Article

Preliminary study on the inner pitcher structure of five *Nepenthes* species from Sabah, Borneo

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Abstract

Nepenthes pitchers are quite diverse in both their macromorphological and micromorphological structures. The inner pitcher structures of five *Nepenthes* species from Sabah, Borneo, were examined in cuticular and sectional studies to clarify their differences in micromorphology. The key differences observed in the inner surface of pitchers among the five *Nepenthes* species are 1) the existence and density of lunate cells below the peristome, 2) the shape and size of the digestive glands, and 3) the shape and size of the lunate cells above the glands. Given that the glands of carnivorous plants play roles in sensing the arrival of potential prey, secreting digestive fluid, and absorbing nutrients from digested insects, the differences in morphologies of the glands and lunate cells might have some relationship with the ecology inside the pitchers. The factors behind these differences are discussed here from an ecological point of view.

Key words: *Nepenthes*, carnivorous plant, pitcher structure, digestive gland, lunate cell, Borneo

Introduction

The term "carnivorous plant" is not a name for a monophyletic group of plants, but for wide range of plants that are distinguished most by their strange life-styles. They share similar ecological features such as the ability to grow in poor soils, to trap insects, digest the trapped insects, and then absorb the consumed nutrients. However, the morphologies and mechanisms of their traps are quite diverse, e.g., adhesive traps that stick to insects like cockroach catchers, snap traps that clip insects, or pitchers to that lure insects inside. These traps are now recognized to have independently evolved from different lineages of plants (Albert et al., 1992), which helps explain why carnivorous plants have such great variation.

Some carnivorous plants have a rich diversity even within a related group. One such example is the *Nepenthes* species.

Nepenthes L. is the only genus of the family Nepenthaceae, and contains about 80 species (Cronquist, 1981). Their distribution ranges from the eastern India to northern Australia, including New Caledonia, and Madagascar as well. The center of the diversity is in Borneo in Southeast Asia, where about 30 species are recognized (Clarke, 1997).

Nepenthes pitchers are reported to be modified leaves (Juniper et al., 1989). The pitchers retain water inside and trap insects, sometimes actively, attracting them by scent or nectar secreted from the lids or outside of the pitchers. The water inside usually contains strong acid and enzymes that digest the trapped insect. The water, a digestive fluid, is secreted by digestive glands inside the pitchers. The glands are also reported to play other roles at the same time, such as sensing the arrival of potential prey and absorbing nutrients from digested insects (Juniper et al., 1989). The procedures to trap and digest insects and the basic structures of the pitchers are similar among the *Nepenthes* species, but the morphologies of the pitchers are so different that they

can be used as a key to identify the species. A great variation is also recognized in the pitchers' micromorphology (Kurata, 1976). However, it is still unknown why such diversity has developed within the *Nepenthes* species.

Although many recent studies of the *Nepenthes* describe the macromorphologies of the pitchers (Juniper et al., 1989; Phillipps and Lamb, 1996), few studies of their micromorphology exist. Owen and Lennon (1999) presented a detailed description of the microstructure and development of *N. alata* pitchers, but they did not describe the variety among different *Nepenthes* species. Kurata (1976) provided pictures of the inner pitcher surface among 16 species, but the images are of low resolution and not suited for detailed comparative studies. Additional data is still needed to clarify the differences among *Nepenthes* pitchers and explain the reasons for the differentiation.

To provide more detailed information for the micromorphological studies of *Nepenthes* pitchers, we carried out comparative studies on five Bornean species. We observed the inner structure of the pitchers, focusing on their cuticle (cell wall morphology of epidermis) and cross section, because morphology of the epidermal cells might provide important functional information on the interaction between the pitchers and the organisms inside them. The aim of this study is to describe the inner surface structure as a preliminary observation, which could lead us to find the differences we need to study further to investigate the reasons for pitcher diversification, especially from the ecological point of view. We also made an attempt to see if there is any relationship between the inner pitcher characters we recognized and their ecological conditions using data in the literatures.

