

### III. Methods

#### 1. Morphological analyses

##### **Morphological observations**

For light microscopic observations, urediniospores and teliospores were scraped from the specimens and mounted in a drop of lacto-phenol solution on a microscopic slide. For each specimen, fifty spores were randomly chosen and observed for the selected morphological features listed in Fig. 3.1. under an Olympus BH 2 microscope. The urediniospores and teliospores dimensions were measured by Image Analyzer (Leica Qwin) software connected to an Olympus BH 2 microscope. Spore-wall color was determined under an Olympus BH microscope without filter. The light intensity was adjusted by controlling the voltage of the lamp. The color was described according to Rayner (1970). Number and distribution of germ pores in urediniospores were determined by the aniline-blue squash method (Jennings et al. 1989). The distribution pattern of the pores was categorized according to Cummins and Hiratsuka (2003). The shape of urediniospores and teliospores was categorized according to Stearn (1995).

For scanning electron microscopy (SEM), urediniospores and teliospores were dusted on a double adhesive tape on a specimen holder and coated with platinum-palladium at 25 nm thick by a Hitachi E-1030 Ion Sputter Coater. The coated specimens were observed under a Hitachi S-4200 scanning electron microscope operating at 15 kV.

### **Statistical analyses**

Seven morphological characteristics of urediniospores and teliospores were measured under a Leica Qwin image Analyzer and subjected to analyses. From a specimen, 50 spores were randomly chosen and each character was study. Statistical analyses including multivariate analyses of measured continuous numerical variable were performed using the software package Systat™ version 5.2 (Wilkinson, 1989) run on a Macintosh Power Mac G4. Discrete numerical or qualitative attributes or host species were superimposed on two-dimensional or three-dimensional scatter diagrams generated from the analysis to detect possible group(s).

## **2. Molecular phylogenetic analyses**

### **DNA extraction**

Genomic DNA was extracted from about 150-200 urediniospores or teliospores from a single uredinium and telium. Spores were crushed between two sterile glass slides and suspended in 20 µl extraction buffer containing 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% Proteinase K, 0.01% SDS, incubated at 37°C for 60 min, and then at 95°C for 10 min (Suyama et al. 1996; Virtudazo et al. 2001).

### **PCR amplification**

From this crude extract, 2-3 µl were used directly for each PCR amplification. Amplification was done using 40 µl PCR reactions each containing 0.2 µM of each

primer, 1 unit of TaKaRa Ex Taq DNA polymerase (Takara, Japan), and the supplied dNTP mixture (containing 2.5mM of each of dNTP) and Ex Taq reaction buffer (containing 2mM Mg<sup>2+</sup>). PCR was carried out using a Gene Amp PCR System 9700 (Applied Biosystems) under the following the conditions: 95°C for 3 min, then 35 cycle of 95°C for 30s, 55°C for 1 min, 72°C for 1 min, and final step of 72°C for 10 min (Virtudazo et al. 2001). PCR of D1/D2 region was amplified using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (O'Donnell 1993; Fig. 3.2). For ITS and 5.8 S regions, the primers used at the beginning of this study were ITS1F and ITS4 (White et al. 1990; Gardes and Burns 1993). However, because amplification using these primers was successful for only a few specimens, I chose the primers that worked better for *Uromyces* spp. The primers were ITS5-u (5'-AAGGTTTCTGTAGGTG-3') and ITS4-u (5'-GGCTTTTCCCTCTTCAT-3') (Pfunder et al. 2001) (Fig. 3.2). PCR products were run on 1% agarose gels containing 0.5 µg/ml ethidium bromide in TAE (40 mM Tris, 40 mM sodium acetate, 1 mM EDTA, pH 8.0) or TBE (89 mM Tris, 89 mM borate acetate, 2 mM EDTA, pH 8.0) buffers.

