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FULL PAPER

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# Morphological and phylogenetic analyses of *Uromyces appendiculatus* and *U. vignae* on legumes in Japan

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**Abstract** *Uromyces appendiculatus*, inclusive of three varieties, is distinguished from *U. vignae* primarily by the position of urediniospore germ pores and putative host specificity. However, opinions concerning these morphological and physiological features as taxonomic characters have varied greatly, and distinction of these species has often been confused. To clarify the taxonomy of these two species, morphological features of urediniospores and teliospores of 225 rust fungus specimens on species of *Phaseolus*, *Vigna*, *Apios*, *Lablab*, and *Dunbaria* were examined by light microscopy and scanning electron microscopy. Forty-five specimens were subjected to molecular phylogenetic analyses. As a result, the position of germ pores in urediniospores and the teliospore-wall thickness were considered as good characters to separate three morphological groups. In molecular analyses, the specimens fell into two and three clades based on the nucleotide sequence at D1/D2 domain of LSU rDNA and ITS regions, respectively. One of the D1/D2 clades corresponded to one morphological group whereas another D1/D2 clade included two other morphological groups. In contrast, each of the three ITS clades corresponded to a separate morphological group. Neither morphological groups nor molecular clades were host limited. It is suggested that the three morphological groups that corresponded to three distinct ITS clades constitute distinct species.

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**Key words** D1/D1 · ITS · Rust fungi · Taxonomy · Uredinales · *Uromyces appendiculatus* · *U. vignae*

## Introduction

*Uromyces appendiculatus* (Pers.) Unger and *U. vignae* Barclay are autoecious and macrocyclic rust fungi that occur on beans, cowpea, and related legumes throughout the world (Cummins 1978; Duke 1981; Guo and Wang 1986; Hiratsuka et al. 1992; Thurston 1998).

*Uromyces appendiculatus* was first found in Europe in 1795 on *Phaseolus vulgaris* L. (cited from Arthur 1934) and *U. vignae* in India in 1891 on *Vigna vexillata* (L.) A. Rich. (Barclay 1891). Fromme (1924) considered that *U. vignae* was distinct from *U. appendiculatus* as it possessed superequatorial germ pores in the urediniospores and occurred on a different host genus. However, Arthur (1934) believed that the morphological distinction between the rust fungi on *Phaseolus* and *Vigna* was not sufficient to warrant specific separation and classified them under a single species, *U. phaseoli* (Pers.) Winter, with two varieties, var. *typica* Arth. and var. *vignae* (Barclay) Arth.

In Japan, Ito (1922) regarded the rust fungus found on species of *Phaseolus* and *Vigna* as *U. appendiculatus*, with morphologically similar urediniospores on both hosts. However, Hiratsuka (1937) separated the rust fungi on *Phaseolus* and *Vigna* but did not state a reason for regarding them as distinct species. Later, Ito and Murayama (1943) described a rust fungus on *Apios fortunei* Maxim. as a new species, *U. dispersus* Hiratsuka f. ex S. Ito & Murayama. Hirata (1952) separated a rust fungus on *V. angularis* (Wild.) Ohwi & Ohashi var. *angularis*, *V. angularis* var. *nipponensis* (Ohwi) Ohwi & Ohashi and *V. umbellata* (Thunb.) Ohwi & Ohashi from *U. appendiculatus* and described it as a new species, *U. azukicola* S. Hirata.

Hiratsuka (1973) changed his opinion and followed Arthur's (1934) taxonomic treatment of *U. phaseoli*; thus,

**Table 1.** Specimens of rust fungi used for morphological observations

| Host plants | Locality in Japan (no. of specimens) |
|---|---|
| *Vigna angularis* (Willd.) Ohwi & Ohashi var. *angularis* | Hokkaido (5), Honshu (30), Kyushu (10), Shikoku (1), Okinawa (4) |
| *V. angularis* var. *nipponensis* (Ohwi) Ohwi & Ohashi | Honshu (10), Kyushu (3), Okinawa (1) |
| *V. umbellata* (Thunb.) Ohwi & Ohashi | Honshu (6), Kyushu (3), Shikoku (1) |
| *Apios fortunei* Maxim. | Honshu (5), Kyushu (3), Shikoku (3) |
| *Dunbaria villosa* (Thunb.) Makino | Honshu (1) |
| *V. radiata* (L.) Wilczek | Hokkaido (1), Kyushu (1), Shikoku (1), Japan (1) |
| *Phaseolus minimus* Roxb. | Okinawa (2) |
| *P. vulgaris* L. | Hokkaido (16), Honshu (49), Kyushu (5), Shikoku (4), Okinawa (2), Japan (3) |
| *V. unguiculata* (L.) Walp. ssp. *unguiculata* | Honshu (22), Kyushu (9), Shikoku (4), Okinawa (5) |
| *V. unguiculata* ssp. *sesquipedalis* (L.) Verdc. | Honshu (4), Kyushu (2), Shikoku (1) |
| *V. marina* Merr. | Okinawa (3) |
| *Lablab purpureus* (L.) Sweet | Honshu (1), Okinawa (3) |
| Total | 225 |

*U. appendiculatus*, *U. azukicola*, *U. dispersus*, and *U. vignae* became varieties of *U. phaseoli*. Hiratsuka et al. (1992), however, followed Cummins's (1978) circumscription of *U. appendiculatus* and *U. vignae* and recognized three varieties under the former species. Since Cummins's (1978) taxonomic treatment, *U. appendiculatus* has been said to differ from *U. vignae* in the position of urediniospore germ pores, the teliospore-wall ornamentation, and putative host specificity (Cummins 1978; Fernandez and Heath 1985; Guo and Wang 1986; Hiratsuka et al. 1992).

The various taxonomic conclusions have not aided the identification of the rust species that occur on *Phaseolus* and *Vigna* across the world, and reported host specificity has been a source of confusion (El-Gazzar 1981; Duke 1981). Therefore, identification of *U. appendiculatus*, with three varieties, and of *U. vignae* has often been difficult and even erroneous. The difficulties encountered have necessitated a reevaluation of morphological features and physiological specialization of these fungi.

In addition to morphological and physiological analyses, molecular phylogenetic analysis has become a useful tool to study the taxonomic distinction and relationships among closely related and morphologically similar rust fungi. For morphologically indistinct fungal species, several ribosomal DNA regions and mitochondrial DNA have often been chosen for the analyses because of their high degree of variability. Several research groups have already applied this tool to rust fungi and discussed their phylogeny (Zambino and Szabo 1993; Kropp et al. 1997; Vogler and Bruns 1998; Pfunder et al. 2001; Virtudazo et al. 2001; Maier et al. 2003; Weber et al. 2003; Chung et al. 2004).

