5. DISCUSSION

5.1. Taxonomy and Ecology

Perusal of taxonomic literature on *Bacopa monnieri* is found to corroborates the observation of Philcox (1979) that there is much confusion regarding the nomenclature of the species. It is noted that about 40 synonyms were used to denote the species and incorrect names are still appearing in scientific literature. The species was first described as ‘Moneria’ by Patrick Browne in March 1756, before the publication of the species as *Lysimachia monnieria* by Linneaus in June, 1756. Although Philcox (1979) in his study ‘Clarification of the name *Bacopa monnieri* (Scrophulariaceae)’ concluded that *B. monnieri* (L.) Pennell is the correct name of the species, later studies pointed out that as regards the priority of publication, Wettstein’s naming should get preference and the correct citation of the species is the following.


Another important aspect to be noted with regard to the taxonomy of *B. monnieri* is its relatively recent shift to the family Plantaginaceae from Scrophulariaceae. The basis of this shift is grounded in molecular phylogenetic studies. According to this school of thought, the conventional Scrophulariaceae is a heterogeneous mix of species on phylogenetic point of view and shifted this species to its more appropriate family, the Plantaginaceae and thoroughly reorganized both the

According to recent estimates, the genus *Bacopa* includes 68 species worldwide, mostly from the Americas (The Plant List 2013). Three species were reported from India including Kerala (Hooker 1872; Drury 1982; Gamble 1923; Sasidharan 2004; Nayar et al. 2006; Nayar et al. 2014) viz. *Bacopa monnieri* (L.) Wettst., *B. hamiltoniana* (Benth.) Wettst. and *B. floribunda* (R. Br.) Wettst. No new taxa or new reports of the extant species under the genus *Bacopa* has been known in the recent past from the region. *B. caroliniana* (Walter) B. L. Rob., an aquatic plant with fragrant leaves and stem is a recently introduced species in India, considering its ornamental value. Lack of reports of new species from the vast ecologically diverse Indian region could be due to absence of evolutionary divergence of the species or due to lack of thorough taxonomic investigation on the genus *Bacopa* Aublet.

*B. monnieri* is pantropical in distribution and prefers aquatic or marshy habitats (Lansdown et al. 2013). As part of the present study 71 populations (Appendix I), from its distributional range in the State of Kerala were located and found that it grows in shallow waters and mostly in marshy ecosystems. The habitat of most of the accessions is marshy and that of certain others aquatic. It is interesting to note that some of the accessions prefer brackish water ecosystems too. The accessions which were collected from the saline conditions were difficult to nurture in the Field Gene Bank at JNTBGRI. The accessions studied also include representatives from varying altitudinal
ranges, near sea level (Karumadi, Alappuzha) to 1462 m (Munnar, Idukki). In this context, it may be noted that the maximum elevation of the place of occurrence of the species reported earlier is 1500 m (Lansdown et al. 2013). During the field surveys it was observed that in Kerala, the habitat of the species – wetlands – is under great threat due to anthropogenic factors, leading to their destruction and consequent extinction of the genotypes. It is also noted that this species is a poor competitor and its growth is suppressed by grassy weeds. Inflow of saline water to aquatic fresh water habitat of the species also leads to destruction of the populations. High demand of the species and consequent over-exploitation also leads to shrinkage of the germplasm.

5.2. Morphology

The study encompasses detailed analysis of morphological features of the 60 accessions of the species which enabled their characterization, identification and documentation of its genotypes. *B. monnieri* is well known for its medicinal importance as brain tonic especially in the treatment of mental disorders. Its therapeutic utility is primarily due to bacoside A, bacoside B and bacopaside I. High demand of the species for production of pharmaceuticals of traditional systems of medicine, herbal medicines and extraction of bioactive principles, cause over exploitation of the species from the wild, leading to depletion of its populations, endangerment of the genotypes and consequent gene erosion. This warrants development of strategies for conservation of the existing genetic resource of the species. In order to circumvent imminent gene erosion, comprehensive knowledge of genotypes is a dire necessity, especially from the
point of view of crop improvement, cultivation, conservation and utilization. But no serious studies aimed at identification of genotypes and assessment of intraspecific variability of the species have been underway so far.

There is difficulty in proper identification of the genotypes of the species, largely due to reticulation of multiplicity of characters and character states. For mitigating this problem, a detailed genotype wise characterization with respect to the exomorphic characters has been attempted, facilitating easy identification of its genotypes. The study on morphological variation accomplished here involves 15 qualitative and 25 quantitative characters. The morphological data on vast number of genotypes from diverse regions in Kerala can yield valuable information on variation of the traits of the species, its range, magnitude and pattern, and in addition its intraspecific classification.

5.2.1. Variability in the qualitative and quantitative characters of the accessions

Qualitative characters

The accessions were subjected to analysis of variation of 15 qualitative characters (List I), of which the characters such as, disposition of primary branches, stem colour, node-centric anthocyanin pigmentation, leaf colour, leaf shape, flower colour, corolla throat colour, corolla base colour, stigma colour, style colour, ovary colour, capsule shape and seed colour displayed considerable variation. The characters such as corolla type and fruit shape showed less variation. The disposition of primary
branches varied from erect, sub-erect to prostrate, of which sub-erect is the most common among the accessions (Table 4.). Stem colour shades noticed are green, pale green and yellowish green, of which pale green predominating. Based on the magnitude of intensity of node-centric anthocyanin pigmentation, the accessions can be clustered into four classes such as those with no anthocyanin pigmentation, low, medium or high. About fifty three percent of the accessions lacked anthocyanin pigmentation. Based on the leaf shape, the accessions can be broadly grouped into three, of which 45 % of the accessions fall under the oblong leaf shape class, followed by obovate (31.67 %). The oblanceolate type was least frequent (23.33 %). Shape of leaf tip varies from round to obtuse to retuse, of which rounded (81.67 %) is the most common.

There are two types of corolla lobbing, unequal and equal and the latter observed only in 10 accessions. Flower colour falls under three character states such as violet, pale violet and white, pale violet (61.67 %) being the most common. Corolla throat colour occurs in four states such as dark purple, purple, light purple and white. Colour of the corolla base also varied noticeably (yellow, yellowish green, light yellowish green and white), the light yellowish green being the common type (65 %). Colour of the stigma showed very low variation with only two character states, dark green to light green and majority of the accessions fall under dark green, whereas colour of the style showed high variation with four character states such as green, light green, pale yellowish green and white, the pale yellowish green being the commoner.
Colour of ovary varies from green, light green to yellowish green and the common one is light green. There are only two capsule shape types, obovate and oblong, of which the oblong predominated. Seed colour varied from dark brown, brown, to light brown with brown more common. However, shape of the seeds is apparently pliable.

**Quantitative characters**

Twenty five quantitative morphological characters were considered for the morphological analysis (List I). All the characters, barring long stamen length, showed remarkable variation and among the lot the No. of dentations/leaf, leaf thickness and No. of seeds/capsule showed highest variation followed by leaf area, internode length, pedicel length and bracteole length (Table 7). Characters showing low variation are leaf thickness, short stamen length and style length.

Internode length in the accessions showed a range of 0.84 – 2.76 cm and stem thickness from 0.10 – 0.25 cm. With regard to various leaf parameters, the accessions showed conspicuous variation, leaf length 1.50 – 2.80 cm, leaf width 0.50 – 1.1 cm, leaf area 48 – 159 mm², leaf thickness 0.02 – 0.06 mm and leaf dentation 0 – 6 nos. The variation observed in the flower characters is also notable. The pedicel length showed a range of 1.10 – 3.80 cm, flower diameter 0.90 – 2.00 cm, bracteole length 0.10 – 0.45 cm, first sepal length 0.40 – 0.75 cm, first sepal width 0.25 – 0.45 cm, second sepal length 0.40 – 0.75 cm, third sepal length 0.40 – 0.70 cm, fourth sepal length 0.30 – 0.60 cm, corolla length 0.85 – 1.8 cm, corolla width 1.0 – 2.15 cm, short
stamen length 0.15 – 0.25cm, style length 0.40 – 0.60 cm and ovary length 0.10 – 0.20 cm. There is no variation for long stamen length among the accessions.

Among the variation registered for fruit characters the maximum was for the No. of seeds/capsule (09 – 198 nos.), the others are fruit length 0.23 – 0.52 cm, fruit breadth 0.11 – 0.30 cm. Biomass yield of the accessions varied from 6.2 to 11.98 % and 15 accessions showed a comparatively greater biomass yield of more than 9 %.

