論文の内容の要旨

論文題目　**Phylogenetic analysis of pitcher plants *Nepenthes* with molecular evolution of the key digestive enzyme nepenthesin**

（食虫植物ウツボカズラの系統解析と消化酵素ネペンテシンの分子進化）

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*Nepenthes* are the genus of the monotypic family Nepenthaceae, which widely distributed in the Asian tropics mainly in South-east Asia and are endemic to the Sunda shelf region (Ellison et. al., 2003; McPherson, 2009). *Nepenthes* inhabit a wide array of habitat types, ranging from coastal mangrove forests, to the margins of lowland evergreen rainforests, to high altitude montane forests and scrub vegetations (McPherson, 2009; Jebb and Cheek, 1997). *Nepenthes* live in nutrient-poor soils and have overcome this resources deficiency by taking most of their nutrients from the digestion of preys trapped in their pitchers with their enzymatic fluid (Ellison et. al., 2003), containing an aspartic proteinase called nepenthesin, which is the only known extracellular proteinase of plant origin (Athauda et. al., 2004; Hatano and Hamada, 2008). Therefore, this enzyme is interesting from various points of view, such as physiological role, structure-function relationships and molecular evolution.

Phylogeny and phytogeography of *Nepenthes*

The study of phylogenetic relationships in the genus *Nepenthes* had previously been investigated using molecular phylogenetic analysis based on DNA sequence of plastid trnK intron (Meimberg et. al., 2001). But, comparative analysis between *Nepenthes* trnK intron with its translocated copy had demonstrated a topological incongruence, thus it was evaluated that the phylogeny of the trnK intron could not represent true phylogeny of *Nepenthes* (Meimberg et. al., 2006; Meimberg and Heubl, 2006). In the present study, nucleotide sequence of the Internal Transcribed Spacer (ITS) were examined to resolve the phylogenetic relationships within the genus *Nepenthes*. It was reported that the ITS region from *N. ventricosa* and *N. alata* showed many variable characters that are potentially informative for resolving *Nepenthes* phylogeny (Alejandro et. al., 2008). In addition, the study of adaptive evolution of nepenthesin as the key enzyme for nitrogen uptake for *Nepenthes*, has never been conducted. Hence, the objectives of this study were as follows : (1) to clarify the phylogenetic relationships of *Nepenthes* based on ITS nucleotide sequences and the comparison with the current classification, (2) to study the correlation between *Nepenthes* phylogeny with their habitats and morphological characteristics, and (3) to study evolutionary adaptation of nepenthesin to different

habitat types of *Nepenthes*.

In total, 55 ITS sequences from 54 *Nepenthes* species were analyzed. *Ancistrocladus robertsoniorum* (Ancistrocladaceae, GQ443551) and *Dionaea muscipula* (Droseraceae, AB675913) were used as outgroups for phylogenetic analysis, since those two families have been recognized as sister groups to Nepenthaceae (Albert et. al., 1992; Cuenoud et. al., 2002). Phylogenetic analysis were conducted using four different methods, those were: (1) Maximum parsimony method using PAUP version 4.0b10; (2) Bayesian analysis using MrBayes version 3.1.2; (3) the Neighbor Joining method using MEGA version 5.05; and (4) the Maximum Likelihood method using Treefinder. Bootstrap values were calculated from 1000 replicates.

The trees reconstructed from the four methods were essentially consistent, which reveal 1 basal branch and 7 subclades and the most basal taxa was *N. pervillei* from Seychelles, which is concordant to the previous study of *Nepenthes* trnK intron phylogeny (Meimberg et. al., 2001). Each subclade on the tree topology of parsimony analysis was supported by a high bootstrap value of more than 80%. Subclade I contains a taxon from Misool island and two non-endemic taxa (*N. mirabilis* and *N. gracilis*). Subclade II comprises taxa which are distributed in Maluku islands, New Guinea, and the outlying areas (Australia, India and Sri Lanka), and also includes a non-endemic taxon (*N. ampullaria*). Subclade III consists of 2 taxa exclusively from Sulawesi with the exception of *N. tentaculata* which also occurs in Borneo. Subclade IV and V which form an unresolved polytomy, contain taxa that are restricted to the Philippines and a taxon which is endemic to Borneo (*N. campanulata and N. hirsuta*, respectively). Subclade VI contains exclusively species from Borneo. Subclade VII comprises exclusively 13 taxa from Sumatra, 4 taxa from the Peninsular Malaysia and Thailand, an endemic taxon to Borneo (*N. macrovulgaris*) and a taxon which distributed in Borneo and Sumatra (*N. reinwardtiana*).

