The taxonomic relevance of Naphthoquinones in tropical pitcher plants (*Nepenthes* L., Nepenthaceae)

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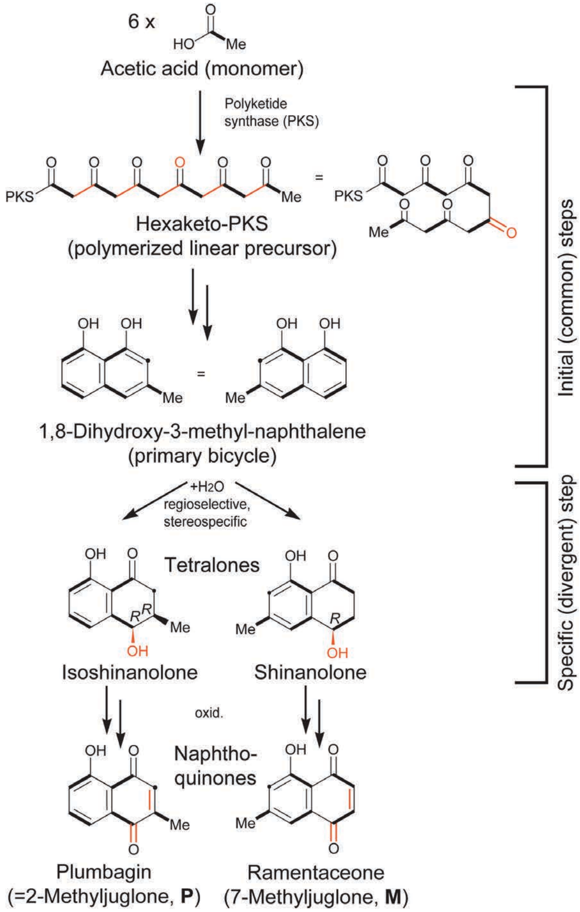
Abstract: The distribution of the naphthoquinones ramentaceone and plumbagin was studied among 50 taxa of the genus *Nepenthes*. Naphthoquinone patterns support classifications based on homology of plastid and/or nuclear genes to some extent, with plumbagin predominant in sections *Nepenthes*, *Urceolatae, Tentaculatae*, and *Regiae*, ramentaceone predominant in sections *Insignes* and *Villosae*, and both isomers present without clear predominance in sections *Pyrophytae* and *Montanae*. Only 9 of 96 studied species contained both isomers in the same plant. Naphthoquinone data from artificial hybrids of known parentage allowed conclusions on the biosynthesis of these compounds and the heredity of the respective enzymatic steps.

Introduction

The taxonomic utility of the distribution of the naphthoquinone isomers ramentaceone (7-methyljuglone, **M**) and plumbagin (2-methyljuglone, **P**) in the genus *Nepenthes* L. (Nepenthaceae) was already indicated by the first systematic screening (Schlauer *et al*. 2005). In some respects, the distribution of the isomers throughout the genus resembles patterns found in the closely related family Droseraceae (Culham & Gornall 1994; Schlauer *et al*. 2017; Schlauer *et al*. 2018; Schlauer *et al*. 2019a-c; Schlauer & Fleischmann 2021; Schlauer & Fleischmann 2022). All *Nepenthes* species investigated so far share the same chromosome count of 2n = 80 (Heubl & Wistuba 1998), while chromosome counts in Droseraceae are more diverse, with most species (ca. 53%) having 2n = 20 chromosomes or counts based on the base number x = 10 (followed by species with counts based on x = 7 ≈ 18% and x = 6 ≈ 16%, Kondo & Lavarack 1984; Kondo & Segawa 1988; Hoshi & Kondo 1998; James *et al*. 1997; Rivadavia 2005), so *Nepenthes* may be polyploid (octoploid with respect to a base count of x = 10). Together with the fact that Nepenthaceae are dioecious (obligately outcrossing) and genetically compatible across species boundaries, a comparatively high degree of hybridity can be expected for most taxa, especially where several different ones co-occur (Scharmann *et al*. 2021). Nevertheless, most *Nepenthes* species contain just one of the possible two naphthoquinone isomers (Schlauer *et al*. 2005). Taxa containing both isomers can thus be assumed to be of comparatively recent hybrid origin, similar to the situation in *Drosera* (Schlauer & Fleischmann 2016).