5.2.2. Estimation of genetic parameters in the 60 accessions

5.2.2.1. Genotypic and phenotypic coefficient of variation

Genotypic and phenotypic coefficient of variation (GCV and PCV), heritability, genetic gain, genotypic and phenotypic correlation coefficients with regard to eight agrobotanic characters of *B. monnieri* were estimated based on the data pertaining to the 60 accessions (Table 8.). The GCV and PCV values of the characters are suggestive of the magnitude of variation as well as influence of environment on the variation of the traits. The GCV of various traits provides a measure of the extent of genetic variability residing in the genotype (Kavitha et al. 2007). The PCV values are consistently higher for all the characters than their GCV values, which is suggestive of considerable influence of environment on the expression of the characters. The values of GCV is maximum for bacoside A % and minimum for stem thickness, while for PCV the value is maximum for leaf thickness and minimum for biomass yield. The PCV value of leaf thickness (51.52) is remarkably higher than its GCV value of 16.02
which implies high influence of environment in determining leaf thickness, whereas
difference between GCV and PCV is marginal in the case of percentage content of
bacoside A, bacopaside I and total bacosides, suggesting little influence of environment
on the expression of these characters. The relatively narrow difference between values
of GCV and PCV appears to be reflected in the high heritability profiles noticed for
these characters as against leaf thickness. Comparable findings were on record on a few
other important crop species such as Soya bean (Johnson et al. 1955), Rice (Chauhan
and Tandon 1984), Piper nigrum (Mathew et al. 1999), Mucuna pruriens (Haridas
2014) etc.

5.2.2.2. Heritability and Genetic gain

Success of breeding programmes depends on heritability and genetic gain of the
characters concerned, in addition to their variability of the traits in the gene pool of the
species. Heritability \( (h^2) \) in broad sense is an approximate measure of sets of genes
exhibiting dominance. It is an important parameter useful for judging the extent of
expression of a character. The estimates of heritability act as predictive instrument in
expressing the reliability of phenotypic value. Heritability is a good index of
transmission of characters from parents to its progeny. The estimates of heritability
help the plant breeder in selection of elite genotypes from diverse genetic populations.
Therefore, high heritability helps in effective selection of a given character. Heritability
with respect to eight agrobotanic characters is estimated in the present study.
Heritability showed wide variation ranging from 0.0968 (leaf thickness) to 0.9743 (bacoside A %). Relatively high heritability (>80 %) is recorded for characters such as internode length, bacoside A %, bacopaside I % and total bacosides %. Reddy et al. (2013) classified heritability < 30 % as low, 30 – 60 % medium and > 60 % high. In such a consideration, in the present study five characters showed high heritability, two characters showed medium and one low. The narrow difference between values of GCV and PCV appears to correspond with high heritability profiles noticed for those characters.

The genetic gain values ranged from 10.27 (leaf thickness) to 61.12 (bacoside A %) for the eight agrobotanic characters. High values are shown by characters such as percentage content of bacoside A, bacopaside I and total bacosides. High genetic advance coupled with high heritability estimates offers the most suitable condition for selection. Estimates of heritability and genetic advance are more reliable and meaningful than considering individual parameters (Nwangburuka and Denton, 2012). The present study reveals high heritability coupled with high genetic advance for the characters such as Percentage content of bacoside A (h² = 0.9743, GG = 61.1228), total bacoside (h² = 0.9737, GG = 53.6754) and bacopaside I % (h² = 0.9486, GG = 56.8909) are positive factors for crop improvement.
5.2.2.3. Correlation

The data of genotypic and phenotypic correlation coefficients of eight agrobotanic character pairs showed higher genotypic correlation coefficient values for all the character pairs. This is suggestive of less influence of environmental factors in inhibiting strong inherent relationship between the characters. Comparable findings were reported on a few other important crop species like *Piper nigrum* (Mathew et al. 1999), Soyabean (Johnson et al. 1955), Rice (Chauhan and Tandon 1984), *Mucuna pruriens* (Haridas et al. 2013) etc. Six character pairs such as internode length and stem thickness, leaf area and stem thickness, leaf thickness and stem thickness, percentage content of bacoside A and bacopaside I, bacoside A and total bacosides, and bacopaside I and total bacosides showed highly significant genotypic correlation (at 1 % level). In one character pair (percentage content of bacoside A and total bacosides) phenotypic correlation coefficient value is high. However, one character pair (internode length and biomass yield) exhibited significant negative genotypic correlation. Reddy et al. (2010) reported positive correlation of bacoside content with many morphological characters such as shoot length, leaf length etc. but no such correlation has been observed in the present instance.

5.2.3. Selection Index

Selection index is a measure used for identifying superior genotypes. High selection indices can be considered as one of the criteria for selection of parent
genotypes for crop improvement programmes. Incidentally, estimation of selection indices of *B. monnieri* was attempted for the first time here. The selection indices (Table 10) of the accessions ranged from 42.04 (Bm 1) to 68.55 (Bm 3). High selection indices are exhibited by six accessions (Bm 32, Bm 28, Bm 21, Bm 42, Bm 47, Bm 3) which may be considered as candidates for parentage of hybridization programmes.

5.2.4. **D^2 Analysis**

D^2 analysis is employed for grouping genotypes based on their genetic interrelationship. The study is also useful for assessing genetic diversity in the genepool of a crop. It has been pointed out that for developing better varieties, it is desirable to classify, the germplasm on the basis of genetic diversity and to make crosses between genotypes of divergent groups which share maximum diversity (Narasimhayya and Rao 1974). The maximum amount of heterosis of recombination can be expected from crosses involving parents belonging to most divergent clusters. In the present study, the sixty genotypes of *B. monnieri* were grouped into 12 clusters (Table 11). Cluster III emerged as the largest with 19 accessions followed by Cluster II (17 nos.), Cluster I (7 nos.), Cluster IV (5 nos.), Cluster V (3 nos.), Cluster VI and VII (2 each) and Cluster VIII, IX, X, XI and XII are single member clusters. Clustering of the genotypes reveals the notable genetic divergence of *B. monnieri* in the gene pool. A similar observation in the species has been made by Mathur et al. (2003) in their study on 27 accessions collected from ecologically divergent locations in India. Their
study grouped the accessions into seven clusters based on principal component and canonical variable analyses.

In the present study the Clusters IV and X displayed highest intercluster distance (3378.6) and the lowest (180) between the Clusters IV and IX. The intercluster distance is a measure of genetic divergence. Cluster VII registered maximum intracluster distance (154.11) and the minimum by Cluster VI (58.5). The divergence between the clusters is reflected in their cluster Means (Table 13). The most divergent clusters Cluster IV and X differ significantly with respect to Mean values of seven agrobotanic traits considered for the study. The clusters having high intercluster distance and low intracluster divergence is suggestive of good grouping of genotypes of the species. Clusters IV and X having high intercluster distance possess divergent genotypes and putative parents may be selected from these two clusters for hybridization programmes in the crop to exploit hybrid vigour.

In a similar study, Mathur et al (2003) collected 27 accessions of *B. monnieri* from semi-temperate, subtropical and tropical environments at geographically distinct locations in India and examined their genetic variability in a semi-temperate environment based on 13 qualitative and 24 quantitative characters, including bacoside A content of herbage. They grouped the accessions into 7 clusters comprising 2 to 6 accessions in them where clusters IV and V and cluster VII as the most divergent. No reports are available on the clustering of genotypes of *B. monnieri* accessions occurring in Kerala.
5.2.5. Hierarchial cluster analysis

It is well known that most plant species are endowed with an enormous amount of intraspecific variation. Biosystematic studies were employed for intraspecific classification of many crop species (Baun 1981; Fursa 1981; Fursa and Filov 1982; Hanelt et al. 1993). Most of the intraspecific classifications are based on easily recognizable characters of gross morphology, and variation in such characters used to establish taxonomically formal and informal classifications (Hanelt and Hammer 1995). A variety of methods have been followed for classifying different genotypes of crop species as in Cassava (Rogers and Flemming 1973), Yams (Martin and Rhodes 1977), Taro (Sreekumari 1992), Velvet bean (Haridas 2014) etc.

The present morphological characterization of 60 accessions of *B. monnieri* revealed that considerable variation exist among them with respect to a number of qualitative and quantitative characters. The Ward’s minimum variance cluster analysis was attempted here for sorting out the accessions based on their similarity between each other in regard to the characters. The results (Table 15) and dendrogram (Fig4.3) classified the entire assemblage of accessions into ten clusters at the similarity level of 33.8 %. Among the ten, the Cluster X includes 12 accessions and the Cluster IV is a single member cluster.

Intraspecific classification of crop species in which cultivated forms display reticulate relationship poses a problem (Rogers and Flemming 1973). This is also true
with regard to *B. monnieri* in which the 60 genotypes exhibit profound degree of reticulate morphological variation. The heirarchial cluster analysis performed here assumes importance, as it yields clues for selecting genotypes having divergent characters for the purpose of hybridization programmes to exploit hybrid vigour. The composition of the cluster formed by $D^2$ analysis and heirarchial cluster analysis has not brought out any recognizable similarity. This may be possibly due to the apparent difference in the type of the characters involved and also to the nature of the analyses. Moreover, the difference in the composition of clusters formed by $D^2$ and heirarchial cluster analyses implies substantial influence of environment on the expression of the characters and hence on their phenotype, further confirmed by estimates of GCV and PCV of the eight agrobotanic characters, which showed consistent predominance of the latter over the former.

