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FULL PAPER

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Life cycle and nuclear behavior in three rust fungi (Uredinales)

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Abstract *Kuehneola japonica* has a microcyclic life cycle with a regular alternation of generations. Single basidiospore inoculations onto *Rosa wichuraiana* resulted in teliospore production, indicating its homothallic nature. Dikaryotization in a vegetative mycelium in the host seemed to occur through nuclear division that was not followed by septum formation. Karyogamy and meiosis took place through teliospore and metabasidium development; this fungus was considered to reproduce genetically homogeneous progenies. *Puccinia lantanae* and *P. patriniae* were also microcyclic in their life cycle; however, these fungi differed from *K. japonica* in the mode of nuclear behavior. In the former two fungi, both vegetative and reproductive cells were uninucleate. No karyogamy was observed, and nuclear division in the metabasidium development was thought to be mitotic. In *P. lantanae*, a basidiospore was formed on a sterigma, whereas a whiplike hypha emerged from each metabasidium cell in *P. patriniae*. Inoculations of *Justicia procumbens* with a single basidiospore of *P. lantanae* resulted in teliospore production. The fungus seemed to remain uninucleate, either haploid or diploid, throughout the life cycle. Thus, reproduction was considered to be apomictic.

Key words Apomixis · Homothallism · *Kuehneola japonica* · *Puccinia lantanae* · *Puccinia patriniae*

Introduction

Rust fungi exhibit a diverse array of life cycles and accompanying nuclear behaviors through teliospore (probasidium) and metabasidium development. Most rust species producing only a telial stage associated or not associated with a spermogonial stage are believed to have been derived from macrocyclic parental species, producing spermogonial, aecial, uredinial, and telial stages through various pathways (Jackson 1931; Hennen and Buriticá 1980). These microcyclic species constitute a larger proportion of the rust biota in arcto-alpine regions or oceanic islands, whereas macrocyclic species predominate in temperate regions. The life cycle producing only the telial stage is said to be reduced in these arcto-alpine or island species. In contrast, many tropical species producing only a telial stage are considered to be unexpanded in their life cycle (Hennen and Buriticá 1980). The microcyclic life cycle in the rust fungi includes those with two different evolutionary backgrounds.

Rust species with a reduced life cycle are most diverse in nuclear behaviors and development of metabasidia and basidiospores (Jackson 1931; Petersen 1974; Walker 1928). Some of the best examples have been depicted by Gardner (1981, 1987, 1988, 1994, 1996) and Hodges and Gardner (1984). Possible pathways of life cycle reduction and interpretations of various patterns in nuclear behavior and associated morphological changes in metabasidium and basidiospore development are still controversial. For better understanding of the evolution of the microcyclic life cycle and nuclear behavior, detailed studies on a broad range of rust species are indispensable.

This article reports the life cycle, nuclear behavior, and morphology of the metabasidia and basidiospores of three rust fungi with a reduced life cycle that are distributed in Japan.

Materials and methods

Fungal species and isolates examined

Kuehneola japonica (Dietel) Dietel on *Rosa wichuraiana* Crép., Ibaraki, Kasama, Mt. Sashirosan, 18 October 1993, Y. Ono 2942; Ibaraki, Higashiibaraki-gun, Oarai-machi, 12

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August 1998, Y. Ono 4231. *Puccinia lantanae* W. G. Farlow on *Justicia procumbens* L., Ibaraki, Kuji-gun, Diago-machi, Shimonomiya, 1998 and Mito, 1999 (no voucher specimen). *Puccinia patriniae* P. Hennings on *Patrinia villosa* (Thunb.) Juss., Gumma, Tone-gun, Katashina-mura, Oshimizu, 16 September 1999, Y. Ono 4390.

The fungi were collected together with the host plants, and the infected plants were transplanted in a clay pot and maintained at Ibaraki University campus in Mito. The voucher specimens have been deposited in the Herbarium of Systematic Mycology, Ibaraki University (IBA).

Fixation and staining

Fresh fungal spores were scraped from the sori on the host plants and floated on a drop of distilled water or 0.1% water agar on a microscopic slide. The slide preparation was incubated in the dark at either 18°C or 20°C for 12–24 h, depending on the fungal species. The incubated slide preparation was then air-dried and fixed with Carnoy's fluid (absolute ethanol : chloroform : glacial acetic acid = 6 : 3 : 1) at about 20°C for 30 min and stained with propidium iodide solution according to a standard protocol (Fujita and Minamikawa 1990; Nagle 1996; Mukai 1996): the fixed materials in a vial were hydrated through an ethanol series (75% for 2 min, 70% for 2 min, 50% for 2 min, and distilled water for 2 min), soaked in phosphate-buffered saline solution (Sigma, St. Louis, MO, USA) for 2 min, incubated in RNase A (type I-AS; Sigma, 100 mg/ml) solution at 37°C for 1 h, rinsed with Tris-ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.4), and stained with a solution composed of 5 mg/ml propidium iodide in 10% Tris-EDTA buffer and antifade [1.25% 1,4-diazabicyclo (2.2.2) octane; Sigma) in the dark at 20°C for 1 h. The stained materials were mounted with the propidium iodide solution as described above and the cover slip was sealed with fingernail polish. The preparations were examined immediately or stored at 4°C in the dark until examined (maximum, 1 month).