Materials and Methods

Five *Nepenthes* species were collected mainly from swamp forests in western Sabah, Malaysia, during expeditions organized by the Universiti Malaysia Sabah from 1998 to 1999. The specimens used for this study are listed in Table 1 with their exsiccatae data (for detailed information of the habitat and general morphology of the five species, except for *N. reinwardtiana*, see Takahashi and Ahmad, 2000). The pitchers were fixed in 70% ethanol in the field. Voucher specimens are stored in the herbarium at the Museum of Nature and Human Activities, Hyogo, Japan (HYO).

For the observations on cuticles, ca. 1 cm^2 samples were taken from the leaf blade (the part before the tendril and pitcher) in *Nepenthes ampullaria* and *N.*

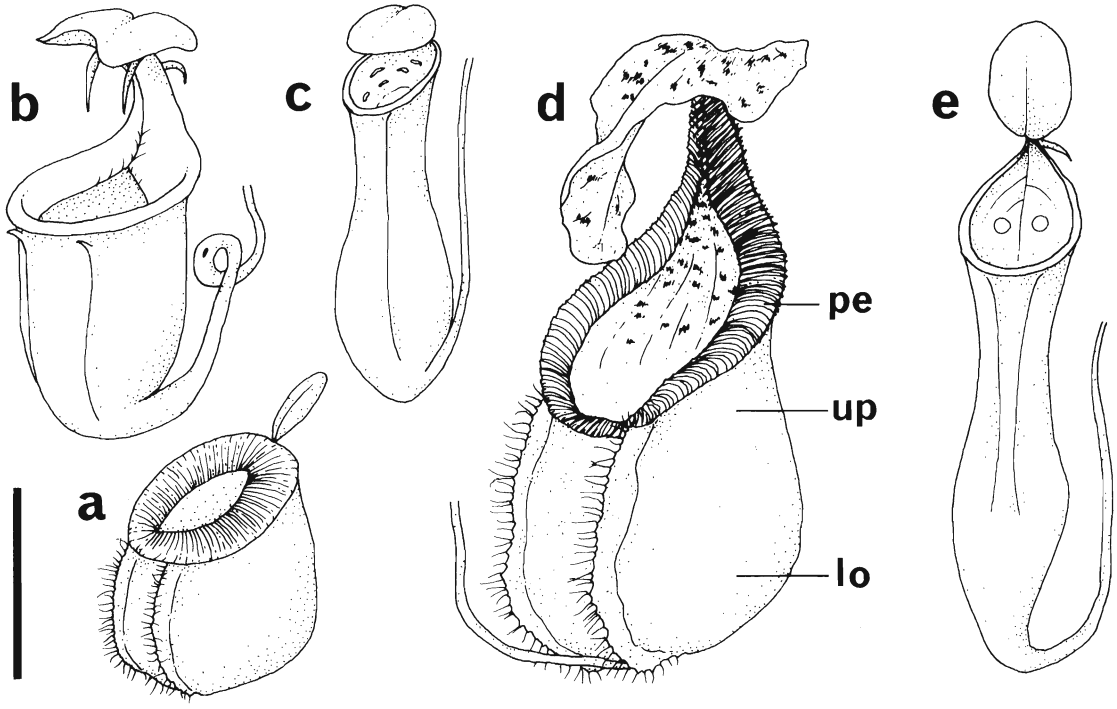

Fig. 1. Pitchers of the five examined *Nepenthes* species. **a:** *N. ampullaria*. **b:** *N. bicalcarata*. **c:** *N. gracilis*. **d:** *N. rafflesiana*. **e:** *N. reinwardtiana*. Bar = 5 cm. *Figure abbreviations:* **pe**, peristome; **up**, upper part; **lo**, lower part.

Table 1. Exsiccatae samples for cuticular and sectional studies of the five *Nepenthes* species.

