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RESEARCH ARTICLE

Phenome analysis of twelve wild grown seedlings from the forest patches of Dinajpur districts of West Bengal, India with special reference to the family Apocynaceae (*sensu stricto)*

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Abstract Seed and seedling morphology of twelve species of Apocynaceae have been studied. Seeds have been collected, characterized and grown in the seedbed of the garden of Botany Department to raise seedlings for comparison with natural ones for authentication. Seedlings have also been collected from the forest floor in appropriate season. Seedlings are characterized following literatures of previous workers. From distinctive characters, table and key to the taxa have been made. Numerical value has been considered in tabular form for different seedling traits. Numerical data are used for the preparation of the dendrogram by UPGMA method. Dendrogram has been analyzed supporting the placement of species in Apocynaceae (*sensu stricto*). ANOVA and regression of different quantitative traits have also been addressed to strengthen the artificial key for identification and to partially justify Apocynaceae *sensu stricto.*

Key words: *Apocynaceae, ANOVA, phenogram, regression, seedling.*

Introducation

In any plant's life, emergence and establishment of seedlings is the most critical phase of early development (Silvertown *et al.* 1993). Plant species differ greatly in seed and seedling traits; and these traits are often associated with regeneration and / or adaptation in particular habitats (Kitajima & Fenner 2000, Leishman *et al.* 2000). Seedlings raised from larger sized seeds get their resource initially from storage reserves for their growth and development (Hladik and Miquel 1990, Garwood 1996, Kitajima 1996, Green and Juniper 2004). Similarly, seedlings having photosynthetic cotyledons start producing food using sunlight as energy source earlier than those with reserve cotyledons (Kitajima 2002). Therefore, phanerocotylar seedlings rose from seeds of smaller species having paracotyledons demand high light environment for autotrophic growth (Garwood 1996, 2009). Reserve cotyledons, on the other hand, provide resources to support seedling energy demands during times of stress and may be an adaptation to growing in low light (Ibarra-Manríquez *et al.* 2001). However, phanerocotylar seedlings with thick paracotyledons again better adapted to lighter environment and negatively correlated to with seed size (Kitajima 1996).

Of various types of groupings of seedlings, the most comprehensive treatment for Neotropical seedlings, Garwood (2009) used three cotyledon traits abstracting types of seedlings: (a) emergence (cryptocotylar vs. phanerocotylar); (b) position (epigeal or hypogeal) and (c) function (foliaceous or reserve). Based on these parameters, Garwood, 2009 recognized 5 morphological groups: phanerocotylar, epigeal, foliaceous (PEF); phanerocotylar, epigeal, reserve (PER); phanerocotylar, hypogeal, reserve (PHR); cryptocotylar, hypogeal, reserve (CHR) and cryptocotylar, epigeal, reserve (CER).

Robert Brown was one of the most important contributors to recognize asclepiads as more advanced than the members of Apocynaceae *sensu stricto*, because of the presence of pollinia. So, he recognised asclepiads and Apocynaceae of

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Jussieu's (1989) Apocineae as two separate families *i.e.* Asclepiadaceae and Apocynaceae respectively. But today, following cladistic procedure, the Apocynaceae and Asclepiadaceae are mostly again united into a single family Apocynaceae having subfamilies, *i.e.* Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae and Asclepiadoideae (Endress 2004)

Considering Brown and recent workers, a study of seedling traits of twelve species has been designed within eight forest patches of Dinajpur districts of West Bengal, India, to observe whether these two families are combined into one as a Apocynaceae *sensu stricto* or not. Identification of seedlings in natural habitats is crucial for conservation as *in situ* or *ex situ.* Besides, seedling taxonomy may establish a predictive value for inclusion or separation of any taxa parallel to other schools of thought.

The study is conducted in six natural and semi-natural forest patches of South Dinajpur districts of West Bengal within the period of June, 2013 to September, 2014. The total forest area is 2.95 sq.km (Mitra and Mukherjee 2013) which falls under northern tropical mixed deciduous type (Kamilya 2011) with mean annual rainfall 1847.8 mm and mean annual temperature varying from 23 – 29^{o} C.

Materials and methods

Seeds and seedlings of the studied taxa are collected in natural conditions from different forests at different times. The seeds are sown in Departmental Garden of Botany of Balurghat College and seedlings are raised. The natural seedlings are then compared with the raised ones for identification. Herbarium sheets are made by dried seedlings and diagnosed following Duke 1965, Burger 1972, Vogel 1980 and Paria 2006. Seedlings are then described up to 7th to 11th leaf-stage and an artificial key to the taxa has been made for the identification of seedlings in natural habitats. For phenome analyses of all collected seeds and seedlings, all the major phenotypical traits have been considered. A photoplate displaying photographs of all the taxa studied in their natural habitats is given below for better comprehension about their phenotypic traits (Plate – 1).