### 5.3. Anatomy

Metcalfe and Chalk (1979) illustrated the aspects of plant anatomy, which revealed the taxonomic significance of anatomical characters in plant classification at various levels in Dicotyledons. The anatomical study on *B. monnieri* undertaken here is confined to stem and leaf. The leaf anatomical study involved epidermis with emphasis on stomatal types, their distribution, stomatal index and other structural details of stomata, leaf venation and trichomes.
5.3.1. Stem

Stem anatomy of *B. monnieri* was studied by Warrier et al (2001), Gupta et al. (2004) and Tandon and Sharma (2010). Warrier et al (2001) and Gupta et al (2004) have described the stem as largely circular in outline. The present study noted considerable variation in stem outline in the 60 accessions from circular, circular-dumboidal to dumboidal, of which dumboidal and circular-dumboidal showed highest frequencies in the accessions. The circular stem type is only in 4 showing lowest frequency among the accessions (Table 16).

According to Warrier et al (2001) and Gupta et al (2004) stem comprise uniseriate epidermis of tubular cells, well developed parenchymatous cortex of about 20 – 22 layers with large intercellular spaces, single layered endodermis with starch grains, pericycle, collaterally arranged xylem and phloem with indistinct cambium and parenchymatous pith with intercellular spaces. Tandon and Sharma (2010) also reported similar structural details, but noted that the stem possess outer thick walled epidermis covered with thin cuticle, and the central region with narrow parenchymatous pith. The present study showed that cortical layers varying from 14 – 23 cell layers in broader diameter and 9 – 18 across the shortest. The diameter of the broad area varied from 2300 – 3660 µm and the short 1960 – 3400 µm in different accessions. The stelar diameter ranged from 650 – 1100 µm and phloem thickness 30 – 120 µm. The No. of xylem strands ranged from 25 – 52 and the No, of xylem vessels/strand varied in the accessions ranging from 1 – 3 to 3 – 6. The cambium
element was narrow and indistinct, which apparently may be the reason for limited secondary thickening of the stem. In all the accessions studied, the pith was large and parenchymatous, with intercellular space.

5.3.2. Leaf

The water balance of the plant is mainly controlled by leaf architecture and the turgour controlling pores (stomata), which control the rate at which water vapour is lost by transpiration. Therefore, attempts to understand the role of leaf architecture and the role played by leaf veins in water distribution as well as by the stomata assume importance. A perusal of the literature reveals that there has been only little attempt having paid previously to the study of the major functional attributes of leaves of *B. monnieri* barring, certain isolated and sporadic studies on stomata (Kannabiran and Ramassamy 1986; Tandon and Sharma 2010; Warrier et al. 2001; Gupta et al. 2004; Sultana 2012 and Aparna et al. 2015). Earlier studies have reported only four stomatal types (tetra-, aniso-, dia- and anomocytic) as occurring in the species (Kannabiran and Ramassamy 1986), whereas the present study revealed five types (diacytic, anisocytic, anisotricytic, tetracytic and anomocytic). All the types present in almost all the accessions but, in different frequencies. In 34 accessions the predominant stomatal type is anisocytic, in 16 accessions anisotricytic, in 3 frequency of anisocytic and tetracytic is same while in one accession, anisotricytic and tetracytic evenly distributed (Table- 18).
5.3.2.1. **Distribution of stomata**

In leaves of most herbaceous plants stomata are found in both the upper and lower surfaces but usually fewer stomata on the upper surface. Such leaves are termed as amphistomatic. The adaptive significance of amphistomaty is unclear, although hypostomaty is considered to be a phylogenetically primitive character and, as plants adapted to growth in more open sunlit habitats amphistomaty may have evolved almost simultaneously (Mott et al. 1982). It has been hypothesized that amphistomaty is responsible for maximum photosynthetic rates usually seen in plants growing in sunny habitats and possessing thick leaved species (Mott et al. 1982). This appears to be so in all the accessions of *B. monnieri*.

A possible relation between chromosome number and stomatal frequency has been suggested from evidences in *Zea mays* in which the tetraploids have fewer but larger stomata than the diploids. In coffee plants, the No. of stomata decreases as the No. of chromosome increases (Mishra et al. 2011). This aspect has been looked into in the present accessions, which differed in chromosome constitution. It was observed that there was an apparent association between chromosome number and stomatal frequency and stomatal size such that in hyperploid taxa there was an apparent increase in size of stomata and decrease in their number (Bm 11, Bm 19, Bm 37, Bm 41, Bm 50, Bm 54 and Bm 55)
It is reported that stomatal frequency or density can vary significantly within leaves, plants or individuals of single species within a community and can be modified by environmental factors, leaf morphology and genetic composition (Schoch and Silvy 1978). In this context, it may be noted that the anatomical studies were carried out on plant materials of the accessions collected from the plants maintained in uniform environmental conditions in the EP. The EP grown plants of the accessions were raised from third generation vegetatively propagated plants maintained in the FGB. These measures were taken for nullifying the effect of environment of diverse locations from where the accessions were collected originally. So, it can be inferred that remarkable variations exhibited by the accessions on the frequency of the five different stomatal types is due to variation between the genotypes of the species. Stomatal frequencies often vary according to the cell size and smaller guard cells are usually associated with higher stomatal frequencies. The spatial distribution of stomata noticed in the lower epidermis varied from 7.6 – 30.6/300 mm² in the 60 accessions of the species.

The stomatal index was considered to be fairly constant within the leaves of a single species. Even though the stomatal number is determined by many extrinsic and intrinsic factors, the stomatal indices are quite constant and can be used in distinguishing different taxa (Poole et al. 1996). This matter was examined in the present material and found that the stomatal indices among the accessions displayed striking variation ranging from 7.90 – 29.59 in the accessions and it is possible that this attribute may be genetically predisposed in the genotypes.
5.3.2.2. Trichomes

A spectrum of trichome types exist in plants recognized under two basic types, the simple and branched. The simple type is divided into (a) glandular (b) eglandular, the former showing unicellular ellipsoidal heads or multicellular globose heads, either sessile or stalked. The branched types occur under 4 subtypes which are mostly of the multiangular stellate type, stalked or sessile. Many angiosperm leaves are devoid of epidermal hairs and in some cases, the two major types coexist. Species specificity is very evident in many cases as noticed by Anjana et al. (2005) in the genus *Solanum* in which they have observed the trichome types of 3 categories such as simple glandular, simple eglandular and branched.

Very little information is available on the epidermal hairs of *B. monnieri* barring a couple of sporadic reports (Metcalfe and Chalk 1950, Tandon and Sharma 2010). Although, the information accrued from the present study consist only one species, the data from the 60 accessions originally belonging to diverse environmental conditions in Kerala is significant. The study envisaged collection of data concerning many attributes of the trichomes present in the species, which was sessile glandular with multicellular heads. The data scored are No. of trichomes per unit area, trichome index and Mean trichome diameter (Table 19). Number of trichomes per unit area varied from 0.4 – 2.4 and the trichome index 0.47 – 2.37 and the Mean trichome diameter 21.24 – 32.78 µm in the accessions
Glandular trichomes have received considerable attention in respect of their capacity to synthesize store and secrete secondary metabolites that help to protect plants against insect predation and other biotic challenges (Wagner 1991; Ranger and Hower 2001; Wagner et al. 2004). But, there was no apparent correlation observed between tricome index and the percentage content of bioactive compounds in the accessions of *B. monnieri*. This may be due to the reason that the bioactive compounds of interest here is not associated with glandular trichomes in the species.

5.3.2.3. Leaf venation

As regards leaf venation, the leaves are enormously diverse and in all groups of plants it display enormous degree of variation both within and between species. Over the years, there has been an increasing degree of awareness and recognition about the importance of leaf venation. Sack and Scofforni (2013) who studied the leaf venation and structure and function in relation to evolution and ecology have reported a wide range of aspects of leaf venation occurring in angiosperms, and reported 10 major structural features that contribute to multiple key functions.

The venation pattern in *B. monnieri* is characterized by three first order veins. The middle first order vein proceeds up to the leaf apex and branches at intervals. The No. of branches of the middle first order vein was found to vary considerably in the accessions (3 – 7). Highest No. of branches of middle first order vein (seven numbers) is present in two accessions (Bm 8 and Bm 54), six in 14 accessions and the lowest
three in seven accessions. Moreover, the pattern of formation of the second order veins from the middle first order vein showed variation in the accessions, in some accessions opposite and in certain others alternate. The accessions also showed variations regarding the nature of the other two first-order veins starting from the leaf base. In some accessions the two other first order veins proceed laterally almost up to the apex, often anastomosing with the second order veins of the middle one and in some others the two first order veins stop half way through and does not proceed up to the leaf apex.

5.4. Cytology

The chromosome study carried out in 30 accessions of *B. monnieri* showed n=32 and 2n=64 in 23 accessions and n=34 and 2n=68 in seven (Table 20). The chromosome numbers reported earlier in the species are n=32 (Raghavan 1959; Lewis et al. 1962; Vasudevan 1975; Barret and Strother 1978; Chandran and Bhavanandan 1981; Darokar et al. 2001; Samaddar et al. 2012) and n=34 (Chennaveeraiah 1953; Carr, 1978). Meiotic behavior in all the taxa studied was normal, both in the n=32 and n=34 accessions. Regular bivalent formation and normal anaphase I separation were noticed in all of them, leading to appreciable pollen fertility. The chromosome data of the present accessions indicate that the species occurs in this region in two cytotypic forms, one with n= 32 and 2n=64, and the other with n= 34 and 2n= 68. The present data together with the earlier reports indicate that the species exists in the Indian
subcontinent in two cytotypic forms such as a tetraploid with n=32 and 2n= 64 and an aneuploid at tetraploid level with n=34 and 2n=68.