Scientific name	Collection no.	Locality
Nepenthes ampullaria Jack	Takahashi et al. 99503	Klias Forest Reserve, Sabah
Nepenthes bicalcarata Hook. f.	Takahashi et al. 99519	Klias Forest Reserve, Sabah
Nepenthes gracilis Korth.	Takahashi et al. 99009	Klias Forest Reserve, Sabah
Nepenthes rafflesiana Jack	Takahashi et al. 99212	Klias Forest Reserve, Sabah
Nepenthes reinwardtiana Miq.	Takahashi et al. 98012	Tabin Wildlife Reserve, Sabah

bicalcarata, and from different parts of the pitchers among the five species (Fig. 1). They were placed into test tubes with ca. 1 ml of 30% H_2O_2 and ca. 0.5 ml of 90% ethanol and then heated in a boiling water bath for about 3 hours. When the samples turned to light yellow or white, they were transferred from the tube to a petri dish with water. The cellular contents of the samples were brushed away with fine artists' brushes to clean the cuticles on both surfaces of the samples. The samples were then returned to a test tube of 90% ethanol for about 15 hours to clean the cuticles. The cuticles were rinsed in 2% ammonia to adjust the pH, and stained in 0.1% Crystal Violet for approximately 30 seconds. The stained cuticles were then mounted in phenol glycerin jelly on a slide. After cleaning off the excess jelly, the coverslips were ringed with nail varnish to retard dehydration.

For the sectional observations, small pieces were cut from the sample pitchers, dehydrated by ethanol-xylol series, and embedded in the paraplast. Sections of about 10μm thick were made with a sliding microtome. The sections were stained with a standard combination of safranin and fastgreen and then mounted in Canada balsam for microscopy.

Results

The cuticles studied here are of the cuticular membrane on the epidermal cells or the stomatal complex that remained through the preparation. Therefore, the characters described here are mostly of the epidermal cells or the stomatal complex whose impression is preserved in the membrane.

The cuticular and sectional features of the inner pitchers are different for each part of the pitcher, as well as within each species. We describe here the features of a pitcher of *Nepenthes rafflesiana* first, then those of the five different species.

Pitcher of *Nepenthes rafflesiana*

1) Cuticular features

Three different parts are recognized in the inner pitcher, i.e., the peristome, upper part, and lower part (Fig. 1d).

The peristome region appears to be the outside structure because it is extending and protruding outward, but it actually is a part of the inner surface. It comprises a series of heart-shaped cells with their long beaks pointing downward and overlapping the cell below (Fig. 2). The cells line up in a very orderly fashion and make the beaks appear as long columns with many thorns along one side.

The inner surface of the pitcher below the peristome divides into two different regions, the upper part and the lower part.

The inner surface of the upper part is covered with epidermal cells with their anticlinal walls undulate to sinuous, and studded with some elliptic cells which have lunate-shaped lips protruding downward (Figs. 3, 4). The letter elliptic cells have been referred to as lunate cells or a transformed stomatal complex (Owen and Lennon, 1999).

The inner surface of the lower part has epidermal cells with rounded anticlinal walls, and many glands that slightly sink toward the inside. Several epidermal cells above the glands protrude and make an eyelid-like structure covering the upper part of glands (Fig. 5). These cells are also referred to as lunate cells (Owen and Lennon, 1999).

The outer surface of the pitcher shows no difference between the upper part and the lower part, which is uniformly comprised of epidermal cells with rounded to straight anticlinal walls. Stellate hairs and sinuous, somewhat disk-like hairs are scattered over the surface as well as stomata are scattered.

2) Sectional features

In the sectional view of the pitcher walls, both surfaces are composed of flat and thin cells in one layer, with an inner part comprising large and round cells (Fig.

