences inoverall number of loci and their coverage of the overall genome, which wouldaffect reliable estimates of genetic relationships among samples (Loarce et al. 1996). In both RAPD and ISSR analysis S. macrosperma had high polymorphismwithin the population which was apparent as the samples were collected frommany different locations. However, in case of S.

chinensis, S. fruticosa, and S. oblonga populations the samples were collected from one location. Despitethe samples within the population originating from one location, a considerablehigh rate of polymorphism was observed which was in correlation with theobservations made earlier by Priya et al.

(2016). Similarly, diversity evaluation of Memecylon species collected from westernGhats of Karnataka by Ramasetty et al. (2016) using RAPD, ISSR and

barcoding genes found high levelof polymorphism in RAPD (65. 4%) and ISSRanalysis (68. 5%). RAPD and ISSR markers were also able to effectively detectlow polymorphism variation in Garciniaxanthochymus species sampled across various states of western Ghats(Anerao et al. 2017) which suggest that RAPD and ISSR are efficientmarkers in for diversity analysis.

In the study by Dev et al. (2015) ITS2 region showed highest interspecific divergenceand 100% efficiency for species identification by nearest distance method whencompared to rbcL, matK and trnH-psbA barcoding regions. The authors alsoobserved reciprocal monophyly among S. fruticosa, S. chinensis, S. agasthiamalana and S. macrosperma in the phylogenetic tree generated from the combined dataset, whichwas also observed in our current results.

The high divergence of S. fruticosa sample SF5 can beattributed to amplification of ITS pseudogene as it is identified by its highrate of substitution especially in the ITS2 region. Furthermore, this fact was validatedby AMOVA, since as compared to RAPD and ISSR, ITS had highest percent ofvariance (80%) in detecting interspecific or among the species divergence whereasthe RAPD had the lowest (39%). From the AMOVA it can be seen that there wasconsiderable variation within and among Salaciaspecies. The variation within the species may due to presence of infragenericvariation in Salacia species. Evidence can be seen from discovery of variety kakkayamana in S. oblonga (Udayan et al.

2014). Also, the high variation among groups was due to thecomponent of genetic variance, as new species S. agasthiamalana (Udayan et al. 2012) S. vellaniana (Udayan et al. 2013) were discovered in western Ghats of Kerala.

From the study of Dev et al. (2015) of Salaciaspecies sampled from Kerala, S. oblongaand variety kakkayamana showed 100%homology, while S. fruticosa, S. vellaniana, S.

chinensis, S. malabarica, S. agasthiamalana samples formed monophyletic group and S. macrosperma and S.

beddomei were closelyrelated sister species as per the phylogram. The results of Tajima’s Dneutrality tests were negative for all the Salaciaspecies population suggesting excess of rare alleles within the population, which may suggest population expansion. However, when the sample SF5 wasremoved and all the individual samples were analyzed across species samples, there exist an equilibrium. Conclusion Comparative study of RAPD, ISSRand ITS for Salacia species has givenan insight into the efficiency of each technique in detecting diversity withinand among the population sampled in the western Ghats of Karnataka.