As naphthoquinone formation is widespread among Nepenthales, species devoid of naphthoquinones are most probably derived from ancestors that produced them. Hybrids of naphthoquinone-free species with naphthoquinone producing species can be expected to contain the quinone

Figure 1: 1 = *N. ampullaria*, 2 = *N. inermis*, 3 = *N. ventricosa*, 4 = *N. talangensis*, 5 = *N. reinwardtiana*, 6 = *N. stenophylla*, 7 = *N. eymae × clipeata*, 8 = *N. rafflesiana*. Photos: S. Hartmeyer.



Scheme 1: Hypothetical biosynthesis of naphthoquinones in Nepenthales (cf. Schlauer *et al.* 2018). Acetate-derived C_2 units shown as bold lines, resulting C_1 units as filled circles.

isomer(s) of the productive species. But it was not clear so far whether the ability to direct naphthoquinone biosynthesis selectively towards one of the isomers (the divergent step in common initial steps in biosynthesis, cf. scheme 1) or if it is inherited independently. The investigation of several artificial hybrids for their quinone patterns should yield some clues to resolve this question.

Materials and methods

All plants used in the present study were cultivated at Andreas Wistuba's and Siegfried & Irmgard Hartmeyer's greenhouses in southern Germany. Small disks (0.5 cm diam.) cut from leaf bases of living plants were investigated as reported previously (Schlauer *et al*. 2017).

Results

Naphthoquinones were detected in the investigated samples as summarized (together with previous results) in Table 1.

Table 1. *Nepenthes* taxa investigated and quinones found.				
Section[a]	Taxon	Provenance/comment[b]	Quinone(s)[c]	Ref.[d]
Nepenthes	*N. danseri*	New Guinea	**P**	1
Nepenthes	*N. distillatoria*	Sri Lanka	**P**	1
Nepenthes	*N. khasiana*	N-India; (SH)	**P**	*, 1
Nepenthes	*N. lamii*	New Guinea (Doorman Top)	**P**	1
Nepenthes	*N. madagascariensis*	Madagascar	**P**	1
Nepenthes	*N. masoalensis*	Madagascar	**P**	1
Nepenthes	*N. neoguineensis*	New Guinea; (AW)	**P**	*, 1
Nepenthes	*N. paniculata*	New Guinea; (AW)	**P**	*
Nepenthes	*N. pervillei*	Seychelles (Mahé, Morne Seychelloise); (SH)	**P+M**	*, 1
Nepenthes	*N. tomoriana*	New Guinea	**P**	1
Nepenthes	*N. treubiana*	New Guinea; (AW)	**P+M**	*
Nepenthes	*N. vieillardii*	New Caledonia	**0**	1
Urceolatae	*N. ampullaria*	Sumatra	**0**	1
Urceolatae	*N. bicalcarata*	Borneo (Malaysia)	**P**	1
Urceolatae?	*N. gracilis*	cult.	**P**	1, 4
Urceolatae?	*N. hirsuta*	Borneo	**0**	1
Urceolatae?	*N. mapuluensis*	Borneo	**0**	1
Urceolatae?	*N. mirabilis*	cult.	**P**	1
Urceolatae?	*N. papuana*	New Guinea (Doorman Top)	**P**	1
Tentaculatae	*N. glabrata*	Sulawesi	**P**	1
Tentaculatae	*N. hamata*	Sulawesi	**P**	1
Tentaculatae	*N. muluensis*	Borneo (Mulu)	**P**	1
Tentaculatae	*N. tentaculata*	Sulawesi & E Borneo	**P**	1
Tentaculatae	*N. undulatifolia*	Sulawesi; (AW)	**P**	*
Insignes	*N. bellii*	Philippines (Mindanao)	**P+M**	1
Insignes	*N. biak*	New Guinea (Biak); (AW)	**M**	*
Insignes	*N. burkei*	Philippines	**M**	1
Insignes	*N. campanulata*	Borneo	**0**	1
Insignes	*N. insignis*	New Guinea (2 accessions: Biak & type loc.) (AW)	**M**	*, 1; **P** in 2
Insignes	*N. merrilliana*	Philipines (Mindanao); (SH)	**0**	*, 1
Insignes	*N. sibuyanensis*	Philippines (Sibuyan); (AW)	**M**	*, 1
Insignes	*N. ventricosa*	Philippines (“green”)	**M**	1
Insignes	*N. ventricosa*	Philippines; (“porcelain”); (SH)	**P**	*, 1
Villosae	*N. boschiana*	Borneo	**P**	1
Villosae	*N. copelandii*	Philippines (Mt. Apo)	**M**	1
Villosae	*N. edwardsiana*	Borneo; (AW)	**M**	*