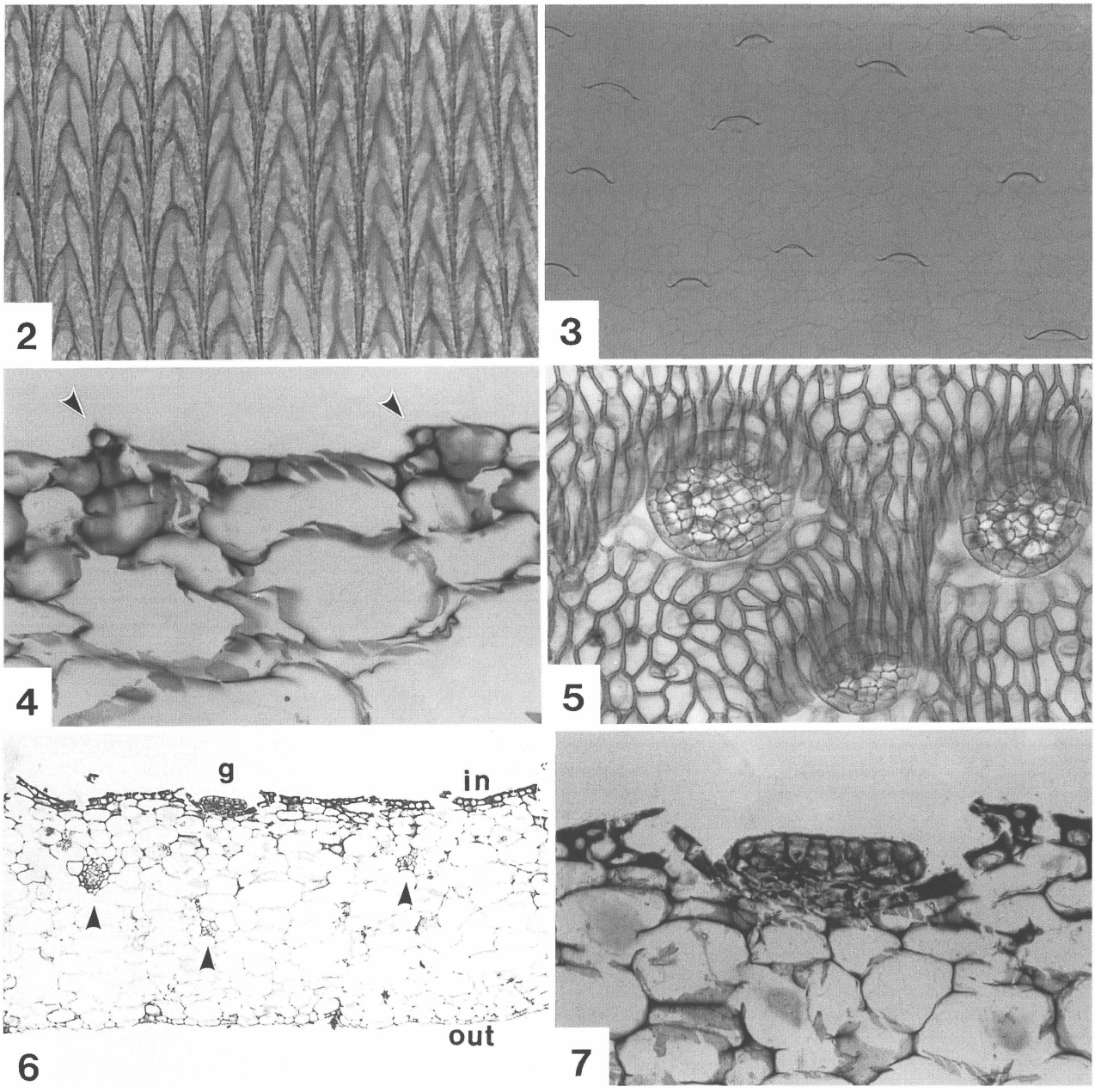

Fig. 2-7. *Nepenthes rafflesiana*. **2:** Peristome, showing closely packed cells with beaks pointing downward. **3:** Lunate cells on the inner surface of upper part of pitcher. **4:** Longitudinal section of the inner surface of the upper part of pitcher (upper part on the right, lower part on the left), showing sectional view of lunate cells (arrowheads). **5:** Digestive glands and eyelid-like lunate cells covering the upper half of the glands. **6:** Longitudinal section of the lower part of the pitcher wall, showing a digestive gland on the inner surface and vascular bundles (arrowheads) scattered among large round cells in the inner part of wall. **7:** Longitudinal section of a digestive gland. Cells at the outermost layer are slightly elongated (upright) and dark stained. *Figure abbreviations*: **g**, digestive gland; **in**, inner surface of pitcher wall; **out**, outer surface of pitcher wall. Magnification: x200 in Figs. 2-4, 7; x150 in Fig. 5; x50 in Fig. 6.