### Sequencing

PCR products were first purified by spin columns (MicroSpin™ S-400 HR Columns). Purified PCR products were reacted with BigDie Terminator v3.0 Cycle Sequencing (Applied Biosystems) under the following conditions: 25 cycles of 96°C for 10s, 50°C for 5s, 60°C for 4 min. Cycle sequencing reaction products (20 µl) were

purified by ethanol precipitation (Fig. 3.3). Data were collected using an ABI PRISM 3100 automated sequencers (Applied Biosystems).

### **Sequence alignment and analyses**

DNA sequences were aligned using Clustal X v1.8 (Thompson et al. 1997). Further visual alignments were done in Sequence Alignment (Se-AL) Editor v.2.0 (Rambaut 2000). Specimen sequences were analyzed together with sequences from GenBank.

The aligned sequence data file is obtainable from the authors. Phylogenetic analyses of the data were done by distance and parsimony methods. The distance matrix for the aligned sequences was calculated using Kimura's two-parameter method (Kimura 1980) and was analyzed with the neighbor-joining (NJ) method (Saitou and Nei 1987) using the program PAUP v 4.0 b (Swofford 1999), excluding positions with gaps and correcting for multiple substitutions. Reliability of the inferred trees was estimated by 1000 bootstrap resamplings using the same program. Parsimony analyses were also done by PAUP v 4.0 b using the heuristic search option with 100 random stepwise-addition sequences to search for the most parsimonious trees. Bootstrap values (Felsenstein 1985) were generated with 1000 replicate heuristic searches to estimate support for clade stability of the consensus trees using the same program.