Reliable information about the chromosome constitution in a group such as chromosome number, meiotic behavior and chromosome morphology (karyomorphology) are important in dealing with chromosome evolution at different levels. Changes in chromosome number and their morphology are fundamental steps, which sometimes are crucial and critical in elucidating evolutionary processes (Jones 1970; Stebbins 1971). Moreover, the chromosome data is a dependable parameter which forms a powerful tool in the resolution of taxonomic problems and in tracing evolutionary changes in plants at various taxonomic levels (Smith 1970). Within groups of plants, both large and small, the trends of chromosome evolution are unambiguous and critical in settling phylogenetic problems. There are unequivocal evidences in cytogenetic literature of different plant groups such as angiosperms, gymnosperms and pteridophytes in which chromosome data having had played decisive role in dealing with such problems. For proper evaluation of the nature of chromosome evolution, it is vital to make use of valid data of different cytological parameters such as the basic chromosome constitution, the patterns, modes and magnitude of factors which affect numerical (polyploidy, aneuploidy) and structural changes of chromosomes at different taxonomic levels.
5.4.1. Basic chromosome number

The parameter of basic chromosome number at the level of species, genus, tribes and families has played crucial role in shaping the prevailing concept of chromosome evolution. This has formed one of the profusely used parameters in formulating phylogenetic speculations, and has also served as a reliable and stable marker of the direction of evolution. Diminution of basic chromosome number, rather than its increase has played significant role in the process of chromosome evolution both at intra- and interspecific and generic levels (Stebbins 1971). Results of cytological studies carried out in the Kerala University, Department of Botany over the past decades have yielded convincing evidence in support of this concept (Philip and Mathew 1988; Mathew and Mathew 1988; Vijayavalli and Mathew 1990; Mathew and Mathew 1999). While agreeing to this in principle, Jones (1979) however, has contended that increase of basic chromosome number also has been prevalent in many plant groups.

Polyploidy, aneuploidy and secondary hybrid polyploidy have played major role in the evolution of basic chromosome constitutions of higher magnitude in many angiosperm families and pteridophytes. The phenomena like polyploid ‘drop’, mostly (Darlington 1956) and polyploid ‘lift’, occasionally (Jones 1970), have been causative for the incidence of unrelated basic numbers in natural groups by dysploidy. These could have been the probable route of origin of high denomination basic numbers in some angiosperms like Magnoliaceae (Mathew et al. 1998) and also in the
pteridophytic genera as *Ophioglossum* (Abraham and Ninan 1954; Ninan 1956), where the high level polyploids are referred to as ‘paleopolyploids’

The chromosome data available in *B. monnieri* show that the numbers such as n=16 and 32 are based on x=16, and n=34 based on x=17, of which the latter could be an ascending aneuploid derivation of x=16. The overall chromosome data in the genus *Bacopa*, the most common basic numbers are x= 10, 12, 16 and 17 (Goldblatt 1984; Cinthya et al. 2014). Of the various basic numbers of the genus *Bacopa*, x=16 and 17 comes under the secondary basic chromosome number category. There is an authoritative contention (Stebbins 1971 and Grant 1982) that basic numbers of order 9 and 10 or above could be secondary or tertiary ones having arisen from lower ancestral ones. This would imply that all the basic chromosome numbers reported in *Bacopa* should be a secondarily originated from a possible x=8 primary one. On such a consideration, the x=17 in the genus would be an ascending aneuploid derivation from x=16. The meiotic behavior in all the present n=32 and n=34 cytotypes was normal with regular bivalent formation and normal anaphase I separation, leading to appreciable pollen fertility. This is suggestive that the n=32 cytotype could be a tetraploid, and the n=34 one an aneuploid derivative from n=32. Meiotic behavior characterized by regular bivalent formation and normal anaphase separation, resulting in high pollen fertility in the 2n=64 accessions of *B. monnieri* is suggestive that the taxa should be allotetraploids, because the factors which distinguish between allopolyploids and autopolyploids are the frequency with which chromosomes
associates into regular bivalents and subsequent anaphase I separation, leading to appreciable pollen fertility, characteristic of allopolyploids as against formation of multivalents in meiosis and irregular anaphase I separation, leading to sharp fall in pollen fertility in autopolyploids (Stebbins 1950). The allotetraploid cytotype of *B. monnieri* should be those arisen from natural crosses of n=8 ones leading to genome mixing, followed by genome duplication resulting in 2n=64 allotetraploid cytotype. In this context, of the existing natural allotetraploid form in this species, reported by Srivastava et al. (2002) assumes importance. They have synthesized an artificial tetraploid of the species from the 2n=16 diploid progenitor. The derived colchiplloid was adjudged as an autotetraploid with 2n=64. Srivastava et al. (2002) observed multivalent association at meiosis in the autotetraploid in varying numbers followed by near normal anaphase I separation, but yet leading to sharp fall in pollen fertility.

5.4.2. Polyploidy

The role of chromosome numerical changes through polyploidy and dysploidy are generally recognized as major factors in plant evolution. The importance of polyploidy in plant evolution and speciation has been stressed by a host of leading cytogeneticists. This is a common phenomenon among plant groups, which often considered responsible for diversity in form and function in plants, and which has been also reckoned as a major speciation mechanism. The phenomenon of polyploidy and its genetic consequence have been the focus of interest among the contemporary cytogeneticists (Soltis et al. 2009; Doyle and Egan 2010). Polyploids often exhibit a
wider range of plant morphological variations than their diploid counterparts, protecting them against their deleterious effects of mutation (Stebbins 1950), and also allows greater polymorphism and adaptability (Stebbins 1985; Wendel 2000). The present polyploid taxa of _B. monnieri_ are widely domesticated, and flourishing in a variety of environmental conditions and surpassing altitudinal differences. The n=32 and 34 accessions of _B. monnieri_ studied here constitute two discrete intraspecific variants of the species. They display a spectrum of intraspecific plant morphological diversity in regard to many vegetative and floral characters, both quantitative and qualitative, such as internodal length, leaf size and shape, flower colour etc. The extent of plant morphological variations has been generally attributed to cytological reasons, mainly chromosome numerical and structural diversity at the intraspecific level. In many cases of angiosperm families in which, more than one intraspecific polyploids occur naturally in native ranges within previously recognized taxonomic species, the chromosomal difference has been found to be associated with recognizable plant morphological difference (Vijayavalli and Mathew 1990). Besides chromosome numerical alterations, gene rearrangements through shift of centromeric position in individual chromosomes at the intrakaryotypic level by incidence, mostly of pericentric inversions, may also cause intraspecific plant morphological diversity. This has been documented in several taxa of the Liliaceae (Mathew and Thomas 1974; Vijayavalli and Mathew 1990) and Asteraceae (Mathew and Mathew 1988). The intraspecific plant morphological difference noticed in the n=32 and n=34 accessions of _B. monnieri_,
could be possibly attributed to genome mixing and doubling accomplished via allopolyplody in the taxon, followed also by aneuploidy from n=32 to n=34. It may also be possible that gene rearrangements through chromosome structural changes have additionally contributed to this, coupled with cryptic structural changes and gene mutations. It may be noted that on account of very small size of chromosomes with indistinct centromeric position, the present taxa were not amenable to detailed karyotype analysis. However, the role of intrakaryotypic structural changes cannot also be ruled out in initiating intraspecific plant morphological difference.

Changes in morphological characters are the consequences of the effect of various ecological factors on the genotype of the species. Stebbins (1971) has suggested that intraspecific variations of morphological characters are dependent upon the environmental modifications, genetic recombinations and mutations. Intraspecific morphological variations is inductive of taxonomic heterogeneity and is considered as one of the fundamental factors in the evolutionary changes (Bateman and Rundall 2006; Blinova 2012). These variations are not only helpful in establishing the connectivity of populations, but also associated with adaptability and evolutionary ability of the taxa. Such variations are more common in widespread species than in local and endemic ones (Grant 1982; Emerson 2002; Whittaker and Fernandez-Palacios 2007). Species growing in different types of habitats also show variations in morphological characters that are accounted for, by differences in ecological conditions (Mamaev et al. 2004). This is very much applicable to *B. monnieri* which flourish in a
variety of ecologically diverse regions. Morphological adaptations among plants to different kinds of climatic and environmental conditions make them more diverse, and such plasticity in the genetic and morphological characters is more prominent among the individuals of different populations than among members of the same population (Svensson et al. 2006; Spaniel et al. 2008). Morphological variation could also occur due differences in environmental conditions (Aroson et al. 1990), geographical differences (White 1971) selection and/or genetic drift (Stuessy et al. 2006). Variations of morphological characters between different accessions have been noticed in *B. monnieri*.