For statistical analysis, to understand the inter–relationships among the taxa, a dendrogram is created using DendroUPGMA software. DendroUPGMA makes clustering from a set of variables from similarity or dissimilarity matrix using Unweighted Pair Group method with Arithmetic Mean (UPGMA) algorithm [Garcia-Vallve *et al.* 1999]. The numerical characters are put in fasta–like format and the system is run in Pearson coefficient to measure linear correlation between the OTUs. The outcome is in newick format where mean branch divergence value between the taxa is displayed from which we finally get the dendrogram. Significant differences of various quantitative traits have also been shown by ANOVA analysis according to Duncan's Multiple Range Test at $p \leq 0.05$. Various quantitative data of seeds and seedlings have also been used for regression analysis.

Results and discussions

Artificial key (valid for the identification of the studied taxa only)

1. Seeds oblong, ovate, rounded or irregular in shape; seedlings phanerocotylar……………2
1. Seeds elliptic or linear; seedlings cryptocotylar…………………….……………..11

2. Seeds oblong or ovate, comose; hypocotyls and first internode may be glabrous, pubescent or scarcely pubescent; first two leaves always exstipulate; seeds lighter in weight (0.007-0.009 gm)…..…..3
2. Seeds round or irregular, not comose; hypocotyls and first internode always glabrous; first two leaves always stipulate or exstipulate; seeds heavier in weight (0.04-0.07 gm) ....…9

3. Seeds oblong; paracotyledons lanceolate or ovate, apex obtuse or acute…………………4
3. Seeds oval; paracotyledons oblong, apex rounded………………………………………5

4. Hypocotyl glabrous, paracotyledons lanceolate, base cuneate, apex obtuse, venation hyphodromous; first two leaves lanceolate, base attenuate; first internode glabrous; paracotyledons significantly smaller (10-13mm × 4.5-6mm)............***Alstonia scholaris*** L.
4. Hypocotyl pubescent, paracotyledons ovate, base truncate, apex acute, venation camptodromous; first two leaves elliptic-ovate,

Plate –1I: Seedlings of: *1. Alstonia scholaris, 2. Carissa carandas, 3. Calotropis gigantea, 4. Cryptolepis buchanani, 5. Ichnocarpus frutescens, 6. Kopsia fruticosa, 7. Pergularia daemia, 8. Rauwolfia serpentine, 9. Tabernaemontana divaricata, 10. Telosma cordata, 11. Wattakaka volubilis, 12. Wrightia antidysenterica*

base cuneate; first internode pubescent; paracotyledons significantly larger (21-28mm × 14-19mm)...... ***Wrightia antidysenterica*** (L.) R.Br.

5. Paracotyledons obovate or elliptic; first two leaves widely elliptic or narrowly elliptic, base cuneate; first inetrnode minutely pubescent……………………...……..................6
5. Paracotyledons oblong; first two leaves ovate, base cordate or rounded; first inetrnode tomentose or pubescent…..........….......................................7

6. Paracotyledons obovate, base cuneate; first two leaves widely elliptic, apex obtuse ....…… ....***Calotropis gigantea*** (L.) W.T. Aiton
6. Paracotyledons elliptic, base rounded; first two leaves narrowly elliptic, apex acute…............. ....***Cryptolepis buchanani*** Roem. & Schult.

7. Paracotyledons with truncate base and three primary veins, venation actinodromous; first two leaves with cordate base, actinodromous venation; first ineternode tomentose…............................... ....***Pergularia daemia*** (Forssk.) Chiov.
7. Paracotyledons with rounded base and one primary veins, venation camptodromous; first two leaves with rounded base, camptodromous venation; first ineternode pubescent……..8

8. Hypocotyl pubescent; first internode round………… ***Telosma cordata*** (Burm. *f.*) Merr.
8. Hypocotyl scarcely pubescent; first internode angular…….. ***Wattakaka volubilis*** (L.*f.*) Stapf

9. Paracotyledons elliptic or ovate, base cuneate or truncate, apex acute, venation hyphodromous or actinodromous; first two leaves base rounded or attenuate……………10
9. Paracotyledons oblong, base rounded, apex rounded, venation camptodromous; first two leaves base cuneate………………………………. ....***Rauwolfia serpentina*** (L.) Benth. *ex* Kurz

10. Seeds round; paracotyledons elliptic, base cuneate, primary vein one, venation hyphodromous; first two leaves stipulate, ovate, base rounded, apex obtuse; first internode round…………………...….. ***Carissa carandas*** L.
10. Seeds irregular in shape; paracotyledons ovate, base truncate, primary vein three, venation actinodromous; first two leaves exstipulate, elliptic, base attenuate, apex acute; first internode angular………………… ***Tabernaemontana divaricata*** R. Br. *ex* Roem. & Schult.