6). Vascular bundles are scattered on the inner part of the walls and helical tracheary elements are occasionally present under the glands. The cellular composition of the walls does not differ between either side except of the glands on the inner surface and stomata on the outer surface.

In the lower part of the pitcher, the digestive glands are settled in shallow depressions on the inner surface. It appears from longitudinal sections that the glands usually consist of four cell layers: 1) an outermost layer consisting of 9 to 10 upright cells; 2) a second layer consisting of 6 to 8 round cells; 3) plus third and fourth, or innermost, layers consisting of 3 to 4 large flat cells (Fig. 7). The cells of the outer layer are stained in a dark color, which becomes lighter on the inner layers.

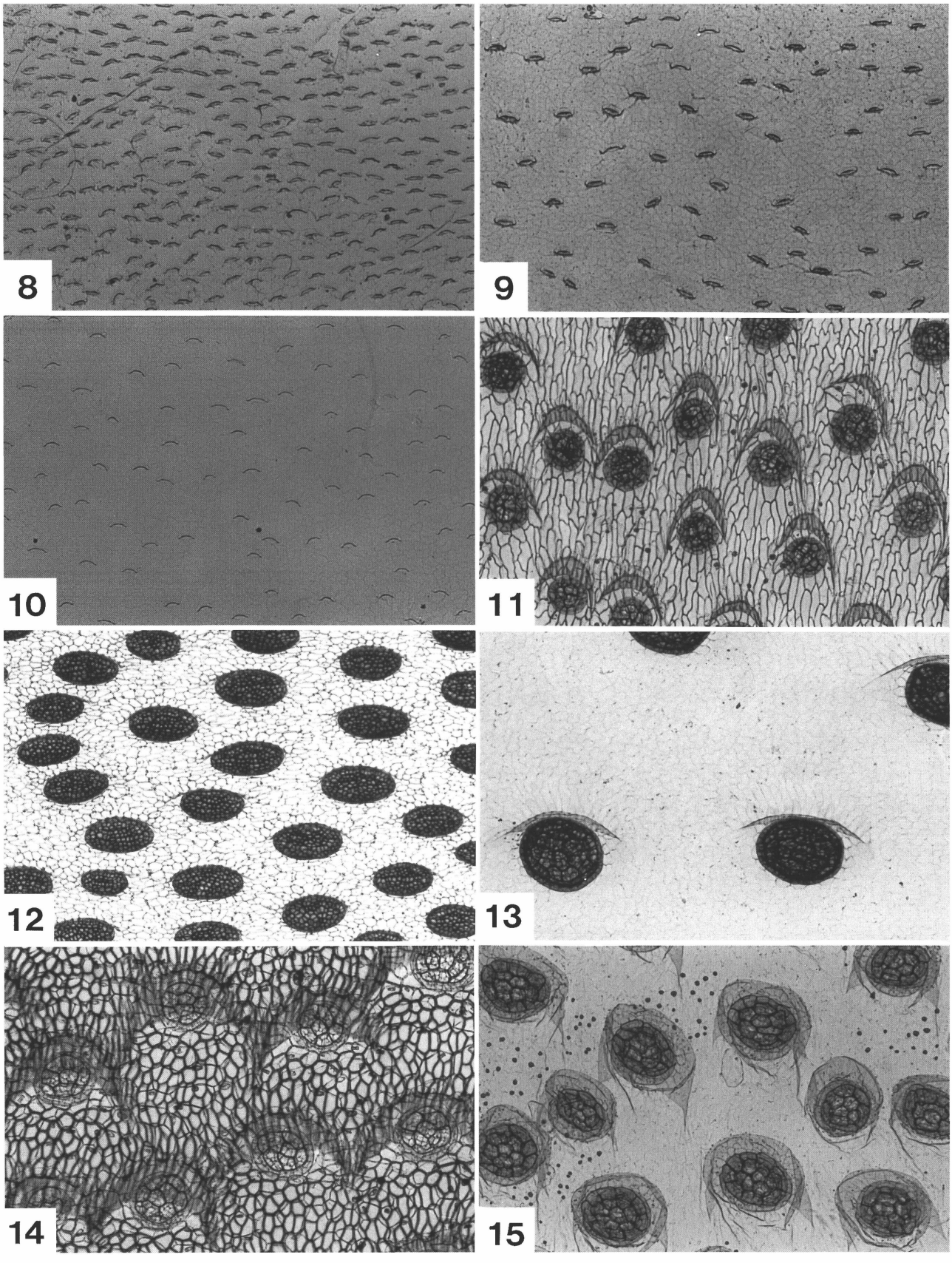

Fig. 8-15. Distribution of the lunate cells of the upper part (Figs. 8-10) and the digestive glands of the lower part (Figs. 11-15) of pitchers of *Nepenthes* species. **8:** *N. gracilis*. **9:** *N. rafflesiana*. **10:** *N. reinwardtiana*. **11:** *N. ampullaria*. **12:** *N. bicalcarata*. **13:** *N. gracilis*. **14:** *N. rafflesiana*. **15:** *N. reinwardtiana*. Magnification: x80 in all figures.

Table 2. Qualitative and quantitative data of the lunate cells and digestive glands in the five *Nepenthes* species.

	N. ampullaria	*N. bicalcarata*	*N. gracilis*	*N. rafflesiana*	*N. reinwardtiana*
Lunate cells below peristome	Absent	Absent	Present	Present	Present
Density of lunate cells (no. of cells / 0.1mm^2)	-	-	47	11	10
Approx. size of glands (minor axis x major axis; μm)	90-110 x 90-110	60-80 x 110-160	140-170 x 180-220	110-130 x 120-180	110-140 x 120-170
Thickness of glands (μm)	40-50	30-40	40-50	50-60	70-90
Density of glands (no. of glands / 1mm^2)	20	32	4	11	14
Density of glands toward bottom	Increased	Increased	Increased	Decreased	Decreased
Depth of lunate cells above glands (μm)	50-60	4-7	20-30	80-90	130-170
Depth of depressions for glands (μm)	50-60	40-50	70-80	70-90	120-140
Cellular composition of glands					
- vertical cell layers	4	4	4	4	6
- no. of cells of outermost layer in longitudinal section	8-10	9-11	12-16	9-10	8-9

Pitchers of the five species

1) Cuticular features