The present study examines morphological features of urediniospores and teliospores in the *U. appendiculatus* and *U. vignae* species complex. Morphologically discrete taxa are assessed by phylogenetic divergence estimated from molecular divergence in the D1/D2 region of large subunit (LSU) rDNA and the internal transcribed spacer (ITS) region including the 5.8 S rRNA gene.

## Materials and methods

### Morphological observations and principal-component analyses

Fresh materials or dry herbarium specimens were used for light microscopic (LM) and scanning electron microscopic (SEM) observations. Most specimens examined were borrowed from the Hiratsuka Herbarium, Tokyo, Japan (HH); the National Fungus Collections, the United States Department of Agriculture, Beltsville, MD, USA (BPI); the Mycological Herbarium of the Institute of Agriculture and Forestry, the University of Tsukuba, Tsukuba, Japan (TSH); the Herbarium of Systematic Mycology, the College of Education, Ibaraki University, Mito, Japan (IBA); and the Herbarium of the National Institute for Agro-Environmental Science, Tsukuba, Japan (NIAES).

Two hundred and twenty-five specimens collected from *Apios*, *Dunbaria*, *Lablab*, *Phaseolus*, and *Vigna* (Table 1) were examined for morphological characteristics in teliospores and urediniospores. Correct host identity and uniform application of the names were mandatory for this study, and these were ascertained from Duke (1981) and Ohwi and Kitagawa (1992). Principal-component analyses of measured continuous numerical variables were performed using the software package Systat version 5.2 (Wilkinson 1989). Discrete numerical or qualitative attributes of host species were superimposed on two-dimensional scatter diagrams generated from the analyses to detect possible groups.

Spores for LM study were mounted in lactophenol and examined following rehydration. For SEM, spores obtained from dry specimens were dusted on double-sided adhesive tape on specimen holders, and then coated with platinum-palladium with a Hitachi E-1030 Ion Sputter Coater. The spores were examined with a Hitachi S-4200 operating at 15 kV.

### Fungal taxa for molecular phylogenetic analyses

Forty-five specimens were selected for sequence analyses at the D1/D2 region of LSU rDNA and the ITS regions in-

**Table 2.** Specimens of rust fungi used for phylogenetic analyses

| Host plants | Locality in Japan | Voucher specimens[a] | GenBank accession no. | |
|---|---|---|---|---|
| | | | D1/D2 | ITS |
| *Apios fortunei* | Kyoto | HH89813 | AB115615 | NA[b] |
| | Miyazaki | HH89814 | AB115636 | NA |
| | Tokyo | HH89673 | AB115635 | AB115727 |
| | Miyazaki | HH89818 | NA | AB115728 |
| | Fukuoka | HH89819 | NA | AB115729 |
| *Dunbaria villosa* | Saitama | HH92766 | AB115638 | NA |
| *Lablab purpureus* | Okinawa | BPI-0019468 | AB115614 | AB115723 |
| *Phaseolus vulgaris* | Ibaraki | TSH-R1734 (IBA-2460) | AB115644 | AB115741 |
| | Yamanashi | HH92821 | AB115646 | AB115739 |
| | Tokushima | HH92832 | AB115645 | AB115740 |
| | Tochigi | HH94638 | AB115647 | AB115738 |
| | Okinawa | HH50721 | AB115634 | AB115726 |
| *Vigna angularis* var. *angularis* | Nagano | HH92876 | AB115642 | AB115734 |
| | Tokyo | HH92788 | AB115640 | NA |
| | Miyazaki | HH92802 | AB115616 | AB115732 |
| | Yamaguchi | HH92014 | NA | AB115730 |
| | Hokkaido | TSH-R16262 | NA | AB115705 |
| | Hokkaido | TSH-R16263 | NA | AB115706 |
| | Hokkaido | TSH-R16264 | AB115621 | AB115707 |
| | Hokkaido | TSH-R16265 | NA | AB115708 |
| | Ibaraki | TSH-R17960 | AB115624 | AB115714 |
| | Tochigi | TSH-R16273 | AB115623 | NA |
| | Ibaraki | TSH-R17959 | AB115620 | AB115713 |
| | Ibaraki | TSH-R17961 | AB115625 | AB115715 |
| *V. angularis* var. *nipponensis* | Kagoshima | TSH-R16267 | AB115622 | AB115709 |
| | Ibaraki | TSH-R16271 | AB115619 | AB115710 |
| | Ibaraki | TSH-R1748 (IBA-7021) | NA | AB115712 |
| *V. marina* | Okinawa | HH50717 | AB115632 | AB115724 |
| | Okinawa | HH50718 | AB115633 | AB115725 |
| *V. umbellata* | Hiroshima | HH92840 | AB115641 | AB115733 |
| | Nagano | HH92772 | AB115639 | NA |
| *V. unguiculata* ssp. *sesquipedalis* | Fukuoka | HH92911 | AB115618 | NA |
| | Kagawa | HH92905 | AB115617 | AB115736 |
| | Ibaraki | TSH-R17968 | AB115630 | AB115721 |
| | Ibaraki | TSH-R17969 | AB115631 | AB115722 |
| *V. unguiculata* ssp. *unguiculata* | Tokyo | HH92902 | AB115643 | AB115735 |
| | Yamaguchi | HH92019 | AB115637 | AB115731 |
| | Ibaraki | TSH-R17967 | AB115629 | AB115720 |
| | Ibaraki | TSH-R17965 | AB115613 | AB115718 |
| | Ibaraki | TSH-R17962 | AB115626 | AB115716 |
| | Ibaraki | TSH-R17958 | AB115612 | AB115711 |
| | Ibaraki | TSH-R17963 | AB115627 | AB115717 |
| | Ibaraki | TSH-R17966 | AB115628 | AB115719 |
| | Shizuoka | BPI-0000967 | AB115648 | AB085196 |
| | Shizuoka | BPI-0000968 | AB115649 | AB085197 |

ITS, internal transcribed spacer
[a]TSH, Mycological Herbarium, University of Tsukuba, Japan; BPI, USDA National Fungus Collections, USA; HH, Hiratsuka Herbarium, Tokyo, Japan; IBA, Herbarium of Systematic Mycology, Ibaraki University, Japan
[b]NA, No analysis

cluding the 5.8S rRNA gene (Table 2). The fabaceous rusts *Uromyces viciae-fabae* (Pers.) Schroet., *U. pisi* (DC.) Otth, and *U. minor* Schroet., together with *U. gageae* Beck on *Gagea lutea* (L.) Ker Gawl. and *Puccinia miscanthi* Miura on *Miscanthus sinensis* Anderss., were chosen as outgroup taxa. GenBank sequences of rust fungi used for phylogenetic comparison are listed in Table 3. Genome DNA extraction followed the methods of Suyama et al. (1996) and Virtudazo et al. (2001).