Table 1. Continued.				
Section[a]	Taxon	Provenance/comment[b]	Quinone(s)[c]	Ref.[d]
Villosae	*N. macrophylla*	Borneo (Trus Madi)	**M**	1
Villosae	*N. micramphora*	Philippines (Mindanao); (AW)	**P**	*
Villosae	*N. pulchra*	Philippines, (possibly hybrid with another species) (SH)	**M**	*
Villosae	*N. rajah*	Borneo	**P**	1
Villosae	*N. truncata*	Philippines (Mindanao); (SH)	**0**	*, 1
Villosae	*N. villosa*	Borneo	**P**	1
Pyrophytae	*N. albomarginata*	cult. ("red"); (SH) & Malaysia (Penang)	**M**	*, 1
Pyrophytae	*N. benstonei*	Malaysia (peninsular)	**P+M**	1
Pyrophytae	*N. gracillima*	Malaysia (Tahan)	**P**	1
Pyrophytae	*N. macfarlanei*	Malaysia (peninsular)	**P**	1
Pyrophytae	*N. macrovulgaris*	Borneo	**0**	1
Pyrophytae	*N. northiana*	Borneo	**P**	1
Pyrophytae	*N. rafflesiana*	cult. (SH)	**P**	*, 1
Pyrophytae	*N. ramispina*	Borneo; (SH)	**0**	*, 1
Pyrophytae	*N. reinwardtiana*	Borneo ("green"); (SH)	**P+M**	*, 1
Pyrophytae	*N. sanguinea*	Malaysia (Genting)	**M**	1
Pyrophytae	*N. smilesii*	Thailand	**P**	1
Pyrophytae	*N. thorelii*	Vietnam	**0**	1; **P** in 3
Montanae	*N. adnata*	Sumatra	**M**	1
Montanae	*N. aristolochioides*	Sumatra	**P**	1
Montanae	*N. bongso*	Sumatra (Singgalang)	**M**	1
Montanae	*N. densiflora*	Sumatra	**P+M**	1
Montanae	*N. diatas*	Sumatra (Bandahara)	**P**	1
Montanae	*N. dubia*	Sumatra (Barisan)	**P**	1
Montanae	*N. eustachya*	Sumatra	**P**	1
Montanae	*N. gymnamphora*	Sumatra; (SH)	**P**	*, 1
Montanae	*N. inermis*	Sumatra (Gadut)	**P**	1
Montanae	*N. jacquelineae*	Sumatra	**M**	1
Montanae	*N. lavicola*	Sumatra	**0**	1
Montanae	*N. longifolia*	Sumatra	**P**	1
Montanae	*N. mikei*	Sumatra	**P**	1
Montanae	*N. ovata*	Sumatra	**M**	1
Montanae	*N. rhombicaulis*	Sumatra	**P+M**	1
Montanae	*N. singalana*	Sulawesi (Mantalingajan & Singgalang)	**P**	1