The peristome features among the five species are quite different even in their macromorphology. We exclude comparison of these features because our study focus is on the micromorphological differences and that any comparison of peristomes in micromorphology might yield a problem of homology. We compared the features of the inner surface only below the peristome in the pitchers of the five species. The results are shown in Table 2.

The epidermal cells of the interior surfaces have smooth periclinal walls and rounded to sinuous or straight anticlinal walls. The shape and size of the epidermal cells often vary, but those of *Nepenthes rafflesiana* and *N. ampullaria* are partly dimorphic, while those of *N. reinwardtiana* are rather uniform through the parts. The epidermal cells are relatively long in *N. ampullaria*.

Below the peristome there is an area where protruding epidermal cells ("lunate cells") are scattered in *Nepenthes gracilis, N. rafflesiana,* and *N. reinwardtiana* (Figs. 8-10). However, *N. ampullaria* and *N. bicalcarata* lack these kinds of cells, instead employing digestive glands distributed just below the peristome. The density of lunate cells is different among the three former species, and in the *N. gracilis* pitcher is conspicuously high (Table 2).

Below the area with lunate cells or directly below the peristome, the area with numerous glands extends to the bottom of the pitchers. The shape and size of the glands are different among the five species (Figs. 11-15). *Nepenthes ampullaria, N. rafflesiana,* and *N. reinwardtiana* have circular glands, while *N. bicalcarata* and *N. gracilis* have rather elliptical ones. The size of the glands is relatively large in *N. gracilis*; medium size in *N. bicalcarata*, *N. rafflesiana*, and *N. reinwardtiana*; and small in *N. ampullaria*. The density of the glands varies among the species, with the least dense in *N. gracilis*. Whether it increases or decreases toward the bottom also depends on the species (Table 2). The shape of the lunate cells above the glands (not the "lunate cells" below the peristome) is diverse among the five species, from almost not protruding at all in *N. bicalcarata* (Fig. 12) to conspicuously deep and almost completely covering the glands in *N. reinwardtiana* (Fig. 15).

