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Incorporating ecological context: a revised protocol for the preservation of *Nepenthes* pitcher plant specimens (*Nepenthaceae*)

C. Clarke[1], J.A. Moran[2]

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Abstract Pitcher plants of the family *Nepenthaceae* are vines or subscandent shrubs which produce modified leaf organs that in most species serve to attract, trap, retain and digest animals for nutritional benefit. The sole genus within the family, *Nepenthes*, is abundant and diverse in Malesia. Previous taxonomic treatments of *Nepenthes* have relied almost entirely on the morphological features of the plants, with characteristics of the pitchers, inflorescences, leaf blades and indumentum being the most informative. Recent ecological research demonstrates that unique morphological characteristics and trap geometries provide useful taxonomic information, but this is often lost or obscured when specimens are prepared for herbaria by pressing them. In this paper, we demonstrate the value of ecological information in distinguishing between controversial montane Bornean taxa and provide a revised protocol for the collection and preparation of *Nepenthes* specimens, which is designed to maximise the amount of ecological information retained in herbarium material.

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INTRODUCTION

The genus *Nepenthes* L. (*Nepenthaceae*) comprises approximately 120 species of vines or subscandent shrubs (Cheek & Jebb 2001, Phillipps et al. 2009, McPherson 2009), the majority of which are confined to the Malesian phytogeographic region. Centres of diversity and endemism include Borneo, Sumatra and the southern Philippines (Cheek & Jebb 2001). All *Nepenthes* are pitcher plants, producing highly-modified, jug-shaped leaves at the tips of tendrils that arise from the apices of the leaf blades (Jebb & Cheek 1997). The pitchers serve primarily to attract, trap, retain and digest animals for nutritional benefit (Lloyd 1942, Clarke et al. 2009). The majority of *Nepenthes* demonstrate marked pitcher dimorphism: juvenile plants tend to produce ovoid pitchers that rest on the ground (and are known as 'lower' or 'terrestrial' pitchers), whereas mature plants produce narrower, funnel-shaped pitchers (called 'upper' or 'aerial' pitchers).

The status of several taxa in *Nepenthes* is controversial, with a number of recently described species being distinguished from others on the basis of apparently minor morphological characteristics, some of which are of uncertain stability (Clarke & Kruger 2006, Cheek & Jebb 2009, Catalano 2009). One factor that contributes to the controversy is Danser's (1928) decision to not recognise sub-specific taxa. His influential monograph provided students of the genus with a rigorous and clearly defined protocol for describing and distinguishing taxa at specific rank. Minor and/or unstable morphological characteristics, such as plant or pitcher size, and variations in colour, were considered insignificant. All major, subsequent taxonomic treatments of *Nepenthes* (Jebb & Cheek 1997, Clarke 2001, Cheek & Jebb 2001) followed Danser's practice, but in the last few years there has been a departure from this approach, with several new taxa being distinguished from others using morphological characteristics that Danser (1928) would have considered to be insignificant (Clarke 2006). In one respect, this is not surprising: Danser's monograph is 82 years old and our collective knowledge of *Nepenthes* has increased substantially over that time. However, current trends in describing and distinguishing new taxa reflect those of the late 1800s, which resulted in many taxa that were poorly described or defined, or were distinguished on the basis of minor and/or unstable morphological differences, and led to considerable confusion among taxonomists and horticulturists alike.

Nepenthes have broad horticultural appeal and a number of enthusiasts now travel extensively through the natural habitats of *Nepenthes*. Their observations have led to the discovery and description of new taxa (e.g., McPherson 2009, Mey 2009, Catalano 2009), which has significantly enhanced our knowledge of the genus. However, horticulturists and enthusiasts often view the taxonomic importance of certain morphological traits differently from taxonomists and this may give rise to multiple, competing interpretations for some taxa. New discoveries are still being published on a regular basis (Lee et al. 2009, McPherson 2009, Robinson et al. 2009), making it difficult to address the issue at present.

However, recent ecological research has demonstrated that some controversies can be resolved through more detailed field observations and careful preparation of herbarium material. This is possible because various morphological and geometric characteristics of *Nepenthes* pitchers play important roles in trap function or specialised methods of nutrient acquisition, and some of these roles have only recently been elucidated. For instance, in *N. albomarginata* T.Lobb ex Lindl., a unique pitcher characteristic, which takes the form of a dense, tomentose band that lines the pitcher orifice, enables this species to target termites as a major source of prey (Moran et al. 2001, Merbach

[1] School of Science, Monash University Sunway Campus, Jalan Lagoon Selatan, 46150 Bandar Sunway, Selangor, Malaysia;
corresponding author e-mail: charles.clarke@sci.monash.edu.my.
[2] School of Environment and Sustainability, Royal Roads University, 2005 Sooke Road, Victoria, BC, V9B 5Y2, Canada.

