MATERIALS AND METHODS

PLACE OF STUDY

Studies of herbarium specimens were conducted in Herbarium Bogoriense (BO) of Puslitbang Biologi-LIPI, Bogor; Herbarium of the Royal Botanical Gardens Kew (K); British Museum, London (BM), and Rijksherbarium, Leiden (L). Observation on living plants, anatomical and cytological investigations were undertaken in Herbarium Bogoriense, while the isozymes was analyzed in PAU, IPB, Bogor. The SEM analysis of the seed coats was undertaken in Rijksherbarium Leiden.

PLANT MATERIAL

For the present study a total of 969 herbarium sheets, including liquid and carpological collections of *Trichosanthes* from several herbaria (BM, BO, BR, CANB, L, LAE, MEL, P, S, SAN, SING, TNS, USA, U, W) were used to provide morphological, distribution and economic botanical data for this study. Living materials of nine species and one variety collected from Java, Sumatra, Borneo, and Irian were cultivated in the premises of Herbarium Bogoriense, and used for anatomical, cytological and isozyme analyses.

Details of the living plant collections used for this study was as follows:

*Trichosanthes borneensis*: MR s.n. (Kalimantan); Fanani s.n. (Kalimantan).
**METHODS OF INVESTIGATIONS**

Morphology, Distribution and Economic Botany — Data and information on morphology, distribution and economic botany were collected from the specimens and literatures. The procedure for morphological observation followed those described by Leenhouts (1968), Rifai (1976) and de Vogel (1985).
Anatomy — Leaf anatomy was investigated by first fixing the leaves (small part of the middle base) in FAA and then embeded them in paraffin. Transverse sections (20 µm thick) were made, stained with safranin and fast green, and then mounted in canada balsam. Paradermal sections were taken from the upper and lower surfaces of leaves, then stained with safranin 1% in water and then mounted in glycerin. Descriptions of the trichome and stomata types were based on the criteria used by Inamdar & Gangadhara (1975, 1976).

SEM of Seed Coat — Seeds of some species (T. auriculata, T. beccariana, T. borneensis, T. cucumerina, var. anguina, T. elmeri, T. emarginata, T. laeoea, T. montana, T. ovigera, T. pendula, T. quinquangulata, T. rotundifolia, T. schlechteri, T. tricuspidata, T. valida,) were soaked in detergent (amaloco) 1% for one night, then rinsed with aceton for 5–10 minutes. The seed then were cut at the middle and put on the cylindrical stub using carbon glue and coated with gold SCD 005 (BAL-TEC) for 4 minutes. The SEM photographs were made by low pressure JEOL–SEM.

Cytology — Root tips from the living materials were pretreated with 0.0002 M 8-hydroxyquinoline solution and kept in the refrigerator for three hours. The root tips separated from root caps were fixed in 45% acetic acid for 10 minutes, macerated in 1 N HCl solution at 60° C for 2–3 minutes, and then squashed in 2% aceto-orcein solution.
Isozyme — Enzyme electrophoresis was performed by horizontal starch gel electrophoresis using 12% of potato starch from SIGMA. The procedures followed those of Wendel & Weeden (1990). About 50 mg pieces of fresh young leaves were crushed in approximately 0.5 ml extract buffer consisting of 250 mM ascorbic acid Na-salt, Tris-HCl buffer (pH 7.5), 0.25 M pvp, 0.1% mercaptoethanol, 20 mM Diethylthiocarbamate, 20 mM sodium metabisulfite, and 200 mM sodium tetraborate. Filter paper wicks were dipped into the leaf extract and then inserted into the starch gel. The gel buffer was 5mM L-histidin, adjusted with NaOH (pH 7.0) or 0.076 M Tris, adjusted with citric acid (pH 8.6). The electrode buffer was 0.410 M citric-acid, Na2 salt, adjusted with free citric acid (pH 7.0), or 0.300 M boric acid, adjusted with NaOH (pH 8). Gel were run at 18-22 mA for 3-4 hours. The gel then was sliced horizontally and stained with three staining enzymes, PRX (peroxidase), AAT (aspartic aminotransferase), ACP (acid phosphatase). The PRX staining mixer was 0.05 M sodium acetat (pH 5.0), 3-amino-9-etil-carbosol, aceton, 3% H2O2, CaCl2. The AAT staining mixer was alfa-ketogluteric-acid, L-aspartic-acid, pvp-40, EDTA, Na2 salt, sodium phosphate. The ACP staining mixer was 0.1 M sodium acetat (pH 5.0), MgCl2, Na-napthyl acid phosphate, fast garnet GBG salt.

Cladistic analysis — Vegetative and generative characters were used for phylogenetic analysis of Trichosanthes, with Gymnopetalum chinensis and G. integrifolia used as the outgroups. A total of 46 morphological characters (Table 5) involving habit, indument, stem, tendril, leaves, probract, flowers, fruits and
seeds were accumulated and analyzed using Hennig86 computer programme. For this analysis, six species (T. dentifera, T. denticrinita, T. ellipsoidea, T. planiglans, T. pulleana, T. refracta) were not included because of incomplete material available.