2) Sectional features

The cellular composition of the glands was also compared in the five species. The results are shown in Table 2.

The cell structure of the glands is basically similar among the five species of *Nepenthes* but there is a slight difference depending on the species (Figs. 7, 16-19). Cell layers comprising the glands usually number four, but there are six in *N. reinwardtiana*. Cells of the

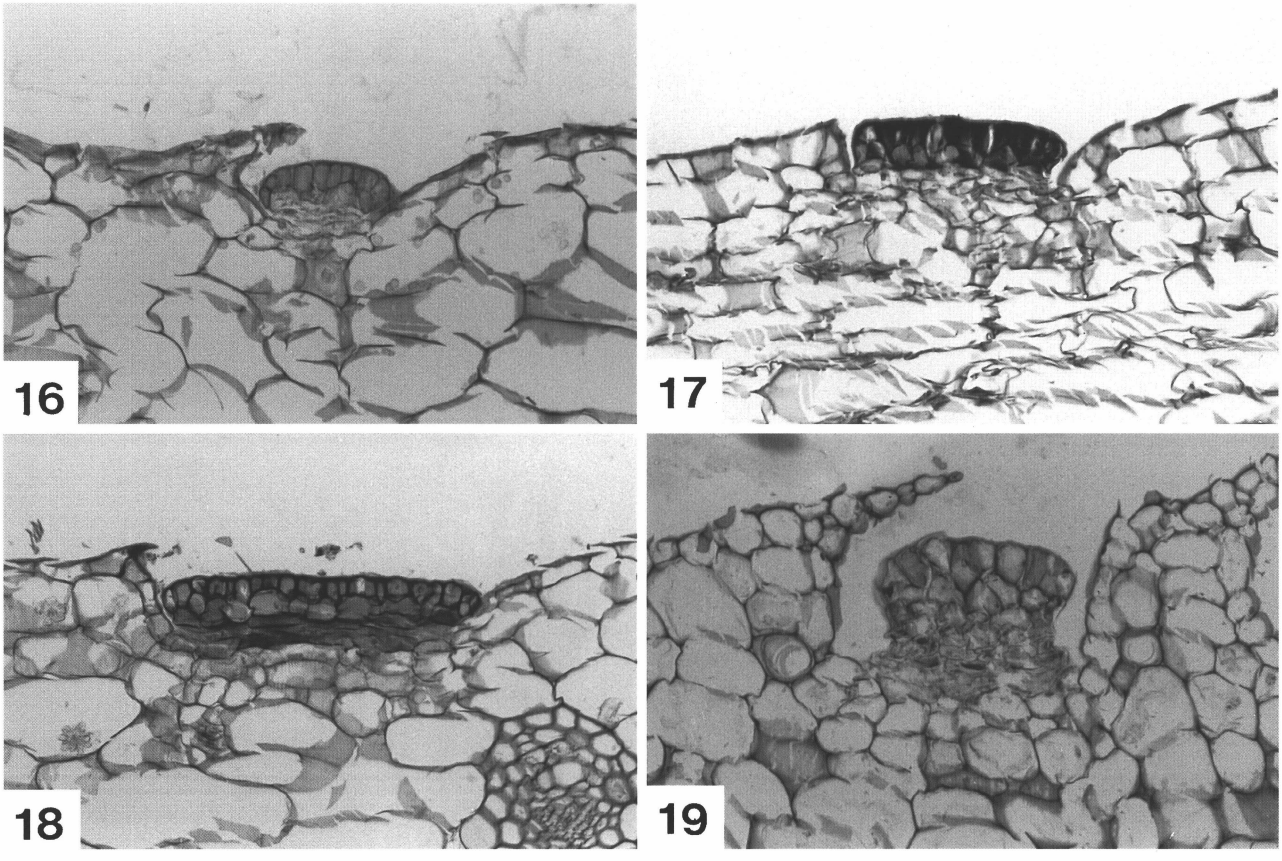

Fig. 16-19. Sectional view of the digestive glands of *Nepenthes* species. **16:** *N. ampullaria*. **17:** *N. bicalcarata*. **18:** *N. gracilis*. **19:** *N. reinwardtiana*. Magnification: x200 in all figures.

outermost layer are usually of 8 to 10 upright cells, but 12 to 16 in *N. gracilis*. The depressions for the glands on the inner surface of pitchers are commonly shallow yet quite deep in *N. reinwardtiana* (Fig. 19).