The fungus-infected lesions of the host plants were free-hand sectioned under a binocular dissecting microscope, fixed, and stained within a screw vial with the same protocol as above.

Microscopy and photomicrography

The slide preparations were examined with an Olympus (Tokyo, Japan) BX50 epifluorescent microscope equipped with U-MWIG cube (a 520 to 550-nm excitation filter BP520–550, a 565-nm dichroic mirror DM565, and a 590-nm barrier filter BA580IF) and UPlanFl 40/0.75 and UPlanFl100/1.30 objective lenses. The microscope was also equipped with a differential interference contrast optic (DIC) unit so that both fluorescent and DIC images were simultaneously observed and photographed. Photomicrographs were taken at a magnification of 670× or 270× on 35-mm Fuji Presto film (ISO 400) with an Olympus PM-20 automatic photomicrograph unit.

Single basidiospore inoculation

Single basidiospores spread on a microscopic slide were isolated with an Olympus ON-M1 micromanupilator under an Olympus IX50 inverted microscope. The isolated basidiospores were individually transferred onto young shoots or leaves of the uninfected host plants. The inoculated plants were kept under saturated humidity in the dark at 20°C for 48 h, and then transferred to a growth chamber at about 20°C with artificial illumination of 12-h-light and 12-h-dark intervals.

Successfully infected parts of the host plants as manifested by production of yellow flecks or by production of sorus primordia were cut into small pieces, free-hand sectioned, and stained as already described.

Results

Kuehneola japonica

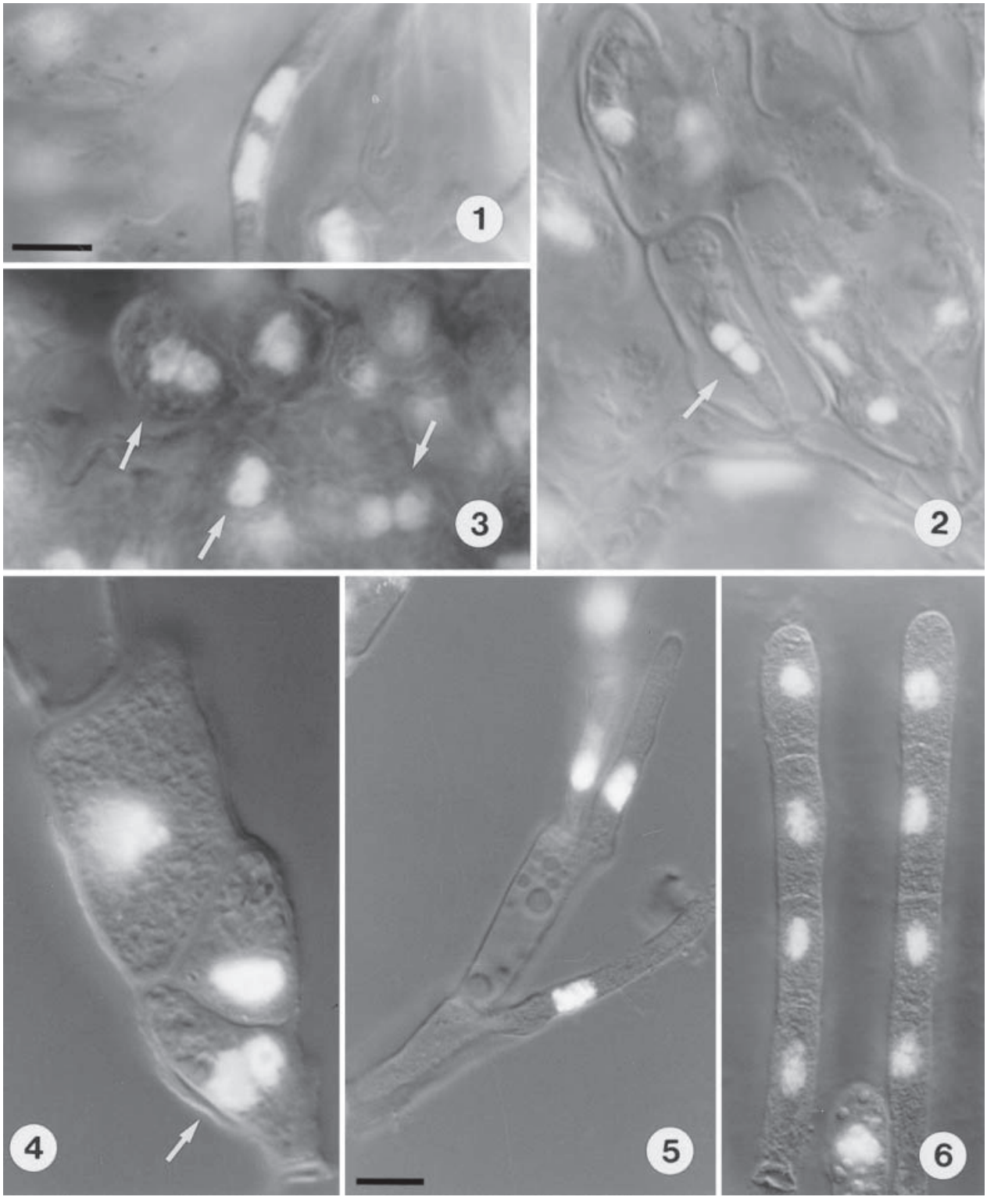
A vegetative mycelium in the tissue of both successfully inoculated and field-collected host plants was binucleate (Fig. 1). The binucleate hyphae ramified and grew intercellularly in the host tissue. The intercellular hyphae gave rise to a cylindrical or club-shaped haustorium in the host mesophyll cells. The haustorium was one-celled, unbranched, and also binucleate (Fig. 2).