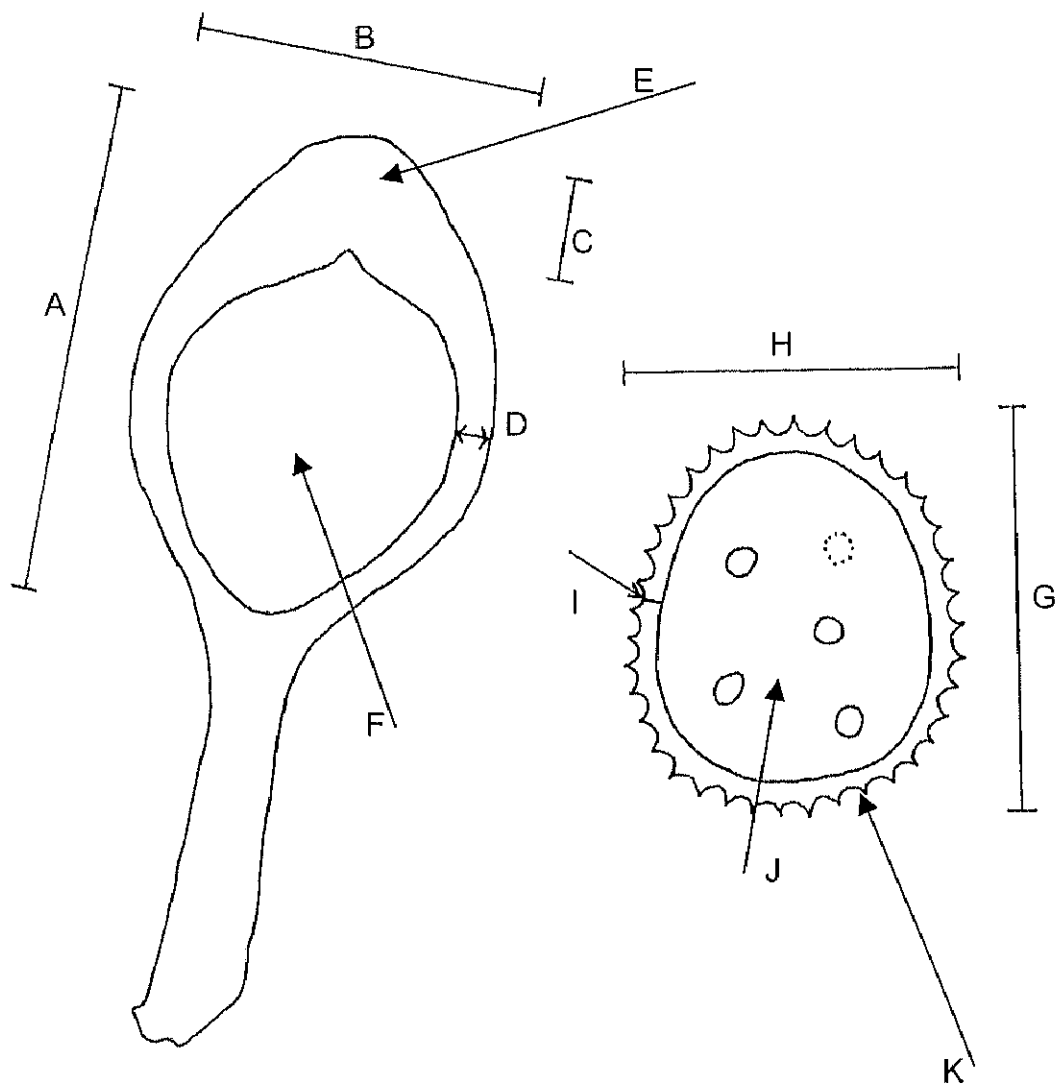
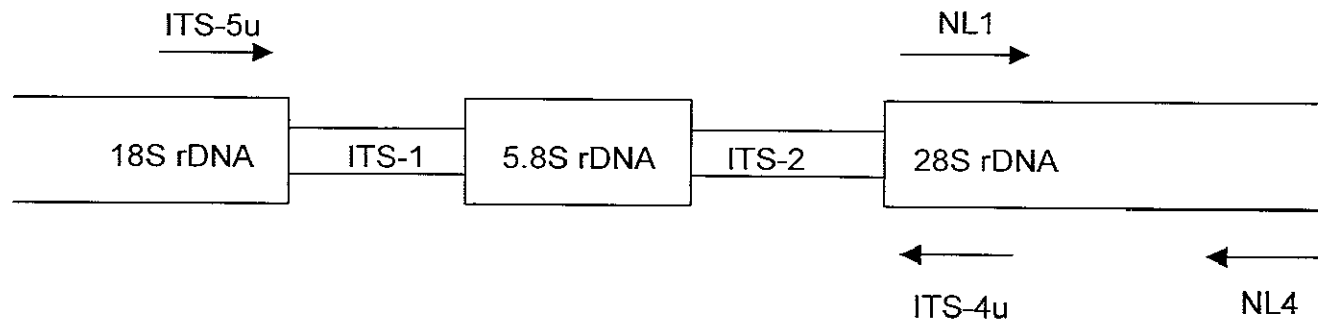


Fig. 3.1. Morphological characteristics of teliospore and urediniospore observed in present study. **A.** teliospore length, **B.** teliospore width, **C.** teliospore apical thickness, **D.** teliospore wall thickness, **E.** with or without papilla, **F.** teliospore surface-structure (SEM), **G.** urediniospore length, **H.** urediniospore width, **I.** urediniospore wall thickness, **J.** number and position of urediniospore germ pores, **K.** urediniospore surface-structure (SEM).



D1/D2 regions:

NL1: 5'-GCATATCAATAAGCGGAGGAAAAG-3'

NL4: 5'-GGTCCGTGTTTCAAGACGG-3'

(O'Donnell 1993)

ITS regions:

ITS-5u: 5'-CAAGGTTTCTGTAGGTG-3'

ITS-4u: 5'-GGCTTTTCCCTCTTCAT-3'

(Pfunder et al. 2001)

Fig. 3.2. Diagram of a portion of the rDNA unit repeat of 28S (D1/D2) and ITS regions. D1/D2 region is amplified and sequenced by the primer pairs of NL1 and NL4 and ITS region is amplified and sequenced by the primer pairs of ITS-5u and ITS-4u.