Discussion

Obvious differences were observed among the five *Nepenthes* species in the following characters of the inner surface of pitchers: 1) existence and density of the lunate cells below the peristome, 2) shape and size of the digestive glands, and 3) shape and size of the lunate cells above the glands.

We recognized some differences in shape and size of the epidermal cells among the species. However, these differences were sometimes observed even within a single pitcher of one species. We are not yet able to ascertain whether they are interspecific or intraspecific characters. Since we had only one sample per species available for this study, more samples are needed to clarify this issue.

The cells below the peristome in some species and the cells above the glands are both named as lunate cells in the literature, but the structures are quite different from one another. The lunate cell below the peristome is a single cell and elongated horizontally. On the contrary, the cells above the glands comprise several cells and are elongated longitudinally. These two "lunate cells" are likely from different origins and not homologous.

The lunate cells below the peristome have been reported to be missing only in *Nepenthes ampullaria* (Owen and Lennon, 1999). However, we observed they are also absent in *N. bicalcarata*. Zeigler (1987) referred to these cells as a transformed stomatal complex, but it is not likely so because the inner pitcher corresponds to the adaxial surface of a leaf (Owen and Lennon, 1999) and no stomata were observed on the adaxial surface of the blade (the part before the tendril and pitcher) in our study. Thus far, it is dubious whether the lunate cells are transformed stomata, although we currently have no other theory for their origin.

As for the differences of the glands and the lunate cells above the glands, we searched for a reason from an ecological point of view. *Nepenthes* pitchers embrace complicated dynamics of prey assemblages and

Table 3. PH, prey, and inhabitants inside the pitchers of the five Nepenthes species (based on Phillipps and Lamb, 1996*; estimated from Kato et al., 1993**).

	N. ampullaria	*N. bicalcarata*	*N. gracilis*	*N. rafflesiana*	*N. reinwardtiana*
PH*	3.72	4.33	2.43	2.55	-
Rates of prey**	F 72%; I 8%; D 6%; O 14%	-	F 95%; O 5%	-	F 90%; D 2%; B 4%; O 4%
Approx. no. of inhabitants per pitcher**	5	-	3	-	10

F = Formicoidea, I = Isoptera, D = Diptera, B = Blattaria, O = others.

inhabitant communities (Kato et al., 1993; Phillipps and Lamb, 1996), so we selected a few ecological factors of the inner pitchers and compared them to the differences of the inner structure among the five species (Table 3).

Acidity of the five species appears to be higher in species with larger glands, while it is lower in species with a higher density of glands. This might be explained if the ability to secrete acid is regulated by the size of the glands, not by their density.

No correspondence was found between the variation of the prey and the structures of the glands or lunate cells (above the glands), for ants are reported to be usually the most common preys (Kato et al., 1993). Owen and Lennon (1999) assume that the lunate cells have a role to prevent insects from using the glands or epidermal cavity as footholds for escape. However, this assumption cannot explain the differences in structure of the lunate cells among the species since there is no reason to differentiate their structure to trap the same insects. Rather, the degree of protrusion in the lunate cells appears to correspond to the number of inhabitants. More data on the inhabitants might contribute to detecting the function of the lunate cells.

In summary, even as a very preliminary view, we can recognize a possibility that the micromorphologies of the inner surface have some ecological functions inside the pitchers. The lunate cells above the glands, especially, might have an unknown meaning to the inhabitants in the pitchers. Intensive ecological studies on the insects and microorganisms in *Nepenthes* pitchers with more information on the microstructures of the inner pitcher surface could provide an interesting view of the relationships between the pitchers and the inhabitants inside the pitchers.

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