The hyphae became aggregated to form a telial primordium beneath the host epidermis. From binucleate teliospore initials (sporogenous cells) produced in the telial primordium (Fig. 3), binucleate teliospore mother cells arose. The teliospore mother cell successively divided 3 to 7 times so as to form a linear 3- to 7-celled teliospore and a subtending pedicel cell. The immature teliospore cells and the subtending pedicel cell were binucleate. As the teliospores mature, the two nuclei in each teliospore cell fused, while the two nuclei in the pedicel cell remained separated (Fig. 4).

The teliospores germinated to become a metabasidium, into which the fused nucleus migrated (Fig. 5). The nucleus divided twice, presumably by meiosis, resulting in four daughter nuclei in the metabasidium (Fig. 6). No clearly condensed chromosomes were observed in the nuclear division (Fig. 7). A septum was laid down between the daughter nuclei on completion of each nuclear division.

From each of four metabasidium cells, a sterigma arose and a basidiospore was formed, into which the nucleus migrated (Fig. 8). After being forcibly discharged from the sterigma, the nucleus in the basidiospore divided once (Fig. 9).

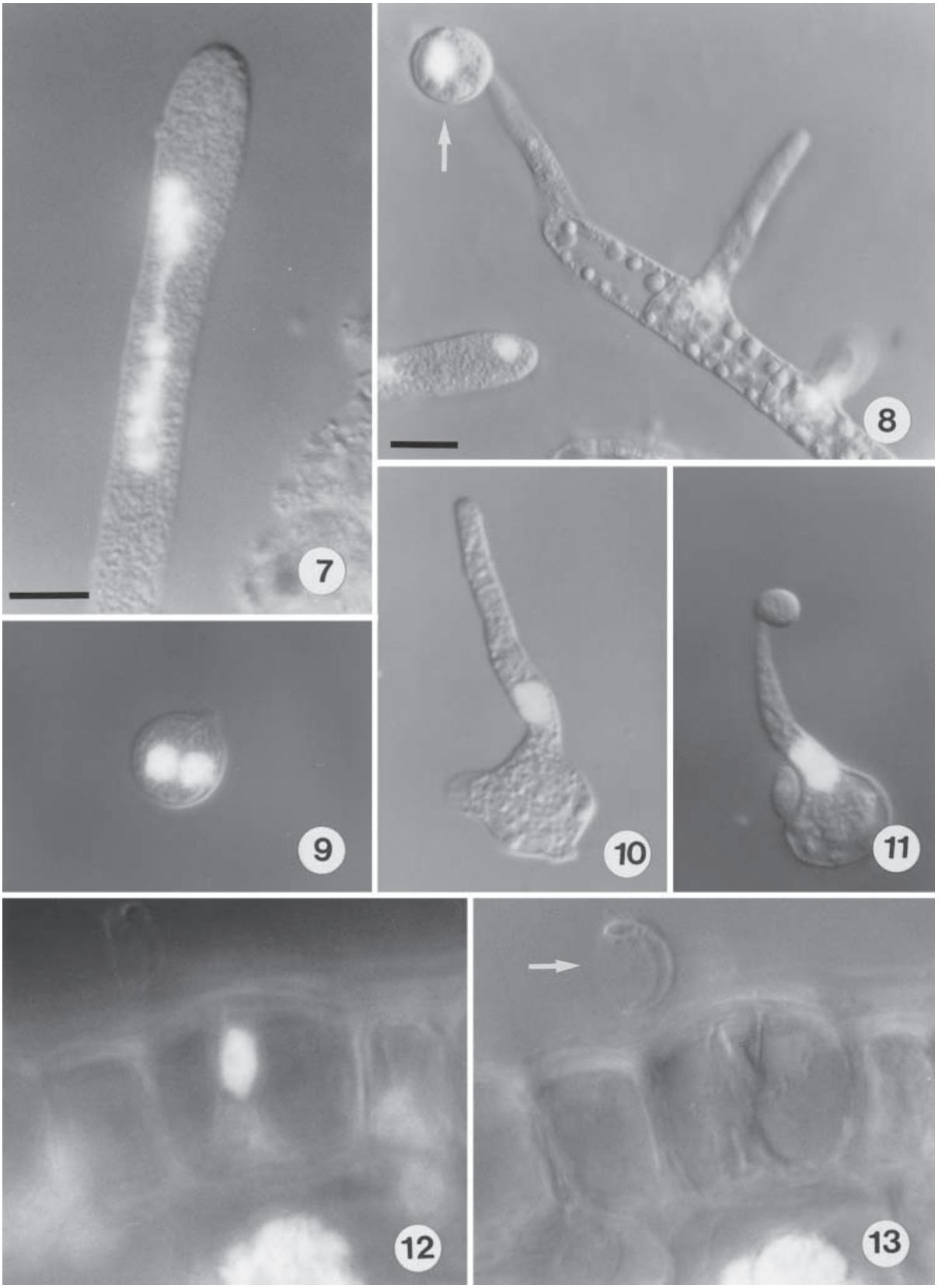
The basidiospore germinated either to a germ tube or to produce a secondary spore. Before the germination, most of the basidiospores became uninucleate by disintegration of one of the two nuclei and the remaining nucleus migrated either into a short germ tube (Fig. 10) or to a secondary spore (Fig. 11). Some basidiospores remained binucleate during germination; however, only one of the two nuclei