Table 1. Continued.				
Section[a]	Taxon	Provenance/comment[b]	Quinone(s)[c]	Ref.[d]
Montanae	*N. spathulata*	Sumatra (G. Tudjuh & Tanggamus)	**M**	1
Montanae	*N. spectabilis*	Sumatra (Pangulubao & Bandahara)	**0**	1
Montanae	*N. sumatrana*	Sumatra	**P**	1
Montanae	*N. talangensis*	Sumatra	**P**	1
Montanae	*N. tenuis*	Sumatra	**P+M**	1
Montanae	*N. tobaica*	Sumatra (Toba)	**P**	1
Regiae	*N. alata*	Philippines	**P**	1
Regiae	*N. burbidgeae*	Borneo	**0**	1
Regiae	*N. chaniana*	Borneo; (SH)	**P**	*, 1
Regiae	*N. clipeata*	Borneo; (SH)	**P**	*, 1
Regiae	*N. deaniana*	Philippines (Palawan)	**P**	1
Regiae	*N. ephippiata*	Borneo	**P**	1
Regiae	*N. eymae*	Sulawesi; (SH)	**P**	*, 1
Regiae	*N. faizaliana*	Borneo	**P**	1
Regiae	*N. fusca*	Borneo (Kinabalu)	**P+M**	1
Regiae	*N. graciliflora*	Philippines; (SH)	**M**	*
Regiae	*N. klossii*	New Guinea; (AW)	**0**	*
Regiae	*N. lowii*	Borneo (Mulu)	**P**	1
Regiae	*N. maxima*	cult. (SH) & New Guinea (Poso)	**P**	*, 1
Regiae	*N. oblanceolata*	Papua; (SH)	**P**	*, 1
Regiae	*N. philippinensis*	Philippines (Palawan)	**P**	1
Regiae	*N.* spec.	Borneo (E Kalimantan)	**P**	1
Regiae	*N. stenophylla*	Borneo (Bario)	**P**	1
Regiae	*N. veitchii*	Borneo; (Bario, "highland"); (SH)	**0**	*, 1
Regiae	*N. viridis*	Philippines (Dinagat); (SH)	**M**	*
Artificial Hybrids				
Regiae × *Insignes*	*N. alata* × *ventricosa*	P × P; *Nepenthes* × *ventrata* (SH)	**P**	*
Regiae × *Villosae*	*N. burbidgeae* × *edwardsiana*	0 × M; (AW)	**M**	*
Insignes × *Regiae*	*N. campanulata* × *maxima*	0 × P; (AW)	**P**	*
Regiae	*N. chaniana* × *veitchii*	P × 0; *Nepenthes* "*pilosa*" × *veitchii* (SH)	**P**	*
Regiae	*N. clipeata* × *eymae*	P × P; (AW)	**P**	*