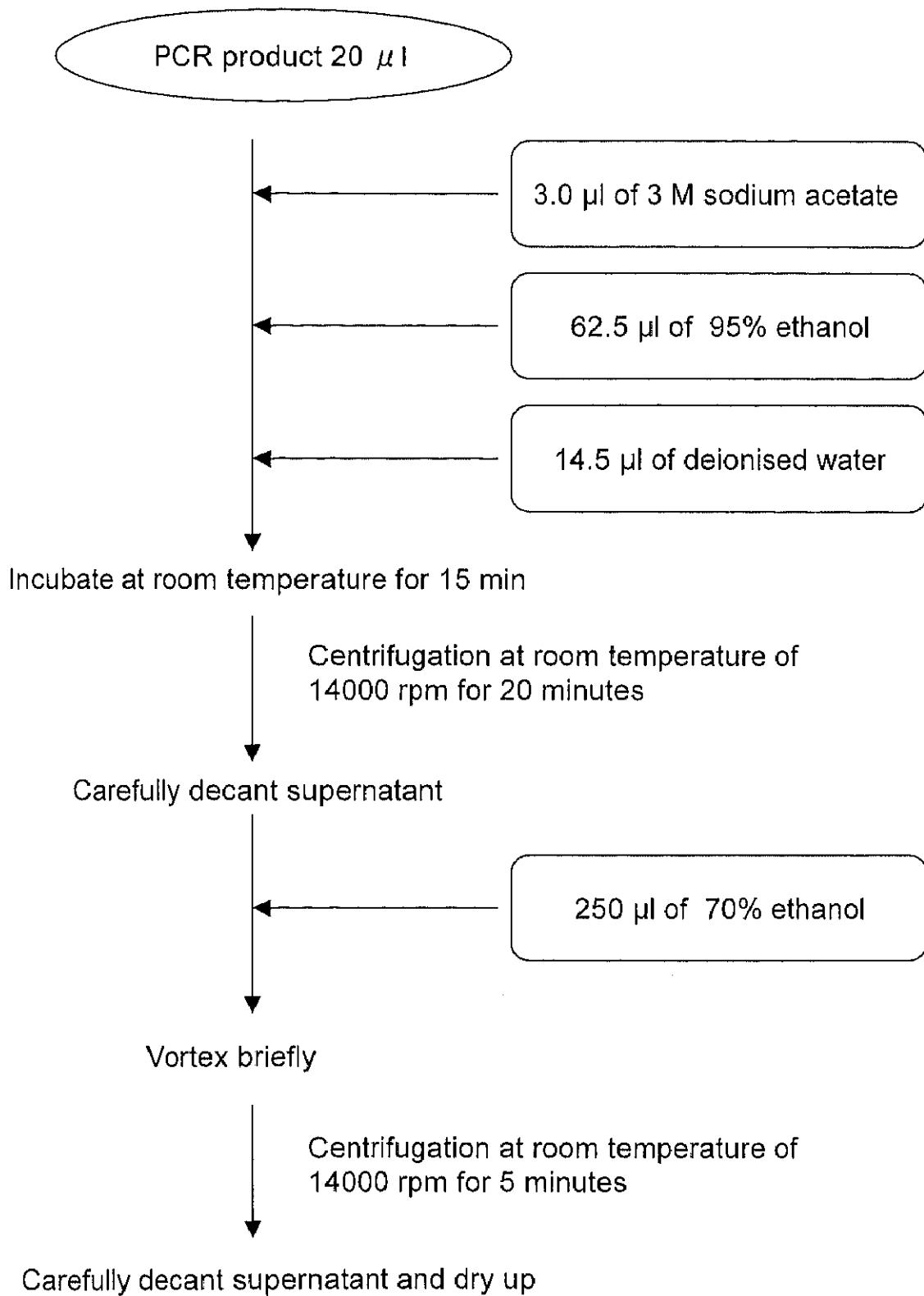


Fig. 3.3. The procedure of ethanol precipitation.