PCR and sequencing of D1/D2 and ITS regions

From the crude extract, 2–3 μl was used directly for each polymerase chain reaction (PCR) amplification. Amplifications were 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 a commercial deoxynucleoside triphosphate (dNTP) mixture (containing 2.5 mM of each dNTP) and Ex Taq reaction buffer (containing 2 mM $Mg^{2+}$). PCR was carried out using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the conditions used by Chung et al. (2004). The

**Table 3.** Additional rust taxa selected for D1/D2 and ITS analyses

| Species | Host plants | GenBank accession no. | |
|---|---|---|---|
| | | D1/D2 | ITS |
| *Uromyces minor* Schroet. | *Trifolium lupinaster* L. | NA | AB115737 |
| *U. fabae* de Bary | *Vicia pannonica* Crantz | AF426199 | NA |
| *U. viciae-fabae* (Pers.) Schroet. | *V. cracca* L. | AB115598 | AB115655 |
| *U. viciae-fabae* | *Pisum sativum* L. | AB115594 | AB115651 |
| *U. viciae-fabae* | *Lathyrus maritimus* Bigel. | AB115599 | AB085193 |
| *U. pisi* (DC.) Otth | *Euphorbia cyparissias* L. | AF426201 | NA |
| *U. pisi* | *E. cyparissias* | NA | AF180165 |
| *U. gageae* Beck | *Gagea lutea* (L.) Ker Gawl. | AF426208 | NA |
| *Puccinia miscanthi* Miura | *Miscanthus sinensis* Anderss. | AJ296546 | NA |

NA, no analysis

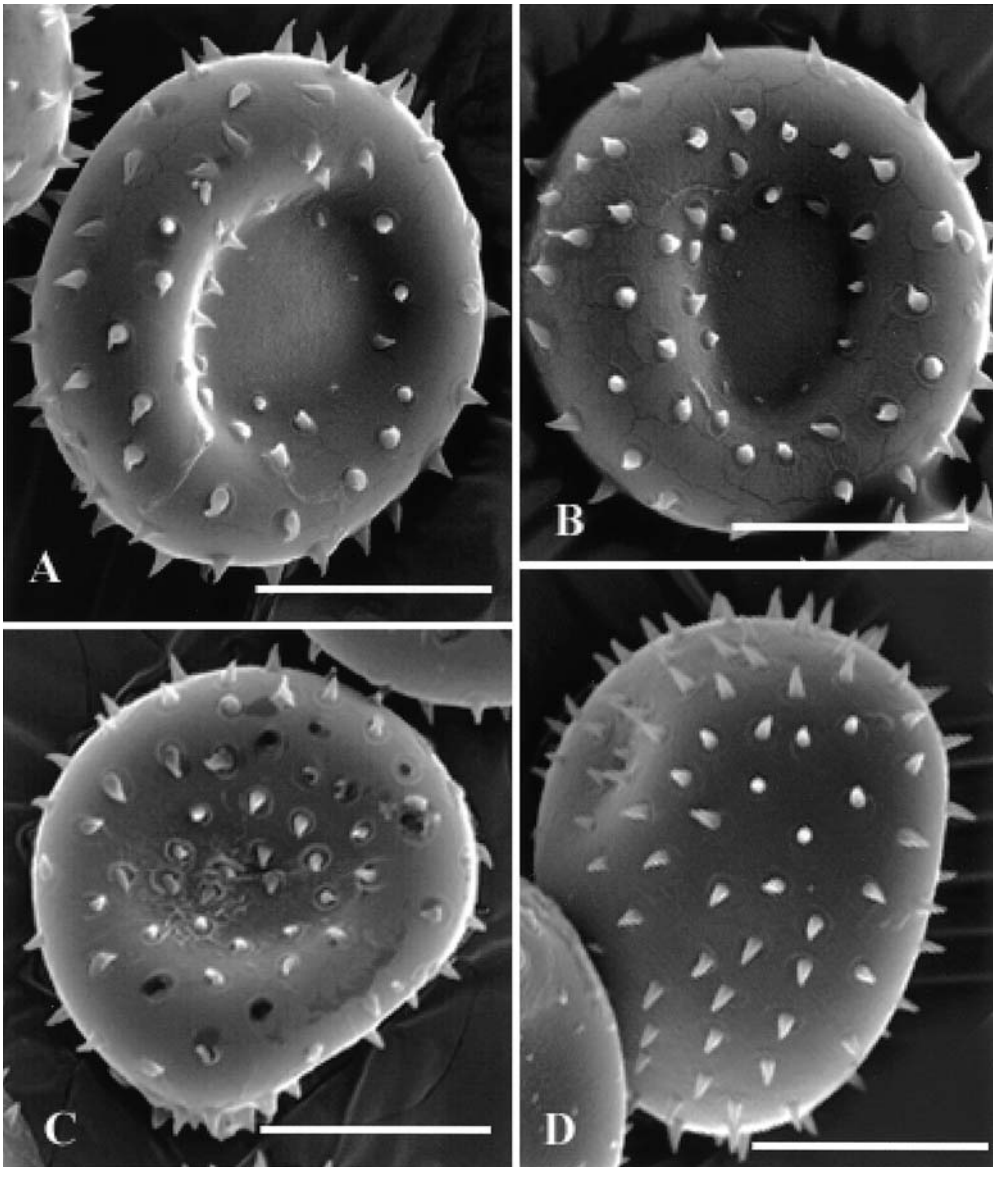


**Fig. 1.** Wall ornamentation of urediniospores from *Phaseolus vulgaris* (**A, B**), *Vigna angularis* var. *angularis* (**C**), and *Vigna unguiculata* ssp. *unguiculata* (**D**). *Bars* 10 μm