Secondarily associated bivalents were noticed at meiosis in varying frequencies (2 - 4) in the 2n=64 and 2n=68 tetraploid accessions of *B. monnieri*. Darlington and Moffett (1930), who first used the term, secondary association of bivalents, had considered it to be due to residual attraction between more distinctly related chromosomes. A pair of bivalents lying in close proximity, but without any material connection is referred to as secondary association of bivalents (Darlington 1965). This phenomenon is more common in polyploids as compared to natural diploids, and the incidence is considered to denote ancestral homology, and hence helpful in tracing the ancestral basic numbers (Stebbins 1950). The occurrence of such association of bivalents at meiosis in the tetraploid accessions of *B. monnieri* adds additional support for their allotetraploid nature.
The chromosomes of the polyploid accessions of *B. monnieri* studied here are characteristically very small sized. There are varying contentions and suggestions concerning the cause and association between chromosome size and ploidy in plants. There is a general tendency of genome and chromosome size being negatively correlated with polyploidy (Stebbins 1971; Mathew and Mathew 1999). Such associations have been noticed in many angiosperm families and gymnosperms. In coniferales, very large sized chromosomes is uniquely and characteristically associated with little incidence of polyploidy (Mathew et al. 2014). Similar association characterized by large sized chromosomes and little polyploidy documented in many angiosperm families like Liliaceae and Amaryllidaceae (Vijayavalli and Mathew 1990) while very small chromosome and high polyploidy as in Piperaceae (Mathew and Mathew 1999). In *B. monnieri*, the chromosomes are exceedingly small sized, and are of moderate ploidy level. This apparently agrees with the contention of Miksche and Hotta (1973) that very small chromosomes and smaller quantity of less repetitive DNA bring about more dynamic genetic and evolutionary versatility and better chances of species diversification.

5.5. **Palynology**

The pollen grain in angiosperms is the highly reduced male gametophyte, and it forms a unique entity both with regard to form and function. It represents an essential genetic bridge between and among generations (Erdtman 1952). As an organ, less influenced by changing ecological conditions, characters of pollen grains are
considered to be more dependable and stable and *ipso facto* serve as a supplementary potential tool in the studies of comparative morphology, that is much more useful in making conclusions in plant taxonomy, phylogeny and evolution than those of any other morphosystems (Saad 1972; Nair 1974). The morphological characters of pollen grains are those relating to the germinal aperture, exine ornamentation, exine strata, size and shape, of which the aperture characters is of primary importance, exine surface pattern secondary, and the other tertiary (Nair, 1965). The aperture characters are based on their form, number and position, all showing variation in plants at various heirarchial taxonomic levels so as to be of use in the identification of genera, species and intraspecific variants. The aperture number and their distribution constitute one among many trends in the evolution of pollen morphology (Walker and Doyle 1975; Chanda et al. 1979) The pattern of pollen wall sculpturing is species specific generally, and in most cases is determined by the sporophyte (Quiros 1975). The exine ornamentation serves as a supplementary factor to the apertural form in reaching taxonomic and phylogenetic conclusions (Nair 1975). The pollen wall has a unique endowment among plant morphosystems being made up of a highly resistant compound, the sporopollenin, and it embodies in it unique architectural features which are remarkably stable and genetically controlled.

During the past few decades, the pace and progress in the study of pollen morphology have been rapid, and this has resulted in the realization of a wider and varied scope of its application. More recently, the application of pollen morphology in
intraspecific taxonomy has received great attention. It has been demonstrated that a statistical evaluation of pollen variation can be utilized as an effective tool in the categorization of intraspecific forms, and also in the understanding of the hybridity status of cultivars within a taxon (Nair 1960a, b). The study of pollen morphology in conjunction with cytology, termed cytopalynology (Lewis 1965) has been shown to be rewarding in biosystematic studies, especially in as much as it yields clues to the ploidy status (Sagoo and Bir 1983; Mathew and Philip 1983; Haridas 2014). There are also reliable reports that palynological data corresponds with phyletic trends (Mathew and Philip 1983) in angiosperms. Scanning electron microscopic (SEM) observations of pollen grains has special utility in the understanding of finer details of pollen morphology with great precision, which has added advantage of assessing the degree and extent of intraspecific variations.

Screening study of palynological features of eight accessions of *B. monnieri* was carried out by LM and SEM observations. The findings emerged from the SEM study of the species are briefly discussed in relation to various palynological attributes of the species, and with reference to the intraspecific variation of some of the pollen features brought out.

5.5.1. **Pollen Aperture**

Almost all palynological discussions are based on the aperture form, their number and distribution and position designated as the NPC system, where N =
number; P = position, C = character (Erdtman, 1969). The aperture character is usually stable and uniform at family, genus and species level in most angiosperms, and such groups are called stenopalynous, and when they do differ, termed euripalynous.

In B. monnieri accessions, the aperture character was strikingly uniform. In all the accessions screened, the aperture was trizonocolporate and the ora mostly lalongate, but with a slightly modified form in a few like irregularly lalongate (Bm 41), and circular lalongate (Bm 42). Ora size was fairly uniform in all, length ranging from 1.3 -2.5 µm and width 2.33 -3µm.

5.5.2. Exine Sculpturing

The pollen wall is made up of two layers, the inner intine, which surrounds the cytoplasm, and the outer exine, which is exceedingly hard and composed of sporopollenin, and hence very resistant. The exine shows morphological features that are of high diagnostic value and this is beautifully and finely revealed by SEM. The exine generally consists of two layers, an inner homogeneous nonsculptured nexine, and an outer variously sculptured layer sexine. The pattern of pollen wall sculpturing is mostly species specific, and known to be determined by the sporophyte (Quiros 1975). The exine surface ornamentation is a significant morphological character aiding a great deal in the categorization of genera and species and even intraspecific forms (Nair and Sharma 1965). The morphoform categorization based on exine ornamentation is particularly useful in the grains of stenopalynous taxa. The exine surface often presents
various ornamentation forms. Two broad categories are (a) the depression type and (b) the excrescence (projection) type. The basic forms among the excrescence type are spinate (spinulate), baculate, clavate, gemmate, verrucate and granulate, and those of the depression types are reticulate (retipilate, faveolate, fossulate, scrobiculate), lophate and striate. The primitive angiosperms have pollen with more or less psilate (smooth) pattern. The phylogenetic trend is considered to be from the psilate to the reticulate to the spiny to the echinate (Nair 1974; Saad 1972; Walker 1976).

The exine ornamentation pattern noticed in the accessions of *B. monnieri* is predominantly and primarily reticulate, exhibiting minor modifications such as homo brochate to heteroberochate, brochus showing gemma like projections, luminae reticulate, almost circular, adjacent luminae fused together and striato-reticulate.

### 5.5.3. Pollen size and shape

Pollen size and shape do not apparently possess much phylogenetic significance. According to Walker and Walker’s (1983) contention, large boat-shaped, granular and monosulcate pollen grains are the primitive type in angiosperms. Variation in pollen size and shape are of less diagnostic value. However, this has value as an index of aneuploidy (Nissen 1950), and for correlating with chromosome number. The size of pollen may be affected by the process of acetolysis, and hence it is discerned as an unstable pollen character. In all the accessions of *B. monnieri*, the
pollen size (P/E values) was fairly uniform (Table 21 and 22) ranging from 0.88 – 1.38 µm.

The shape of the pollen grain is unfixed, and hence this character is often considered as a nonreliable parameter in pollen morphological analysis (Nair 1970). The grain shape is correlated largely to aperture type, which in turn to polarity and symmetry. Pollen shape varies greatly from species to species. There are two basic pollen shape classes such as boat-shaped and globose, both further subdivided depending on the ratio of the equatorial axis to the polar. The pollen outline in polar view may be circular, triangular or in other geometrical shapes, while in the equatorial view, the ratio between the polar and equatorial diameter multiplied by 100 gives an indication of the shape. Based on the calculation, the angiosperm pollen are grouped into nine classes such as peroblate, oblate, suboblate, oblate-spheroidal, spheroidal, prolate-spheroidal, subprolate, prolate and perprolate (Walker and Doyle 1975). The pollen shape noticed among the accessions of B. monnieri was by and large subprolate and with a few exception such as perprolate (Bm 24 and Bm 42) and prolate-spheroidal (Bm 59).
5.6. **Phytochemistry**

The use of plants as medicines goes back to early man. Prior to the World War II, a series of natural products isolated from higher plants became clinical agents, and a number of them are still in use today. The medicinal value of plants is mainly due to bioactive constituents they contain, and which produce a definite physiological action in the human body. The major bioactive compounds identified in plants include alkaloids, flavanoids, terpenes, saponins phenolic compounds etc. Plants contain an enormity of chemical constituents that interact in a complex manner, many of which having therapeutic effect. Phytochemistry deals with the chemical structures of these substances, their biosynthesis, metabolism, natural distribution and biological function (Buchanan et al. 2000; Bowsher et al. 2008). Phytochemicals include biologically active, naturally occurring chemical compounds found in plants which provide health benefits for the humans, further than those attributed to macronutrients and micronutrients (Hasler and Blumberg 1999). These chemicals also protect plants from diseases and damages and contribute to the colour, aroma, flavor and their medicinal properties. More than 4000 phytochemicals have been catalogued and about 150 chemicals studied in detail (Saxena et al. 2013). Despite many remarkable discoveries of bioactive compounds, the impact of phytochemistry on new drug development has declined, and inevitably the pharmaceutical industry turned to synthetic chemicals. Recently, attention of the pharmaceutical industry has switched once more to the natural source for drug discovery and development (Phillipson 2001). Phytochemicals are classified as primary constituents and secondary constituents depending on their
role in metabolism. Primary constituents include sugars, amino acids, proteins, nucleic acids etc. and the secondary constituents include alkaloids, terpenes, flavonoids, ligans, plant steroids, saponins, phenolics etc. The saponins, the compounds of interest in the present study, include compounds that are glycosylated steroids, triterpinoids and steroidal alkaloids.