Table 1. Continued.				
Section[a]	Taxon	Provenance/comment[b]	Quinone(s)[c]	Ref.[d]
Regiae	*N. eymae* × *clipeata*	P × P; P. Debbert, Munich ca. 1989 (SH)	**P**	*
Tentaculatae × Insignes	*N. hamata* × *campanulata*	P × 0; (AW)	**P**	*
Regiae × *Insignes*	*N. lowii* × *campanulata*	P × 0; (AW)	**P**	*
Regiae	*N. lowii* × *ephippiata*	P × P; (AW)	**P**	*
Regiae × *Insignes*	*N. lowii* × *merrilliana*	P × 0; (AW)	**P**	*
Regiae × *Montanae*	*N. lowii* × *spectabilis*	P × 0; (AW)	**P**	*
Regiae × *Villosae*	*N. lowii* × *truncata*	P × 0; (AW)	**P**	*
Regiae × *Pyrophytae*	*N. maxima* × *northiana*	P × P; *Nepenthes* ×*mixta*; (SH)	**P**	*
Montanae × *Villosae*	*N. talangensis* × *truncata*	P × 0; Weil am Rhein 2007 (SH)	**P**	*
Villosae × *Regiae*	*N. truncata* × *ephippiata*	0 × P; (AW)	**P**	*
Regiae × *Villosae*	*N. veitchii* × *edwardsiana*	0 × M; Weil am Rhein 2014 (SH)	**M**	*
Regiae × *Villosae*	*N. veitchii* × *edwardsiana*	0 × M; Weil am Rhein 2018 (SH)	**M**	*
Regiae	*N. veitchii* × *lowii*	0 × P; (AW) and Weil am Rhein 2012 (SH)	**P**	*
Regiae	*N. veitchii* × *maxima*	0 × P; H. Hennern (SH)	**P**	*
Insignes × *Urceolatae?*	*N. ventricosa* × *mapuluensis*	P × 0; (AW)	**P**	*
Insignes × *Urceolatae?*	*N. ventricosa* × *mapuluensis*	P × 0; (AW)	**P**	*
Insignes × *Regiae*	*N. ventricosa* × *maxima*	P × P; (see reference)	**P**	5

[a]Classification according to Clarke *et al*. 2018
[b]Plants investigated in this study: AW = cultivated by Andreas Wistuba; SH: cultivated by Siegfried & Irmgard Hartmeyer
[c]**P**: plumbagin, **M**: ramentaceone, **0**: no quinone detected
[d] * New/additional data from this study
1 Schlauer *et al*. 2005; all investigated specimens cultivated by Joachim Nerz
2 Rischer *et al*. 2002 A hybrid origin of the studied plants cannot be excluded. Possible partners with **P** and putatively overlapping distribution are *N. mirabilis, N. papuana* and *N. maxima* (H. Rischer pers. comm.).
3 Likhitwitayawuid *et al*. 1998
4 Aung *et al*. 2002
5 Shin *et al*. 2007

Discussion

Like in the related family Droseraceae, naphthoquinone patterns are fairly constant within species, and some isomer preference is observed in most sections of *Nepenthes*. **P** is predominant in sections *Nepenthes* (present in 91% of the investigated species, vs. 17% **M**), *Urceolatae* (57% **P**, 0% **M**), *Tentaculatae* (100% **P**, 0% **M**), *Montanae* (68% **P**, 36% **M**), and *Regiae* (74% **P**, 16% **M**), **M** is predominant in section *Insignes* (67% **M**, 22% **P**), and both isomers are present without clear predominance in sections *Villosae* (44% each) and *Pyrophytae* (53% **M**, 38% **P**).

In spite of the anticipated high heterozygosity (frequent introgression, high ploidity) of most *Nepenthes* taxa, only 9 of 96 studied species (9.4%, with no obvious concentration in any section) contain both isomers in the same plant. This proportion is only slightly larger than in Droseraceae (19 of 214 investigated taxa, 8.9%), which may indicate selection against heterozygosity in this trait. Unfortunately, no artificial hybrid between quinone-heterogenous parent species was available in this study. But it is anticipated that such hybrids would contain both isomers like in the related genus *Drosera* (Schlauer & Fleischmann 2016).

All investigated hybrids contained their parents' naphthoquinone isomers, and in particular crosses with naphthoquinone-free species did not yield additional isomers. This strongly suggests that the ability to direct naphthoquinone biosynthesis towards one of the isomers (divergent regioselectivity) is inherited together with the ability to produce naphthoquinones (common polyketide synthesis and subsequent redox/cyclization reactions), and both abilities (that are most probably dependent on separate enzymes, cf. scheme 1) are obviously lost in species that do not contain any isomer.

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