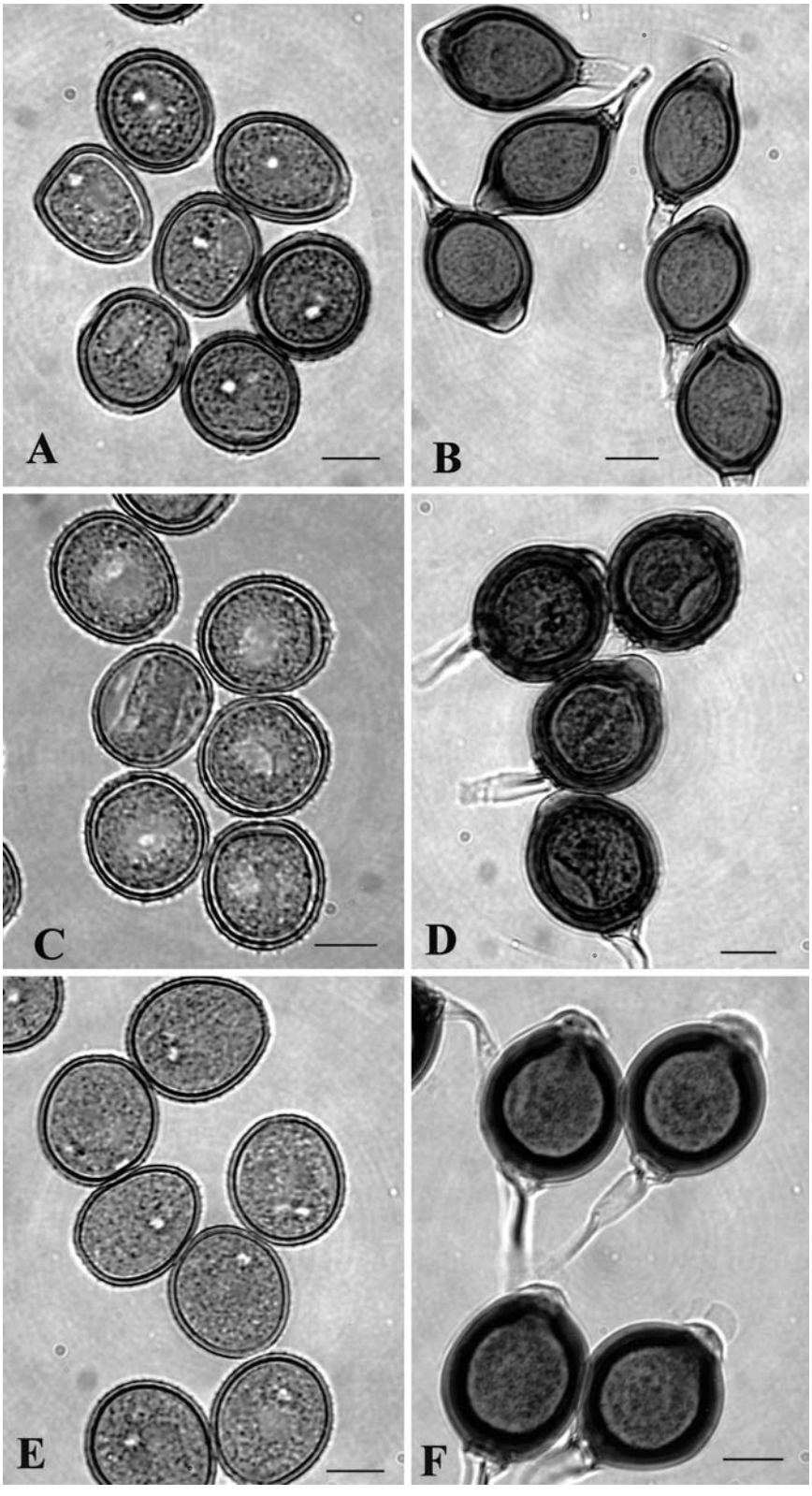


**Fig. 2.** Urediniospores and teliospores in lactophenol. **A, B** From *Vigna angularis* var. *angularis*. **C, D** From *Phaseolus vulgaris*. **E, F** From *Vigna unguiculata* ssp. *unguiculata*. *Bars* 10 μm

D1/D2 region was amplified using primers NL1 and NL4 (O'Donnell 1993). The ITS and 5.8 S regions were amplified using primers ITS5-u and ITS4-u (Pfunder et al. 2001). PCR products were run on 1% agarose gels containing 0.5 μg/ml ethidium bromide in TAE [Tris-acetate, ethylenediamine tetraacetic acid (EDTA)] or TBE (Tris-borate, EDTA) buffers. PCR products were first purified by spin columns (MicroSpin S-400 HR Columns). Purified PCR products

were reacted with BigDye Terminator v3.0 Cycle Sequencing (Applied Biosystems) under the following conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Cycle sequencing reaction products were purified by ethanol precipitation and then analyzed by ABI PRISM 3100 automated sequencers (Applied Biosystems).

### Phylogenetic analyses

Amplified nucleotide sequences were aligned using Clustal X version 1.8 (Thompson et al. 1997). Further visual alignments were done in Sequence Alignment (Se-Al) Editor version 2.0 (Rambaut 2000). The aligned sequences were analyzed together with sequences drawn from GenBank.

Phylogenetic analyses of the aligned sequences were done by distanced 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), including positions with gaps as a fifth character because they may be informative for inferring phylogenies. Reliability of the inferred trees was estimated by 1000 bootstrap resamplings using the same program. Parsimony analyses were done by PAUP version 4.0b (Swofford 1999) using the heuristic search option with 100 random stepwise-addition sequences to search for the most parsimonious tree. Bootstrap (Felsenstein 1985) values were generated with 1000 replicate heuristic searches to estimate support for clade stability of the consensus tree using the same program.

## Results

### Morphological features of urediniospores

Urediniospores of the specimens examined were globose, subglobose, ovoid, obovoid, or ellipsoid, and the urediniospore wall was uniformly echinulate (Fig. 1). Size and shape variation of the spores within individual specimens were as broad as those among the specimens. The mean urediniospore length of individual specimens ranged from 21.3 to 30.9 μm, width from 18.4 to 25.3 μm, and wall thickness from 1.1 to 1.9 μm. The scatter diagram generated from these variables revealed no discrete morphological groups (see Fig. 3A).

However, the position of urediniospore germ pores could be used to sort each specimen into one of three groups. The two (rarely three) germ pores were either superequatorial (Fig. 2E), equatorial (Fig. 2C), or a constant mixture of superequatorial and equatorial (Fig. 2A).

### Morphological features of teliospores

Teliospores of the specimens examined were subglobose, ovoid, or ellipsoid, and the wall ornamentation ranged from irregularly verrucose to slightly verrucose or completely smooth (Fig. 4). The degree of surface roughness varied both within each specimen and among the specimens. In the specimens identified as *U. appendiculatus*, the mean teliospore length ranged from 24.2 to 34.1 μm and width from 17.6 to 27.1 μm. In those identified as *U. vignae*, mean teliospore length ranged from 26.2 to 33.2 μm and width from 19.7 to 27.7 μm. The size variation in each specimen was as large as that among the specimens; thus, no discrete morphological groups were detected by the teliospore size (Fig. 3B). On the other hand, the teliospore-wall thickness varied narrowly within individual specimens and differed among the specimens (Fig. 2B,D,F). By a combination of germ pore position in urediniospores and teliospore-wall thickness, the specimens were sorted into three morphological groups (Table 4).