Phytochemical investigations on *B. monnieri* were carried out by several workers on account of the importance of the plant in the indigenous systems of medicine. Bose and Bose (1931) have isolated an alkaloid in the plant and named it as ‘brahmine’. This was followed by reports of isolation and identification of other alkaloids, nicotine and herpestine (Chopra et al. 1956). The major chemical constituents isolated and characterized in the species using various methods like spectral, 2D NMR and chemical, from the alcoholic extracts of the herb are dammarane type triterpenoid saponins with jujubogenin and pseudojujubogenin as aglycones. The jujubogenin derivatives include a series of compounds, bacoside A1, bacoside A3, bacopasaponin A, bacopasaponin E, bacopasaponin F, bacopasaponin G, bacopaside III, bacopaside IV, bacopaside V (Chakravarty et al., 2003), bacopaside IX - X and bacoside N1. The major pseudojujubogenin derivatives include a line of bioactive compounds such as bacopasaponin B, bacopasaponin C, bacopasaponin D, bacoside A2, bacopaside III, bacopaside I and II, VI – VIII, bacopaside III, bacopasaponin G, bacopasides A, B and C, bacopasaponin H, bacopaside N2, bacopaside XI and bacopaside XII. In addition, many other compounds such as phenylethanoid glycosides, flavonoids, alpha
alanine, aspartic acid, glutamic acid, betulic acid, D-mannitol, stigmastenol, β-sitosterol and stigmasterol have also been isolated and characterized.

Pharmacological effects of *B. monnieri* are mainly attributed to its saponins such as bacosides, bacopasides and bacopasaponins. Bacosides are known for their memory enhancing properties (Singh et al., 1988; Pal and Sarin, 1992). Bacosides A and B were first reported from *B. monnieri* (Chatterjee et al., 1963). The bioactive compounds, bacoside A and bacopaside I (Fig 4.5) are used as chemical markers for the quality control of *B. monnieri* products used for promoting mental health and intellect (Srivastava et al., 2012). The major chemical entity responsible for the nootropic activity of the plant is levorotatory bacoside A (Chatterji et al., 1965), which usually co-occurs with dextrorotatory bacoside B. Later on, the identity of bacoside A has been established as a mixture of four triglycosidic saponins such as Bacoside A3, bacopaside II, bacopasaponin C and its jujubogenin isomer. Sivaramakrishna et al. (2005) have contended that bacoside B is also a mixture of four saponins namely bacopaside N1, bacopaside N2, bacopaside IV and bacopaside V. However, identity of bacoside B appears ambiguous as it may be formed during the extraction of the plant via hydrolysis of saponins of bacoside A (Deepak and Amit 2013).

Optimized extraction, standardization and quantification protocols are critical for commercial use of medicinal plant extracts and formulations. Since *B. monnieri* is used for cognitive problems, standardization of its extracts and products is significant and vital. Various extraction methods were adopted for maximum yield of the bioactive compounds (mainly bacosides) from *B. monnieri*, and these
include (i) Soxhlet extraction with solvents such as ethanol (Tandon and Sharma, 2010), 80% methanol (Shahare and D’ Mello 2010) and 100% methanol (Sharma et al., 2013) (ii) hot extraction by refluxing with 100% methanol (Deepak et. al. 2005) or 70% methanol (Sivaramakrishna et al. 2005; Murthy et al 2006) (iii) Sonication with methanol (Ganzera et al., 2004) or with 1:1 ethanol-water mixture (Bhandari et al. 2006b).

The methods used for the quantification of saponins in the *B. monnieri* extracts and products include, ultraviolet spectroscopic methods based on hydrolysis of bacosides to aglycones having absorption maximum at 278 nm (Pal and Sarin 1992), High Performance Liquid Chromatography (HPLC) coupled with nuclear magnetic resonance and mass spectrometry (Murthy et al. 2006; Sharma et al. 2013) and High Performance Thin Layer Chromatography (HPTLC) densitometric methods (Shahare and D’ Mello 2010; Tandon and Sharma 2010). Among these analytical methods, HPTLC is widely used for the estimation of Bacopa saponins. Bhandari et al. (2006b) developed a microanalytical HPTLC technique for the determination of bacoside A in which, small quantity (≥2mg) of powdered plant material was required for the analysis. Standardization of the extracts of *B. monnieri* and its formulations with respect to its active principle, bacoside A was reported using HPTLC (Shahare and D’ Mello 2010). Other HPTLC based methods for estimation of bacoside A in the extracts and its herbal products/formulations were reported by various groups (Tandon and Sharma, 2010; Khandagale and Shanbhag 2012 etc.). Most of the reported HPLC and HPTLC
studies in the species were method developments based only on single or a few accessions and samples (Mathur et al. 2003; Tripathi 2012 and Bansal et al. 2014).

Owing to its pharmaceutical applications, traditional uses and export of its extracts/drugs, plants of *B. monnieri* are being collected extensively and indiscriminately from various parts of India, leading to its overexploitation resulting in depletion of this herbal resource as well as its genetic erosion. In order to achieve conservation and effective utilization of germplasm of the species in cultivation, conventional breeding, genetic transformation and direct utilization of the wild genotype in pharmaceutical industry, an indepth understanding of the genetic resource of *B. monnieri*, especially on its primary gene-pool, which constitutes its intraspecific variants is an important requirement. Although there are some preliminary attempts for assessing variability of the bioactive constituents in the germplasm of the species (Mathur et al. 2001, 2003; Bansal et al. 2014; Roshini et al. 2014) a comprehensive study on the biochemical profile of important bioactive constituents involving a large number of populations occurring in Kerala is not yet attempted.

The present phytochemical study of the species was undertaken aimed at screening of accessions covering almost all ecogeographical zones in Kerala. A precise HPTLC-based method was adopted for the simultaneous estimation of the bioactive saponins, bacoside A and bacopaside I in the sixty accessions, collected and grown under identical ecological conditions in an Experimental Plot at JNTBGRI. Estimation of these two major saponins in the accessions has led to the discovery of elite genotypes of *B. monnieri*. The high yielding
genotypes/chemotypes may be cultivated and utilized for pharmaceutical applications.

HPTLC densitometry analysis of the accessions showed considerable variations in the contents of the bioactive constituents, bacoside A and bacopaside I (Table 23). Bacoside A ranged from 1.31 (Bm 54) to 5.57 %, w/w (Bm 52) and bacopaside I 0.30 (Bm 2) to 1.46 %, w/w (Bm 9). The total content of both the bioactive constituents (bacoside A + bacopaside I) was ranged from 1.92 (Bm 54) to 6.75 %, w/w (Bm 52). The average (total) content of (bacoside A + bacopaside I) in the sixty accessions is 3.71 ± 0.99 %, w/w (n = 60), bacoside A alone is 2.91 ± 0.88 %, w/w (n = 60) and bacopaside I is 0.80 ± 0.23 %, w/w (n = 60). The ratio of bacoside A/bacopaside I in the sixty accessions is 3.64. Eleven accessions collected from relatively low altitudes (<10 m) registered both high as well as low percentage contents of total bacosides. The ten accessions collected from higher altitudes (>700 m) also showed both high as well as low percentage contents of total bacosides. The average total contents (bacoside A + bacopaside I) in the former (<10 m) and latter (>700 m) groups of accessions were 3.60 % and 3.80 %, w/w respectively. The finding of only narrow difference in the percentage content of bioactive contents in the accessions belonging to high and low altitudinal ranges is suggestive that altitude of original collection location has no bearing on the yield of the bacosides. Furthermore, in this study, the fourth generation vegetatively propagated progenies of the accessions grown in uniform environmental conditions appear to imply that the variation in their chemical profiles are by and large determined genetically.
Previous studies also reported variation in percentage content of bacoside A in different accessions of the species collected from diverse geographic locations in the country (Mathur et al. 2003; Deepak et al. 2005; Shahare and D’ Mello 2010; Naik et al. 2012; Bansal et al. 2014; Ahmed et al. 2015). Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow has developed improved varieties like Pragyashakti (bacoside A 1.8 %, w/w), Subodhak (bacoside A 1.6 %, w/w) and CIM-Jagriti (bacoside A 2.1 %, w/w). Indian Institute of Integrative Medicine (IIIM), Jammu, also has developed a variety with 1.8 - 2.2 %, w/w bacoside A content (Gupta 2000).