et al. 2002). Some other species appear to have evolved away from a strictly carnivorous mode of supplementary nutrition, with *N. ampullaria* Jack and *N. lowii* Hook.f. employing modified pitchers to trap significant amounts of leaf litter and tree shrew faeces, respectively (Moran et al. 2003, Clarke et al. 2009, Chin et al. 2010). Generally, less specialised species also appear to fall into three broad groupings:

1. Those that rely on a broad, expanded peristome (a ridge of hardened tissue that forms a 'collar' around the margins of the pitcher orifice) as the primary arthropod trapping mechanism. An example is *N. bicalcarata* Hook.f. (Bohn & Federle 2004);
2. Those that rely more on a well-developed waxy zone on the inner surfaces of the pitchers, for example, *N. gracilis* Korth.; and
3. Those that employ a viscoelastic fluid in the pitchers as a prey retention mechanism, for example, *N. inermis* Danser, *N. jacquelineae* C.Clarke, Troy Davis & Tamin, and *N. rafflesiana* Jack (which also utilises the two strategies outlined above (Clarke 2001, Gaume & Forterre 2007)).

It is becoming apparent to ecologists that the degree of development of the peristome and waxy zone are fundamental to the prey-trapping strategies of many *Nepenthes* species (Gaume et al. 2002, Bohn & Federle 2004, Gorb et al. 2004, Gaume & Forterre 2007, Gaume & Di Giusto 2009). This has important implications for taxonomists: if ecological characteristics can play a key role in distinguishing taxa, it is vital that as much ecological information is recorded and preserved in herbarium collections as possible.

Current 'best practice' for collecting herbarium specimens of *Nepenthes* involves collecting all parts of the plant: rosettes bearing lower pitchers, fragments of climbing stem bearing upper pitchers, and both and male and female inflorescences. *Nepenthes* specimens are difficult to press well, as the pitchers are often greatly distorted or damaged in the process. Furthermore, delicate structures such as the waxy zone on the inner surfaces of the pitchers (or the peristome itself) are particularly susceptible to damage through pressing, and important ecological information that can be derived through examination of the inner surfaces of the pitchers is often obscured in the process.

In this paper, we demonstrate how ecological research can be used to resolve taxonomic uncertainty about the status of a Bornean *Nepenthes* species, and propose revised 'best practice' methods for the collection of *Nepenthes* specimens for herbaria, so that the maximum amount of morphological and ecological information can be preserved.

Nepenthes macrophylla (Marabini) Jebb & Cheek – an example of a controversial taxon that is resolved through its ecological traits

Nepenthes villosa Hook.f. is a spectacular montane *Nepenthes* species that is endemic to Mt Kinabalu and Mt Tambuyukon in northern Sabah, Borneo (Fig. 1a). Along with the giant *N. rajah* Hook.f., it generated substantial public and botanical interest when it was described in 1852 (see Phillipps et al. 2009), as the extraordinary degree of development of the peristome ribs was unlike that of any other pitcher plant known at that time. A few years later Hooker (1859) described a remarkably similar species from the same two mountains: *Nepenthes edwardsiana* H.Low ex Hook.f. (Fig. 1b). This species was distinguished from *N. villosa* by its elongated, tubular pitchers, simpler peristome structure, ebracteolate pedicels and sparser indumentum. Danser (1928) was of the opinion that these characteristics were insignificant, and reduced *N. edwardsiana* to a synonym of *N. villosa*. In contrast, Harms (1936) reinstated *N. edwardsiana*.

More recently, Marabini (1987) described *N. edwardsiana* subsp. *macrophylla*, which is confined to the summit region of Mt Trusmadi, approximately 60 km SE of Mt Kinabalu. This taxon was distinguished from *N. edwardsiana* by its very large, ovate leaf blades and ovoid pitchers with broad, concave pitcher lids and less well-developed peristome teeth (Fig. 1c). Despite these marked differences, Marabini (1987) did not feel that they were significant enough to warrant distinction of the two taxa at specific rank. Jebb & Cheek (1997) disagreed and raised *N. edwardsiana* subsp. *macrophylla* to specific rank, an interpretation that has been adopted by subsequent authors, but not without some reservations (see Clarke 1997).

On the basis of pitcher characteristics alone, the competing interpretations of *N. macrophylla* cannot be resolved objectively. However, Chin et al. (2010) demonstrated that *N. macrophylla* belongs to an extraordinary group of three *Nepenthes* species (the other two are *N. rajah* and *N. lowii*) that trap the faeces of mountain tree shrews (*Tupaia montana* Thomas (Scandentia)) for nutritional benefit. Tree shrews visit the pitchers to feed on nectar produced by glands on the pitcher lid, and the concave structure of the lid results in the nectar being accessible only if the tree shrews sit astride the pitcher (Fig. 1d). *Tupaia montana* marks the location of valuable resources with faeces, and as pitcher nectar appears to be an important food source, these animals frequently defecate into the pitchers (Clarke et al. 2009, Chin et al. 2010).