The results of the present study were compared to the natural division of genus *Nepenthes* made by Danser (2006), based on morphology and phytogeography of the genus, where six species groups were distinguished, namely *Vulgatae*, *Montanae*, *Nobiles*, *Regiae*, *Insignes* and *Urceolatae*. Judging from the co-existance of the species belonging to different groups in the same subclade, it reveals that Danser's grouping did not represent the phylogeny in *Nepenthes*. Moreover, the taxa belonging to *Vulgatae* group are positioned not only at the base of subclade I, II and III, but also at the basal position of subclade IV. This suggests that most of characteristics defining *Vulgatae* group would be plesiomorphic characters. The similar situations are also found in subclade V and VII. There is a different position of three non-endemic taxa (*N. mirabilis*, *N. gracilis*, and *N. ampullaria*) from the present study compared to the previous trnK phylogeny study (Meimberg et. al., 2001), which may caused by the difference in markers used for the analysis, where ITS is located in nuclear genome, whereas *trnK* intron is located in chloroplast which represents the maternal phylogeny of *Nepenthes*. There are some close relationships of *Nepenthes* taxa among two groups of Bornean taxa, one group of Sumatran taxa and one group of Peninsular Malaysia and Indochina taxa, which characterized by the similarity of their habitats, life forms, structure of inflorescence, shape of leaf base, leaf apex, and lamina; shape of lower and upper pitchers and their lids; and the existance of indumentum. ITS nrDNA sequences analysis shows strong evidence for a monophyletic origin of the genus *Nepenthes*. Moreover, the comparison to the natural division of genus *Nepenthes* (Danser, 2006) is for the most part consistent for the *Nobiles*, *Regiae*, *Insignes*, and *Montanae* groups. But, the representatives of the *Vulgatae* group appear scattered in different subclades and are obviously polyphyletic.

To study the correlation between *Nepenthes* phylogeny with their habitats and morphological characteristics, the character states of the altitudinal distribution and habitat types, as well as the upper pitchers along with their lids of each *Nepenthes* species samples, were mapped using MacClade version 4.06. In the view of altitudinal distribution, taxa from the eastern (Australia, New Guinea and the surrounding islands) are mostly lowland species, whereas taxa from the western (Borneo and Sumatra) are mostly highland species. Between these two clusters of altitudinal distribution, Sulawesi and the

Philippines seem to become a transitional zone with their own inhabitant taxa, as both islands had been mentioned to belong to the Wallacea (Van Welzen et. al., 2011). The highland species mostly grow in montane forest and scrub habitats, whereas the lowland species inhabit many different habitats types. Moreover, highland species possess upper pitchers with wholly or partly infundibuliform, which resemble the corolla of flowers (Joel; 1988). All of the highland upper pitcher lids are presumably shield the pitcher opening loosely, because the peristome of the infundibuliform upper pitchers are mostly wider than their own lids, so that it get wet and the pitcher flooding during rain, which facilitate prey wetness-based trapping (Bohn and Federle, 2004; Bauer et. al., 2008; Bauer and Federle, 2009; Gorb et. al., 2007) and fluid viscosity prey retention (Clarke, 2007; Di Giusto et. al., 2008), respectively, which had been reported from the highland *Nepenthes* pitchers from Sumatra (Salmon, 1993; Cheek and Jebb, 2001). Therefore, *Nepenthes* species living at high altitude, trap a higher diversity of prey than species living at lower ones (Adam, 1997). We may assume that *Nepenthes* move from the lowland to the highland for prey, by develop upper pitchers with their lids that attract and trap prey effectively by mimic flowers morphologically (Joel; 1988) and adapt humid and rainy habitats in the montane forest and scrub vegetation habitats, which reveal that pitcher morphology has converged on a suitable features that facilitates the trapping of a high diversity of prey (Biesmeijer et. al., 2005; Ellison and Gotelli; 2001; Moran, 1996), in response to nutrient deficiency, which characterizes *Nepenthes* habitats.

Molecular evolution of Nepenthesin

Nepenthesin from 29 *Nepenthes* species from different altitudinal distribution and habitat types were isolated and sequenced. Their length varies from 1314 to 1317 bps. All the DNA sequences are identical to the aspartic proteinase nepenthesin II from *N. gracilis* (AB114915). The prepro form of nepenthesin II, most composed of 438 amino acids, including 24 residues putative signal sequence, 55 residues putative propeptide and 359 residues mature enzyme. Nepenthesin II gene has no intron, which will produce its protein product rapidly (Jeffares, 2008) for digesting the trapped preys. This rapid production of nepenthesin II may help to avoid putrefaction of trapped preys which result in an accumulation of ammonium that may harm the pitcher to die. This is concordant to the result of immunohistochemical staining of nepenthesin (Athauda et. al., 2004). The plant aspartic proteinase gene has experienced both the gain and loss of introns during the process of evolution (Asakura et. al., 1995). Thus, nepenthesin gene is supposed to has adapted specifically to produce extracellular digestive enzyme by removing its introns during the course of molecular evolution. All nepenthesin II enzymes contain 12 cysteine residues per molecule of protein, which would form 6 disulphide bonds. Aspartic proteinase with such a high disulphide bonds content has never been known before. The high content and specific pairing of the disulphide bonds should contribute greatly to the stability of nepenthesin II (Athauda et. al., 2004). Each nepenthesin II contains different number of acidic and basic residues, which resulted in the difference of calculated pI value of each mature enzyme. All the pI from species living in more than 1 habitat (called generalist) are above pH 3 and most of the pI from species inhabit only 1 specific habitat (called specialist) are below pH 3. To test whether the pI from the generalist and the specialist are significantly different, the one-sample Kolmogorov-smirnov test continued by the independent-samples T test and Levene's test were performed by using SPSS trial version 20. The results conclude that the two data are statistically different. A higher pI value of nepenthesin II is supposed to contribute to render the enzyme much more stable, where the charge repulsion among the dissociating carboxylate groups, which will lead to denaturation, should be less pronounced as the pH is raised (Athauda et. al., 2004). Thus, nepenthesin II from species inhabit more than 1 habitat, appear to be more stable, indicating an evolutionary adaptation of the enzyme to different habitats.