The highest content of bacoside A reported previously from the varieties released from the two Institutes was only 2.2 %, while the present study revealed still higher content in 48 accessions out of the 60, of which 23 accessions registered remarkably higher concentration (3 – 5.57 %, w/w). This include Bm11, Bm 50 and Bm 52 which possessed the highest percentage content of bacoside A >4.5 %, w/w could be considered as elite genotypes.

In the entire 60 accessions the present study noted that bacopaside I ranged from 0.30 (Bm 2) to 1.46 %, w/w (Bm 9) of which, five accessions (Bm 2, Bm 20, Bm 25, BM 46 and Bm 48) showed only very low percentage (< 0.50 %, w/w), while ten (Bm 7, Bm 9, Bm 11, Bm 15, Bm 19, Bm 21, Bm 27, Bm 34, Bm 35 and Bm 52) possessed remarkably higher content (> 1%, w/w). There is no previous reports of variation of bacopaside I content in different accessions of the species.

Out of the sixty accessions screened in this study, three showed total contents of bacoside A and bacopaside I above 5.50 %, w/w (Bm 11 - 6.75 %, w/w;
Bm 50 - 5.58 %, w/w; Bm 52 - 6.74 %, w/w) (Table ). These estimates of total bacosides contents (bacoside A + bacopaside I) are the highest ever reported in the species. Considering the presence of high total bacosides contents, these accessions can be rated as elite genotypes which merit prospects of their multiplication and cultivation on a large scale for pharmaceutical/industrial purposes. This could lead to mitigation of uncontrolled and indiscriminate collection of B. monnieri from the wild and this in turn would help conservation of its natural populations.

### 5.7. Molecular biology

Molecular markers are the best tool for diversity analysis in plants, since they detect variation at the nucleotide level and provide quick and reliable means for determining genetic relationships among genotypes. Remarkable achievements have been made in crop improvement by exploring the genomes of individual crop species, and the present day staggering growth in the field of molecular biology widens the possibilities to exploit polymorphism in breeding programmes. Molecular markers have been proven to be powerful tools in the assessment of genetic variation and in understanding the genetic relationships within and among species (Chakravarthi and Naravaneni 2006). Molecular biological parameters used for classification of genotypes, unlike morphological parameters, are not affected by environment (Staub et al. 1997). The advent of DNA marker technology has revolutionized the field of genetics by enhancing the pace and precision of genetic analysis (Collins 2002; Dodgson et al. 1997; Rafalski and Tingey 1993).
Sixteen ISSR markers have been employed for assessing genetic variability and interrelationships in the 60 accessions of *B. monnieri* in the present study. Sixteen primers generated 2896 bands and out of which 921 bands are polymorphic (31.81%). The estimates of the four parameters (Na, I, H and P) taken into consideration for assessing genetic diversity among the accessions showed that they are recognizably diverse as regards the 13 loci. The cluster analysis using POPGENE and the dendrogram generated revealed that the 60 accessions as differing each other genetically. These observations points to the possibility that the accessions represent 60 different genotypes of the species. The estimated value of No. of alleles per locus (1.13 - 1.69), Shannon’s Information Index (0.08 - 0.45), genetic diversity (0.06 - 0.32) and percentage of polymorphic loci (12.50 - 68.75) showed a wide range among the 60 accessions.

The 60 accessions were clustered into three main groups Cluster I, II and III, of which Cluster I includes 2 accessions, Cluster II, 24 in 12 subgroups and Cluster III, 34 in 21 subgroups. Some of the subgroups are single member clusters. This grouping appears to reveal genetic interrelationship between the accessions and the extent of genetic variability among the accessions of the species.

As per the present study the average level of genetic polymorphism among the accessions is 31.81 %, revealing moderate genetic diversity in the germplasm of *B. monnieri* occurring in Kerala. Apparently similar finding was reported by Bansal et al. (2014) in the accessions of the species from different parts of India. This, as has been pointed out by many of the previous workers, could be due to vegetative
mode of reproduction predominantly occurring in the species, alongside with sexual reproduction.

5.8. Intraspecific variability

*Bacopa monnieri* (L.) Wettst. (Plantaginaceae) is a perennial, herbaceous creeper distributed in the tropical parts of the world. It occurs all over India, including the Andaman & Nicobar Islands and distributed widely in Kerala. It is one of the core species used in Indian traditional systems of medicine such as Ayurveda, Siddha and Unani. *B. monnieri* is used as the source of many medicines in homoeopathy and it is an ingredient of a number of herbal drugs too. It is a highly valued medicinal species mainly due to its rejuvenative and nootropic therapeutic properties. As a result of high demand of the species in pharmaceutical industry, this species is being collected indiscriminately from the wild, causing drastic depletion of the resource as well as gene erosion. The present day degradation and destruction of habitat of the species – wetlands – is yet another reason for extinction of its genotypes. Loss of genotypes of a species is an irreversible set back to the richness of its germplasm and it usually goes unnoticed. A genotype once lost is lost forever, because re-creation of these genetic entities, which has evolved in nature over a long period of time, is not feasible. Loss of genotypes of a species and consequent shrinkage of its germplasm is detrimental to conservation and utilization of *B. monnieri* as well as its genetic improvement and cultivation. In order to achieve conservation and effective utilization of germplasm of a species, an indepth understanding of intraspecific variants of the species, which constitutes its primary gene-pool, is a prerequisite. With this end in view, a field
survey all over Kerala has been undertaken for locating the intraspecific variants occurring in the State. The variants were inventorized, characterized and documented. Assessment of intraspecific variations of the species in the genepool has been accomplished based on biosystematic parameters of diverse biological disciplines such as morphology, anatomy, cytology, palynology, phytochemistry and molecular biology. The enormity of intraspecific variability encountered in diverse characteristics of the plant is summarized in Table 28 and the outstanding instances of variability noticed under various disciplines are briefly discussed.

Morphology: The field survey revealed that the most of the accessions prefer mashy ecosystems and certain others are adapted to freshwater and brackish habitats. The distributional range of the species in Kerala covers altitudinal ranges from near sea level (4 m) to as high as in high ranges (1462 m). Detailed morphological characterization of the accessions in respect of 15 qualitative and 25 quantitative characters showed that they vary in their habit (disposition of primary branches) having prostrate, erect and sub-erect types. Variation in the qualitative characters was assessed visually by identifying their character states. Among the 14 qualitative characters studied, four traits (node-centric anthocyanin pigmentation, corolla throat colour, corolla base colour, style colour) showed four character states each, whereas the other eight characters showed three character states, and two traits (corolla lobe type and capsule shape) exhibited only two character states each. Variations in regard to their quantitative characters were assessed by computing their Mean, Standard deviation and their Critical difference and all the 25 characters except one (long stamen length) showed considerable variation. The range of
variation exhibited by the quantitative characters noted are: stem thickness (0.10 – 0.25 mm), internode length (0.18 – 2.76 cm), leaf length (1.50 – 2.80 cm), width (0.50 – 1.1 cm), area (48 – 159 mm$^2$), thickness (0.02 – 0.06 mm), No. of dentations/leaf (0 – 6 nos.), pedicel length (1.10 – 3.80 cm), flower diameter (0.90 – 2.00 cm), corolla length (0.85 – 1.8 cm), corolla width (1.0 – 2.15 cm), fruit length (0.23 – 0.52 mm), fruit breadth (0.11 – 0.30 mm), No. of seeds/capsule (9 - 198 nos.) and biomass yield (6.2 - 11.98 %).

**Anatomy:** Anatomical study was carried out concerning the stem (internode region) and leaf characters. The major variation noticed are: shape of the stem (circular, circular- dumboidal and dumbiodal), No. of cortical layers 14 – 23 in the broad area and 9 – 18 in the short area, thickness of the broad area 2300 – 3660 µm and short area 1960 - 2800 µm, stelar diameter 650 to 1100 µm, phloem thickness 30 – 120 µm, No. of xylem strands 25 – 52 nos. and No. of vessels per strand 1 – 3 to 3 – 6.

Five different stomatal types occur in almost all the accessions (diacytic, anisocytic, anisotricytic, tetracytic and anomocytic). The frequency of different types of stomata varied considerably in the accessions, the anisocytic was the most predominant type. The other major variations noted are: stomata per unit area (7.6 to 30.6), stomatal index (7.90 – 29.59), Mean stomatal length (12.32 – 24.75 µm) and mean stomatal breadth (2.46 – 4.95 µm). The trichomes also showed some variation as regards the trichome index (0.47 – 2.37) and Mean trichome diameter (21.24 – 32.78 µm).
The leaf venation pattern observed in accessions of *B. monnieri* is characterized by three first order veins originating from the leaf base, the middle one proceeding up to the leaf apex and branching at intervals. The branch number of the first order vein was found to vary considerably across the accessions (3–7). The number and pattern of formation of the second order veins from the middle first order vein also showed variation in the accessions. The nature of the other two first order veins starting from the leaf base also varied. In some accessions the two other first order veins proceed laterally almost up to the apex often anastomosing with the second order veins of the middle one. In some others the two first order veins do not proceed up to the leaf apex, stopping halfway through.