### Principal-component analyses

Principal-component analyses were undertaken with various combinations of numerical variables in urediniospore

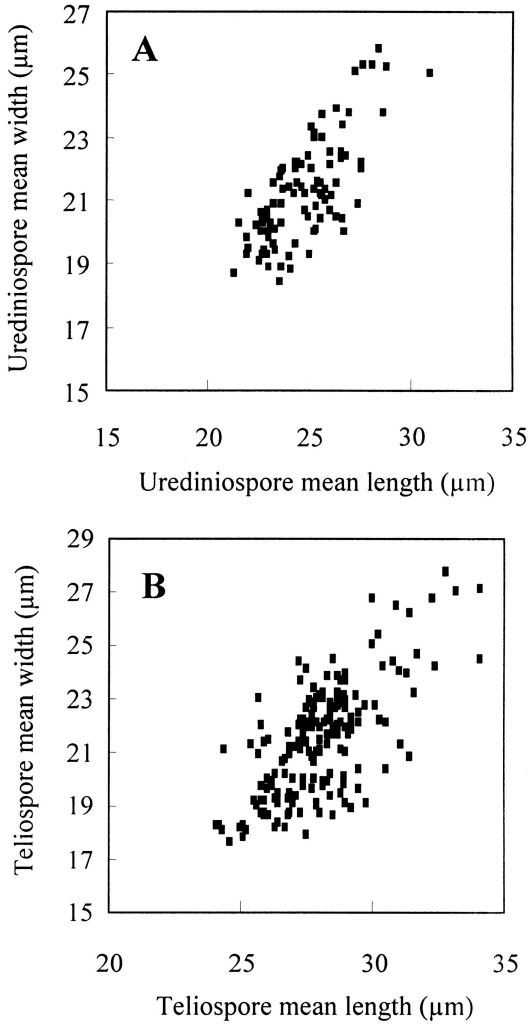


**Fig. 3.** Variation in urediniospore and teliospore size. **A** Urediniospore mean length against mean width. **B** Teliospore mean length against mean width

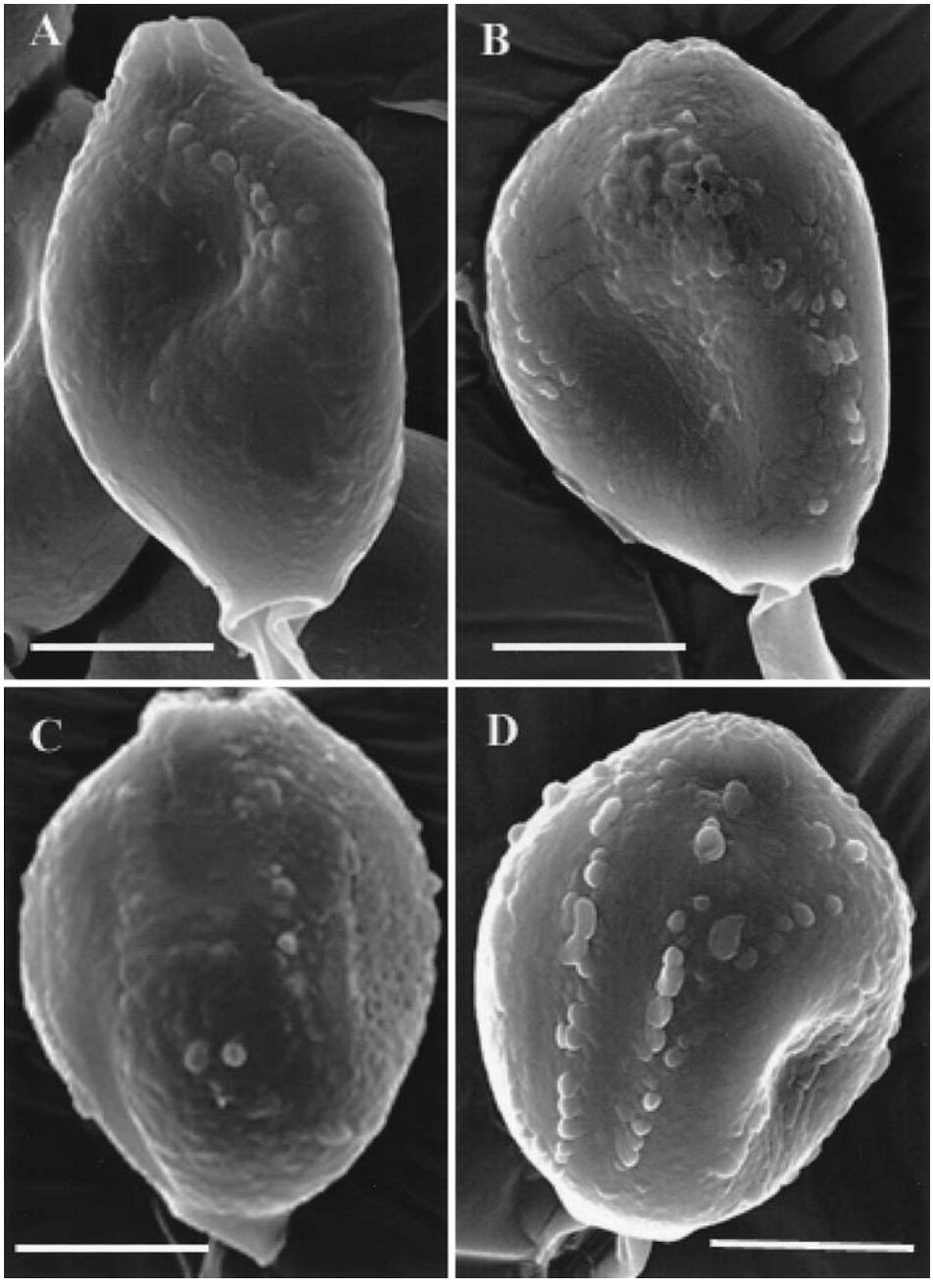


**Fig. 4.** Wall ornamentation of teliospores from *Vigna angularis* var. *angularis* (**A**), *Phaseolus vulgaris* (**B, D**), and *Vigna unguiculata* ssp. *unguiculata* (**C**). *Bars* 10 μm