11. Seeds linear, comose; first two leaves ovate, base rounded; first internode tomentose; seeds lighter in weight (0.009 gm).......***Ichnocarpus frutescens***(L.) W.T. Aiton
11. Seeds elliptic, not comose; first two leaves narrowly elliptic, base cuneate; first internode glabrous; seeds heavier in weight (1.02 gm)…… ***Kopsia fruticosa*** (Roxb.) A.DC.

Phenogram analysis

Two cryptocotylar taxa *viz.*, *Ichnocarpus frutescens* and *Kopsia fruticosa* are initially separated from remaining ten phanerocotylar species having branch point distance of 0.424 as depicted in the Phenogram (Fig. 1). Among the phanerocotylar taxa, *Wrightia antidysenterica* and *Alstonia scholaris* are separated out from the rest of the species by their distinct characters of hypocotyls, paracotyledons and first two leaves mentioned in the artificial key. *Carissa carandas*, *Tabernaemontana divaricata* and *Rauwolfia serpentina* again formed a group from the remaining five species by possessing some distinct characters of seed, hypocotyls, first internode and first two leaves. With the oblong shape of

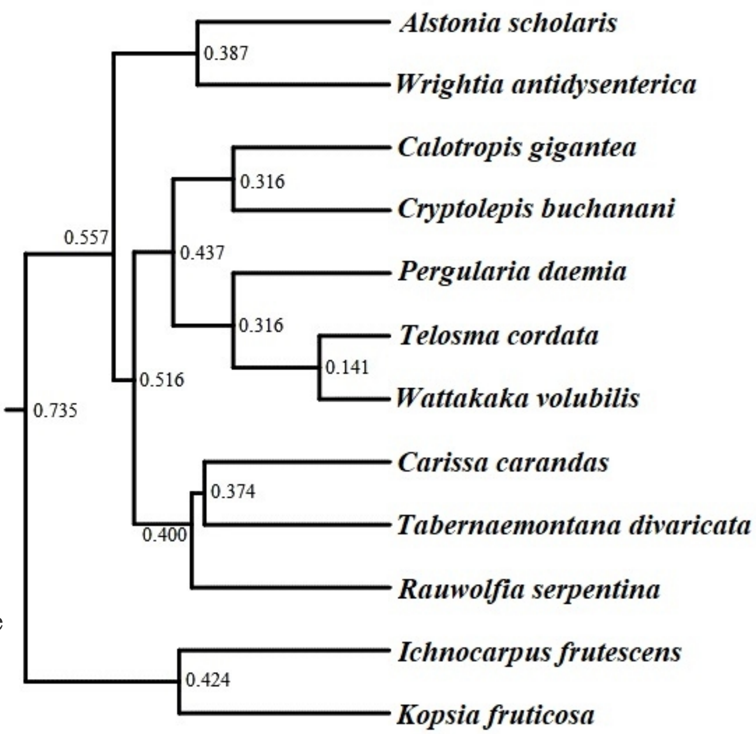

Figure 1: Phenogram based on qualitative and quantitative traits of seedlings

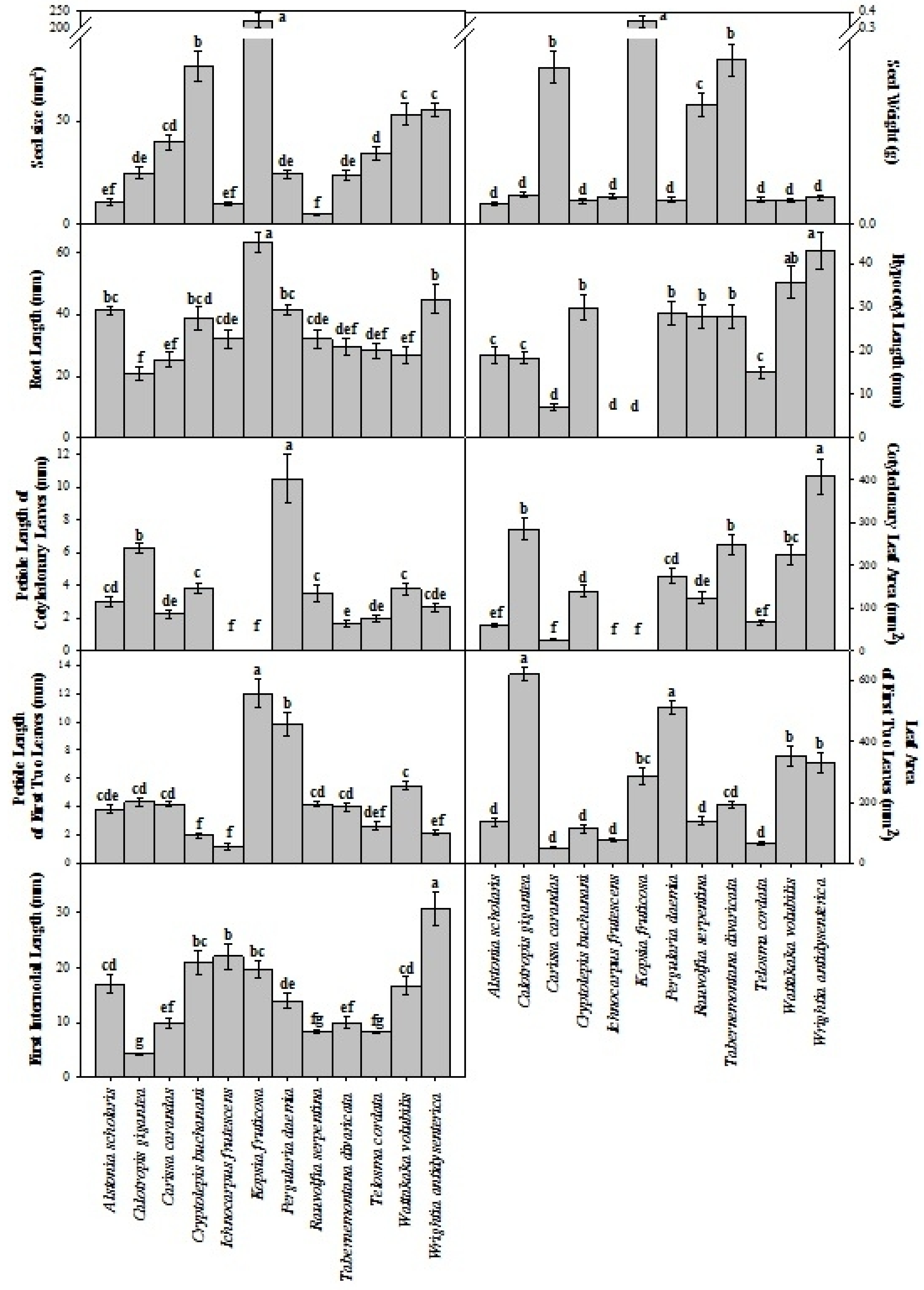

Figure 2: Analysis of different quantitative traits observed in twelve species of family – Apocynaceae *sensu stricto*. Values represent mean ± SE. Bars showing *different letters* indicate significant differences according to Duncan's Multiple Range Test at $p \leq 0.05$.

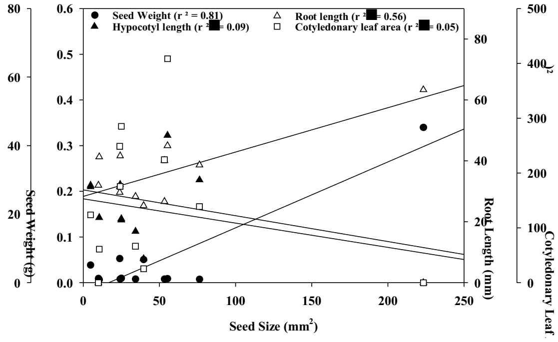

Figure 3a: Simple regression analysis of hypocotyls length, seed weight, root length and cotyledonary leaf area against seed size observed in twelve species of family Apocynaceae *sensu stricto*