Chin et al. (2010) also demonstrated that the trap geometry of *N. villosa* is significantly different to that of *N. macrophylla* and that its pitchers do not trap tree shrew faeces. Field observations by C. Clarke indicate that the same applies to *N. edwardsiana*. Clearly, *T. montana* distinguishes *N. macrophylla* from *N. edwardsiana* and *N. villosa* as a source of food, providing a compelling argument (in addition to the morphological characteristics listed by Jebb & Cheek (1997)) for the recognition of the former taxon as a distinct species. While it is clear that field observations are essential to the elucidation of the relationship between *N. macrophylla* and tree shrews, it is likely that this association would have been detected much earlier if herbarium specimens included longitudinally dissected pitchers and brief notes about their contents, as this information is obscured when the pitchers are pressed.

A 'best practice' method for the collection of Nepenthes specimens for herbaria

Chin et al. (2010) demonstrated that characteristics such as pitcher orifice depth, lid reflexion angle and lid concavity are central to the faeces-trapping syndrome in *N. lowii*, *N. macrophylla* and *N. rajah*. Furthermore, it has become apparent to ecologists that both the development and extent of the wax zone inside the pitchers, and the microstructure of the inner surfaces of the peristome, can play important roles in prey capture (Bohn & Federle 2004, Bauer et al. 2008). Unfortunately, the conversion of a markedly three-dimensional structure (such as a pitcher) to a two dimensional one by pressing herbarium material, causes much of this potentially valuable information to be lost. However, by making several modifications to the traditional method of collecting and pressing *Nepenthes* specimens, much of it can be retained and this should lead to more accurate and better informed interpretations of herbarium material. Accordingly, we propose the following protocol for the pressing and mounting of future collections of *Nepenthes*:

1. Collect stem fragments (separate ones if necessary) bearing both lower and upper pitchers (more than one pitcher of each type should be included in the collection);

Fig. 1 Pitchers of a. *N. villosa*; b. *N. edwardsiana*; c. *N. macrophylla*; d. *N. rajah*; showing *Tupaia montana* feeding at the inner surface of the pitcher lid. — Photo a, c, d: C. Clarke; b: C.C. Lee.

2. Inflorescences of both sexes should be collected if possible and should not be torn from the stems, as the nature of attachment to the stem can provide useful taxonomic information;
3. At least one pitcher of each type should be dissected longitudinally from the midpoint at the front and rear of the pitcher and mounted so that the inner surfaces of both the pitcher and the peristome are facing outwards from the card;

4. When pressing pitchers, try to preserve the angle of lid reflexion and concavity/convexity of the lids. If the lid angle cannot be faithfully preserved, indicate on the label what the approximate, typical angle of lid reflexion is, for both lower and upper pitchers. See Chin et al. (2010) for the measurement methodology;
   4.1. Preservation of pitchers in alcohol can be an effective method of preserving trap geometry. Alternatively (and particularly for very large pitchers) a series of high-quality photographs that accurately depict trap geometry will serve a similar purpose.
5. Collection notes should include information about:
   5.1. The habitat in which the material was collected (e.g. forest type, altitude, substrate);
   5.2. Observations about conspicuous animal visitors to both the pitchers and the inflorescences (invertebrates and vertebrates);
   5.3. Features unlikely to be obvious in preserved material (e.g. fragrance produced by pitchers (specify upper or lower form) or inflorescences (specify sex); viscosity of pitcher fluid (specify upper or lower form);
6. Deposition of material in the herbarium closest to the type locality. This makes it easier for field biologists (especially those based in Malesia) to study herbarium material economically and expeditiously; and
7. If possible, the contents of one upper and one lower pitcher should be extracted, dried and attached to the card in a clearly labelled, paper envelope.

CONCLUSION

Although it has been confirmed that *N. rajah*, *N. lowii* and *N. macrophylla* receive faecal inputs from tree shrews, several other species may also be candidates for this type of mutualism, but most of these are yet to be investigated because they grow in remote areas or herbarium material is scant. Three such species include *N. ephippiata* Danser and *N. attenboroughii* A.S.Rob., S.McPherson & V.B.Heinrich, and *N. truncata* Macfarl. Given the challenges associated with studying these taxa in the field, ecologists are likely to study herbarium material as a precursor to field experiments. By following the protocol outlined above, the chances of obtaining useful background ecological information are substantially improved. For taxonomists, ecological information that can be related to morphological characteristics can assist in deciding upon the status of controversial taxa, as we have demonstrated here for *N. macrophylla*. To date, the nutrient acquisition strategies of less than 10 % of *Nepenthes* species have been investigated. Our knowledge of the ecology of *Nepenthes* remains grossly deficient. Herbarium specimens that accurately preserve important ecological information can assist not only ecologists, but also taxonomists in their interpretations of the function of unique or atypical morphologies.

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