**Cytology:** Cytological study in the 30 accessions of the species displayed two cytotypes, one with n = 32, 2n = 64 and the other n = 34, 2n = 68. Among the 30 accessions 2n=64 predominated

**Palynology:** LM and SEM studies on the pollen grains of the accessions of *B. monnieri* revealed the taxon is stenopalynous with the primary aperture feature being trizonocolporate. However with regard to certain secondary and tertiary pollen features some variation was apparent concerning the exine ornamentations, pollen size and shape. Variation noticed in the ornamentation pattern concern the existence of two exine sculpturing such as reticulate and striato-reticulate. Pollen shapes noticed were subprolate, perprolate and prolate spheroidal. The colpus end also showed some difference in their disposition, those with pointed colpus end and others with blunt. Some variation was noticed in the floor pattern of the colpus, a few accessions showing gemmae like projections and others with undulated
dispositions or irregular projections and or smooth floor. The ora shape also displayed variation, a few accessions showing lalongate and others irregular-lalongate and/or circular-lalongate.

**Phytochemistry:** Variations in percentage content of bioactive principles bacoside A, bacopaside I and total bacoside were studied and found that bacoside A ranged from 1.31±0.04 to 5.57±0.22, bacopaside I from 0.30±0.01 to 1.46±0.02 and total bacosides from 1.92±0.04 to 6.75±0.25). The percentage content of bacoside A in various accessions of the species collected from different locations of India was studied by many authors and reported notable variation of the content. The present study revealed wide range of variation in the percentage content of bacoside A, bacopaside I and total bacosides. And also identified 3 elite genotypes having the percentage content of total bacoside > 5%, the highest so far known.

**Molecular biology:** Genetic diversity studies have been carried out in the 60 accessions of the species using ISSR. Sixteen primers which generated polymorphic bands were used for the analysis. The primers generated 2896 bands, of which and 921 bands (31.81%) were polymorphic. The parameters used for genetic diversity assessment (No. of alleles/locus, Shannon’s Information Index, Genetic diversity and Percentage of polymorphic loci) revealed that all the accessions showed variation between each other. The estimated average level of polymorphism among the 60 accessions (31.81%) revealed that genetic diversity in the gene-pool of *B. monnieri* in Kerala is moderate, and which can be attributed to vegetative mode of reproduction predominant in the species along side with sexual reproduction.
Table 28. Intraspecific variations in respect of the 60 accessions of *Bacopa monnieri* based on ecological, morphological, anatomical, cytological, palynological, phytochemical and molecular biological parameters

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Discipline</th>
<th>Character</th>
<th>Character states/Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ecology</td>
<td>Habitat</td>
<td>Aquatic, marshy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altitude</td>
<td>4 – 1462 m</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salinity</td>
<td>Saline, freshwater</td>
</tr>
<tr>
<td>2</td>
<td>Morphology</td>
<td>Disposition of primary branches</td>
<td>Prostrate, erect, sub-erect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem colour</td>
<td>Green, pale green, yellowish green</td>
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<tr>
<td></td>
<td></td>
<td>Node-centric anthocyanin pigmentation</td>
<td>Low, medium, high, no pigmentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem thickness</td>
<td>0.10 – 0.25 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Internode length</td>
<td>0.84 – 2.76 cm</td>
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<tr>
<td></td>
<td></td>
<td>Leaf shape</td>
<td>Obovate, Oblong, Oblanceolate</td>
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<tr>
<td></td>
<td></td>
<td>Leaf colour</td>
<td>Dark green, Green, Light green</td>
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<tr>
<td></td>
<td></td>
<td>Leaf length</td>
<td>1.50 – 2.80 cm</td>
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<tr>
<td></td>
<td></td>
<td>Leaf width</td>
<td>0.50 – 1.1 cm</td>
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<tr>
<td></td>
<td></td>
<td>Leaf area</td>
<td>48 – 159 mm²</td>
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<tr>
<td></td>
<td></td>
<td>Leaf thickness</td>
<td>0.02 – 0.06 mm</td>
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<tr>
<td></td>
<td></td>
<td>No. of dentations / leaf</td>
<td>0 – 6 nos.</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Discipline</th>
<th>Character</th>
<th>Character states/ Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pedicel length</td>
<td>1.10 – 3.80 cm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flower colour</td>
<td>Violet, Pale violet, White</td>
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<td></td>
<td></td>
<td>Corolla lobe type</td>
<td>Unequal, equal</td>
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<tr>
<td></td>
<td></td>
<td>Flower diameter</td>
<td>0.90 – 2.00 cm</td>
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<tr>
<td></td>
<td></td>
<td>Corolla length</td>
<td>0.85 – 1.8 cm</td>
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<td></td>
<td></td>
<td>Corolla width</td>
<td>1.0 – 2.15 cm</td>
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<tr>
<td></td>
<td></td>
<td>Capsule shape</td>
<td>Obovate and oblong</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fruit length</td>
<td>0.23 – 0.52 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fruit breadth</td>
<td>0.11 – 0.30 mm</td>
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<tr>
<td></td>
<td></td>
<td>Seed colour</td>
<td>Dark brown, Brown or Light brown</td>
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<tr>
<td></td>
<td></td>
<td>No of seeds/capsule</td>
<td>9 – 198 nos</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biomass yield</td>
<td>6.2 – 11.98 %</td>
</tr>
</tbody>
</table>

3 Anatomy

Stem

Shape of stem | Circular, dumbiodal, circular-dumboidal

Thickness (broad area) | 2300 – 3660 µm

Thickness (short area) | 1960 – 2800 µm

No of cortical layers (broad area) | 14 – 23

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<table>
<thead>
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<th>Character</th>
<th>Character states/ Range</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No of cortical layers</td>
<td>9 – 18 (short area)</td>
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<tr>
<td></td>
<td></td>
<td>Stelar diameter</td>
<td>650 to 1100 µm</td>
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<td></td>
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<td>Phloem thickness</td>
<td>30 – 120 µm</td>
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<tr>
<td></td>
<td></td>
<td>No. of xylem strands</td>
<td>25 – 52 nos</td>
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<tr>
<td></td>
<td></td>
<td>No. of xylem vessels/strand</td>
<td>1 – 3 to 3 – 6</td>
</tr>
<tr>
<td></td>
<td><strong>Leaf</strong></td>
<td>Types of stomata</td>
<td>Diacytic, anisocytic, anisotricytic, tetracytic and anomocytic</td>
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<tr>
<td></td>
<td></td>
<td>Stomata per unit area</td>
<td>7.6 – 30.6</td>
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<td></td>
<td></td>
<td>Stomatal index</td>
<td>7.90 – 29.59</td>
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<td></td>
<td></td>
<td>Mean stomatal length</td>
<td>12.32 – 24.75</td>
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<tr>
<td></td>
<td></td>
<td>Mean stomatal breadth</td>
<td>2.46 – 4.95 µm</td>
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<td></td>
<td>Trichome index</td>
<td>0.47 – 2.37</td>
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<tr>
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<td></td>
<td>Mean trichome diameter</td>
<td>21.24 – 32.78 µm</td>
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<tr>
<td></td>
<td></td>
<td>No of 2nd order veins</td>
<td>3 – 7</td>
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<td><strong>4</strong></td>
<td><strong>Cytology</strong></td>
<td>Basic chromosome number</td>
<td>x = 16 and x = 17</td>
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<tr>
<td></td>
<td></td>
<td>Chromosome number</td>
<td>n = 32, 2n = 64 and n = 34, 2n = 68</td>
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<td>SL No.</td>
<td>Discipline</td>
<td>Character</td>
<td>Character states/ Range</td>
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<tr>
<td>5</td>
<td>Palynology</td>
<td>Pollen shape</td>
<td>Subprolate, Perprolate and Prolate spheroidal</td>
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<td></td>
<td></td>
<td>Exine ornamentation</td>
<td>Reticulate and Striato-reticulate</td>
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<td></td>
<td></td>
<td>Colpus end shape</td>
<td>Pointed and Blunt</td>
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<tr>
<td></td>
<td></td>
<td>Colpus floor</td>
<td>Gemmae like projections, undulated disposition, irregular projections, smooth floor</td>
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<tr>
<td></td>
<td></td>
<td>Ora shape</td>
<td>Lalongate, irregular-lalongate and circular-lalongate</td>
</tr>
<tr>
<td>6</td>
<td>Phytochemistry</td>
<td>Bacoside A</td>
<td>1.31±0.04 – 5.57±0.22</td>
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<tr>
<td></td>
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<td>Bacopaside I</td>
<td>0.30±0.01 – 1.46±0.02</td>
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<td></td>
<td></td>
<td>Total bacosides</td>
<td>1.92±0.04 – 6.75±0.25</td>
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<td>7</td>
<td>Molecular biology</td>
<td>Genetic diversity</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>between accessions</td>
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<td>No. of alleles/locus(Na)</td>
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<td>Shannon’s Information Index (I)</td>
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<td>Genetic diversity estimates (H)</td>
<td>0.06 – 0.32</td>
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<td>Percentage of polymorphism (P)</td>
<td>12.50 – 68.75</td>
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