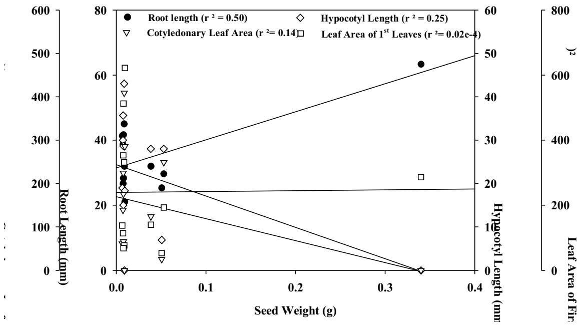

Figure 3b: Simple regression analysis of cotyledonary leaf area, root length, hypocotyls length and leaf area of first two leaves against seed weight observed in twelve species of family Apocynaceae *sensu stricto*

paracotyledons having rounded base and apex, camptodromous venation and cuneate base of first two leaves; *Rauwolfia serpentina* separates out from *Carissa carandas* and *Tabernaemontana divaricata*. The later two taxa are also distinguishable from one another by the conspicuous characters of seeds, paracotyledons, first two leaves and shape of first internodes. Two groups have been displayed by the next five species of which *Calotropis gigantea* and *Cryptolepis buchanani* are quite distinct from rest three taxa since their paracotyledons obovate or elliptic, first two leaves widely or narrowly elliptic with cuneate base and first internodes with minute pubescent hairs. These characters are not met with the rest three. *Calotropis gigantea* and *Cryptolepis buchanani* are also distinctive at their individual juvenile features as presented in the artificial key. In the another extreme, *Pergularia daemia* became distinctive from *Wattakaka volubilis* and *Telosma cordata* by the number of primary veins, venation patterns and base of paracotyledons as well as the few characters of first two leaves and first internode. Again *Telosma cordata* having pubescent hypocotyls and round shape of first internode is distinctive from *Wattakaka volubilis* with scarcely pubescent hypocotyls and angular shape of first internode.

Study of seedlings within such limited number of taxa may draw a partial support towards the union of the two families into a single taxa *i.e.,* Apocynaceae because taxa like *Calotropis gigantea, Cryptolepis buchanani, Pergularia daemia, Telosma cordata* and *Wattakaka volubilis* which were previously treated as the members of Asclepiadaceae by the reproductive characters, mainly for the presence of pollinia, are not distinct in juvenile morphology from Apocynaceae as because they shared some common characters with three members of Apocynaceae like *Carissa carandas, Tabernaemontana divaricata* and *Rauwolfia serpentina* making a same clade at branch point of 0.516. Again except two cryptocotylar taxa, five phanerocotylar members of Apocynaceae and five phanerocotylar taxa of Asclepiadaceae became merged together having a branch value of 0.557.

Within this limited scope, we also support the reunion of these two families into Apocynaceae with three subfamilies *i.e.,* Rauwolfioideae, Apocynoideae and Asclepidioideae. Endress *et al.* (2014) also supported this reunion and divided three major subfamilies into 25 tribes and 49 subtribes. More taxa we should study at juvenile stages in this family, and then we may get parallel realignment of seedling taxa with recent classification systems.

ANOVA analysis

Artificial key has been made using some major quantitative traits. Whether quantitative traits have equal weightage for the distinction of taxa at different taxonomic levels or not, we have analyzed them by ANOVA following Duncan's Multiple Range Test at $p \leq 0.05$. From Fig. 2 it is evident that different quantitative traits of seeds and/ or seedlings have significant differences in the form of bar diagrams having height indications of a to g by retrogressive series. Thus variability of taxa not only depends on their qualitative but also on their quantitative traits sharing thus equal weightage which is the fundamental principle of numerical taxonomy.

Regression analysis

Regression analysis has also been done by various quantitative traits of seeds and seedlings. In Fig. 3a it is evident that hypocotyl length more or less decreases against seed mass except one. Root length almost decreased with increase of seed weight and seed size. Cotyledonary leaf area (= cotyledonary photocynthetic index) decreased along with the increase of seed size or seed mass. Similarly the Fig. 3b depicts the positive correlation of root length with the leaf area of first two leaves and hypocotyl length shows parallel behavior with root length, cotyledonary leaf area and leaf area of first two leaves.

Conclusion

Juvenile morphological traits are equally important as reproductive traits for the designation of taxa in natural habitat. Display of dendrogram is based on both qualitative and quantitative traits. Dendrogram analysis depicts the interrelationship of taxa in their juvenile morphology. Ignoring reproductive distinctiveness, two families further aligned together based on juvenile characters that have been highlighted in the form of dendrogram even considering few taxa only.

ANOVA and regression analysis focused on significant interrelationships of various quantitative traits and consideration of these traits further strengthen the separation of taxa in the form of artificial key. Further study of seedlings of many other taxa within these two families may create an opening for considering seedling as a separate discipline for managing the two families as Apocynaceae *sensu stricto.*

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