**Table 4.** Morphological groups based on characteristics of urediniospores and teliospores

| Morphological groups | Urediniospores | Teliospores | Host plants (no. of specimens) |
|---|---|---|---|
| | No. of germ pores and position | Individual mean of wall thickness | |
| I | 2 (rarely 3), equatorial and superequatorial | 1.4–2.3 μm (rarely more than 2.3 μm) | *Vigna angularis* var. *angularis* (46)<br>*V. angularis* var. *nipponensis* (14)<br>*V. umbellata* (10), *V. radiata* (4)<br>*Dunbaria villosa* (1), *Apios fortunei* (18),<br>*Phaseolus minimus* (2), *P. vulgaris* (9) |
| II | 2, equatorial (rarely superequatorial) | 2.4–3.3 μm | *P. vulgaris* (71)<br>*V. angularis* var. *angularis* (3) |
| III | 2 (rarely 3), superequatorial (rarely equatorial) | 2.6–3.4 μm | *V. unguiculata* ssp. *unguiculata* (41)<br>*V. unguiculata* ssp. *sesquipedalis* (7)<br>*V. marina* (3), *L. purpureus* (4)<br>*V. angularis* var. *nipponensis* (1)<br>*P. vulgaris* (1) |

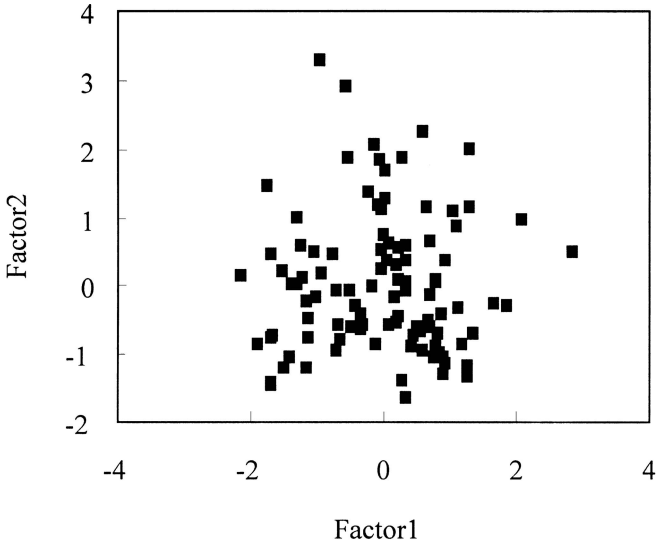


**Fig. 5.** Two-dimensional diagram of the specimens using principal-component analysis with mean values of urediniospore length, width, and wall thickness, and teliospore length, width, wall thickness, and thickness of the apical papilla

and teliospore features. The analysis presented here (Fig. 5) employed mean values of urediniospore length, width, and wall thickness, teliospore length, width, and wall thickness, and thickness of the apical papilla. After the Varimax rotation, the calculated factors 1 and 2 explained 42.1% and 21.0% of the total variance, respectively. The scatter diagram with factor 1 as the horizontal axis and factor 2 as the vertical axis did not reveal discrete groups (Fig. 5). Analyses employing other combination of variables showed similar results.

### Phylogenetic relationships inferred from D1/D2 region of LSU rDNA and ITS regions

The nucleotide sequences of the D1/D2 regions ranged from 597 to 617 bases, which were subjected to phylogenetic analyses. Of the 657 total bases including gaps, 43 sites were variable and 93 sites were parsimony informative. A neighbor-joining tree constructed from the D1/D2 region showed that the 45 specimens fell into two major clades with 84% bootstrap support (Fig. 6). The D1/D2 phylogram had a consistency index (CI) of 0.766, a retention index (RI) of 0.842, retention consistency (RC) of 0.645, and a tree length of 205. Between the two groups, the specimens grouped in the D1/D2 group I had higher T nucleotide repeats (10–11 bases) at D2 than the D1/D2 group II.

The ITS regions including the 5.8S rRNA gene analyzed in the study ranged from 625 to 640 bases. Of the 687 total characters including gaps, 107 sites were variable and 124 sites were parsimony informative. In a neighbor-joining tree constructed from the ITS regions including the 5.8S rRNA gene, the 45 specimens fell into three major clades with 100% bootstrap support (Fig. 7). The ITS phylogram had a CI of 0.678, an RI of 0.802, a RC of 0.544, and a tree length of 398. The specimens constituting the ITS I, II, and III groups possessed sequence homology at 98%–99%, 95%–100% and 97%–100%, respectively. The ITS1 region showed higher sequence divergence (32.4%–35.1%) than the ITS2 region (27.2%–29.3%).

## Discussion

The rust fungi on *Phaseolus*, *Vigna*, *Apios*, *Lablab*, and *Dunbaria* that were examined in this study were sorted by the position of urediniospore germ pores and teliospore-wall thickness into three morphological groups. Taxonomic importance of the position of urediniospore germ pores in the *U. appendiculatus* and *U. vignae* species complex has been well recognized (Cummins 1978; Guo and Wang 1986; Hiratsuka et al. 1992). In addition, the degree of verrucoseness of the teliospore wall was stressed to differentiate the two species (Cummins 1978). The teliospore-wall thickness was considered as important to separate varieties in *U. appendiculatus* (Hiratsuka et al. 1992). Disagreement and confusion over the taxonomy of the *U. appendiculatus* and *U. vignae* species complex has stemmed from the evaluation and judgment of whether these morphological features can be a good taxonomic character to circumscribe the species or subspecific taxa. Thus, Cummins (1978) recognized *U. appendiculatus* and *U. vignae* as distinct species while Arthur (1934) and Hiratsuka (1973) merged the two species under the name of *U. phaseoli* with two varieties, var. *azukicola* and var. *dispersus* (cf. Hirata 1952; Ito and Murayama 1943). Our study confirmed that the rust fungi on *Phaseolus*, *Vigna*, *Apios*, *Lablab*, and *Dunbaria*, classified either as *U. appendiculatus* and its varieties or as *U. vignae*, can be distinguished in three morphologically circumscribed groups regardless of their position in the taxonomic hierarchy.

No other morphological features, either singly or in combination, detected discrete groups in the specimens examined. Teliospore-wall ornamentation has been considered to be a good taxonomic character to differentiate between *U. appendiculatus* and *U. vignae* (Cummins 1978). However, our study showed that the ornamentation was highly variable both within a specimen and among the specimens examined. In contrast to our findings, Guo and Wang (1986) and Hiratsuka et al. (1992) considered that teliospore-wall thickness was not a useful taxonomic character to separate *U. appendiculatus* from *U. vignae*.

It has long been a belief that the rust fungi have high host specificity, and thus that *U. appendiculatus* and its varieties and *U. vignae* can be separated by their host genera and species. This view is expressed in the most recent rust flora of Japan (Hiratsuka et al. 1992), in which *U. appendiculatus* var. *appendiculatus* is restricted to *P. vulgaris*, *U. appendiculatus* var. *azukicola* (Hirata) Hiratsuka, f. to *V. angularis* var. *angularis*, *V. angularis* var. *nipponensis*, and *V. umbellata*, *U. appendiculatus* var. *dispersus* (Hiratsuka, f.) Hiratsuka, f. to *A. fortunei*, and *U. vignae* to *V. unguiculata* and *L. purpureus*. Our study, however, showed

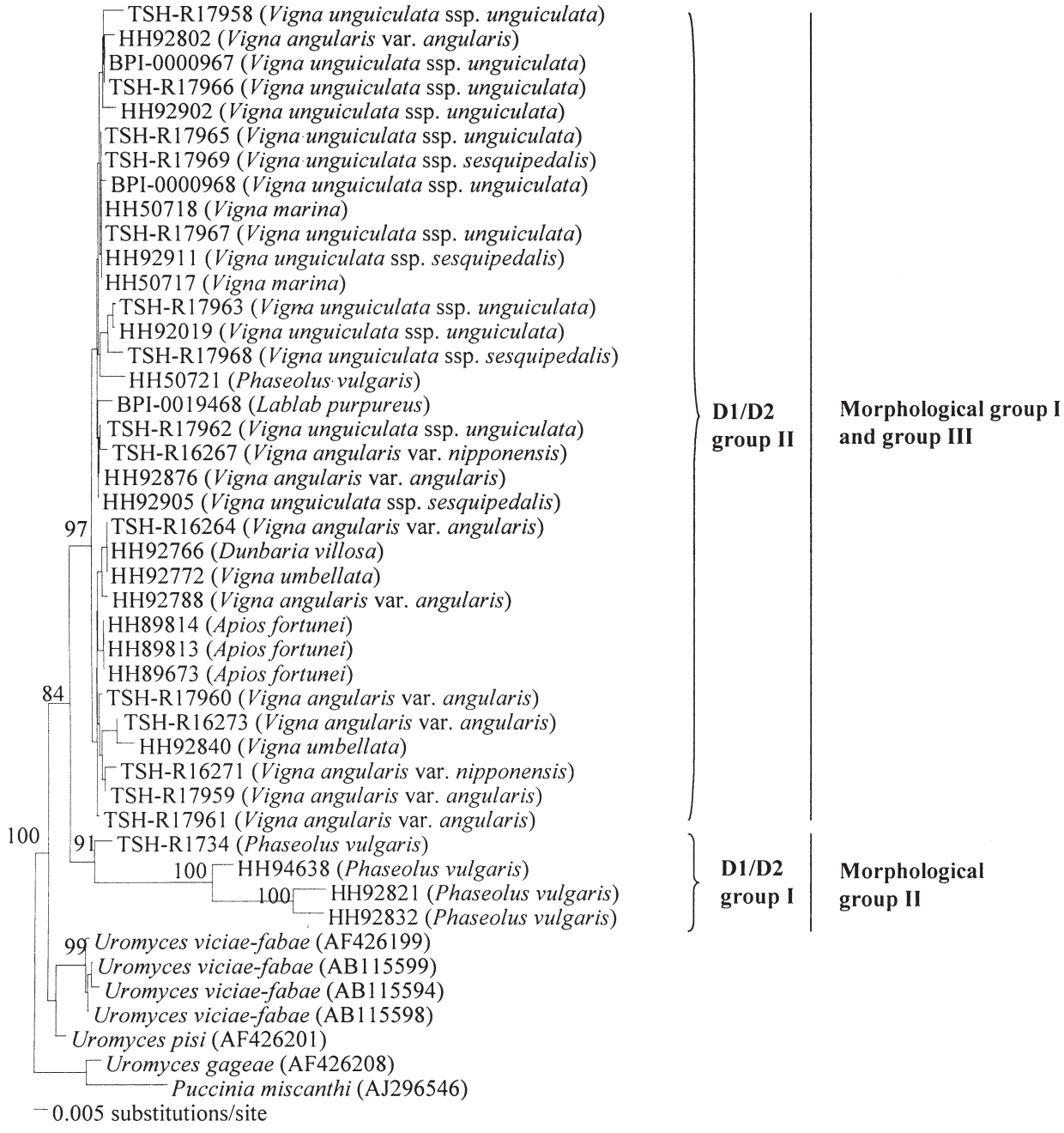


**Fig. 6.** A neighbor-joining tree inferred from sequences of large subunit (LSU) rDNA (D1/D2) region using Clustal X. Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. Length of branches is proportional to number of base changes, indicated by the *scale* at the bottom

that three morphologically circumscribed groups of rust fungi were not host limited (see Table 4). Thus, morphological group II occurs on *V. angularis* var. *angularis* and *Phaseolus vulgaris*, morphological group I occurs on *V. angularis* var. *angularis*, *V. angularis* var. *nipponensis*, *V. radiata*, *V. umbellata*, *A. fortunei*, *D. villosa*, *P. vulgaris*, and *P. minimus*, and morphological group III occurs on *V. unguiculata* ssp. *unguiculata*, *V. unguiculata* ssp. *sesquipedalis*, *V. marina*, *V. angularis* var. *nipponensis*, and *P. vulgaris*. These results disagree with the belief of a strict restriction of these rusts to particular host genera.

The results indicate that *U. appendiculatus* and *U. vignae* may parasitize both *Phaseolus* and *Vigna* species, although no extensive cross-inoculation experiments have been conducted to prove this perspective. Chung et al. (2003) reported that a rust fungus from *V. angularis* var. *angularis* infected and sporulated on *V. unguiculata* ssp. *unguiculata* and a rust fungus from *V. unguiculata* ssp. *unguiculata* infected and sporulated on *V. angularis* var. *angularis*. These results indicate that the host specificity of the fungi classified under *U. appendiculatus* var. *azukicola* and *U. vignae* is not genetically fixed at the level that the two species can be

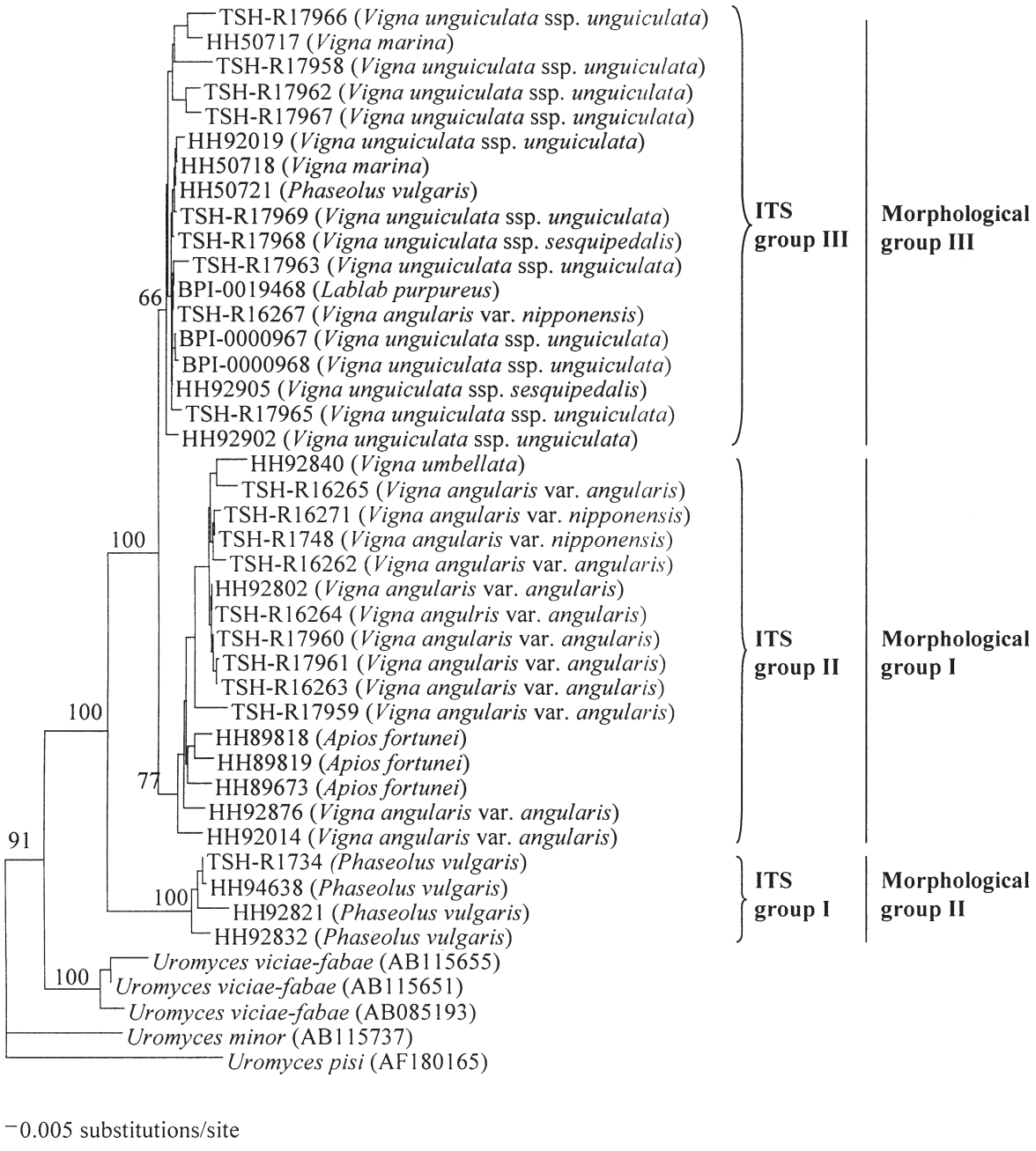


**Fig. 7.** A neighbor-joining tree inferred from sequences of internal transcribed spacer (ITS) and 5.8 S rDNA regions using Clustal X. Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. Length of branches is proportional to number of base changes, indicated by the *scale* at the bottom

distinguished in Japan. Eckenwalder and Heath (2001) discussed the evolutionary significance of the infection process and host specificity of *U. appendiculatus* and *U. vignae* on species of *Phaseolus* and *Vigna*.

A single or a few morphological features alone cannot be judged by themselves as a good taxonomic character to designate rust fungus taxa to a specific rank in the taxonomic hierarchy, even though a few morphological features can circumscribe the taxa. An appropriate assessment of taxonomic characters can be done by comparing independent characters that are not causally related. In this study, we compared the specimen groups circumscribed by morphological features and those in phylogenetic trees inferred from rDNA sequences. The three morphological groups corresponded to the three distinct clades generated from the analysis of nucleotide sequences at the ITS region including 5.8 S rRNA gene (see Fig. 7). In contrast, the specimens in morphological groups I and III scattered over the D1/D2 group II whereas the morphological group II corresponded well with the D1/D2 group I (see Fig. 6).

Topologies of the D1/D2 trees generated by a neighbor-joining method (see Fig. 6) and by a maximum-parsimony method (data not shown) were similar. The level of divergence between the D1/D2 groups I and II was not high, whereas the D2 sequences showed high T nucleotide repeat at the sites from 546th to 556th base. The high T nucleotide repeat at the D2 sequence distinguished clade I from clade II. As with the D1/D2 trees, the topology of the ITS trees were similar both in a neighbor-joining method (Fig. 7) and a maximum-parsimony method (data not shown). The ITS sequences were more diverse than were the D1/D2 sequences. The ITS tree and the D1/D2 tree are different in that the specimens in the ITS groups II and III scatter over the D1/D2 group II whereas the same specimens constitute both the ITS group I and the D1/D2 group I (Figs. 6, 7). The discordance between the two inferred trees may indicate that either one or neither of them reflects the true phylogenetic relationships. It is argued, however, that sequence variation in the D1/D2 region is often insufficient to distinguish biological species (O'Donnell and Cigelnik 1997; Maier et al. 2003), whereas sequence variation in the ITS region is usually large enough to separate taxa at a species level (White et al. 1990). Furthermore, the agreement of morphological groups I, II, and III to the ITS groups II, I, and III is highly indicative that the three groups are distinct at the species level.

It is appropriate to take a practical plant pathological view into consideration for our taxonomic/phylogenetic results. The ITS group I/morphological group II occurs on *P. vulgaris* (Fig. 7) and shows morphological features of what has been recognized as *U. appendiculatus*. The ITS group II/morphological group I occurs on *V. angularis* var. *angularis*, *V. angularis* var. *nipponensis*, *V. umbellata*, and *A. fortunei* (Fig. 7) and shows morphological features of what has been recognized as *U. appendiculatus* var. *azukicola* and var. *dispersus*. The ITS group III/morphological group III occurs mostly on *V. unguiculata* and its variety and occasionally on *V. marina*, *L. purpureus*, and *P. vulgaris* (Fig. 7) and shows the morphological features of what has been recognized as *U. vignae*.

When considering the specimens of three morphological groups that have not been subjected to molecular analyses, the ITS groups/morphological groups are not restricted to a particular host genus as discussed previously. Thus, it is concluded that three rust fungus species occur on *Phaseolus*, *Vigna*, *Apios*, *Lablab*, and *Dunbaria* in Japan and the three species are referable to *U. appendiculatus*, *U. azukicola*, and *U. vignae*. Before drawing further taxonomic and nomenclatural conclusions, however, it is necessary to examine the nomenclatural types of these three species and allied taxa.

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