Figs. 1–6. *Kuehneola japonica*. **1** Binucleate vegetative mycelium. **2** Binucleate haustorium (*arrow*) in a host mesophyl cell. **3** Binucleate telial primordium cells (*arrows*). **4** Diploid, uninucleate telial cells and a subtending haploid, binucleate pedicel cell (*arrow*). **5** Diploid nucleus moving into a developing metabasidium. **6** Haploid, uninucleate metabasidium cells. *Bars* **1–4** 5 μm; **5, 6** 10 μm

migrated either into a germ tube or to a secondary spore. No tertiary or quaternary spores were observed.

Single-basidiospore inoculations resulted in infection and teliospore production, although the rate of successful infection was very low (7 successful infections with the telium production of 100 single-basidiospore inoculations). The inoculation was successful only when the spores were placed on young etiolated shoot tips, and no infection was



Figs. 7–13. *Kuehneola japonica*. **7** Presumed anaphase of meiosis I in a developing metabasidium. **8** Uninucleate basidiospore (*arrow*) formed on a sterigma arising from a metabasidium cell. **9** Binucleate basidiospore. **10** Germinating basidiospore with a single nucleus. **11** Secondary spore production from a uninucleate basidiospore. **12** Uninucleate infection hypha in a host epidermal cell. **13** Collapsed basidiospore (*arrow*) on host epidermis and infection hypha in host epidermal cell (same as in Fig. 12 but not fluorescent). *Bars* **7, 9–13** 5 μm; **8** 10 μm

obtained when the spores were inoculated on fully expanded leaflets of a compound leaf.

Upon inoculation on the host, a short germ tube from the basidiospore penetrated the host cuticle and cell wall. The penetration hypha entered into the epidermal cell and did not become enlarged. The nucleus migrated into the penetration hypha (Figs. 12, 13). From the penetration hypha, intercellular hyphae arose. The intercellular hyphae became binucleate at an early stage of the infection; however, the mode of dikaryotization was not determined.

Puccinia lantanae

Intercellular hyphae in the host mesophyll were mostly uninucleate (Fig. 14). Binucleate hyphae were seldom observed. Haustorial cells were also uninucleate. Cells of hyphal aggregates that would give rise to a telial primordium were still uninucleate (Fig. 15). Teliospore initials arose from a cell layer beneath the host epidermis. The teliospore initial was uninucleate (Fig. 16) and seemed to originate directly from the telial primordium cell without dikaryotization and immediate karyogamy in the teliospore initial. The teliospore initial divided to form a distal teliospore mother cell and a proximal cell, both of which remained uninucleate (Fig. 16). The teliospore mother cell divided to become a one-celled teliospore and a subtending pedicel cell. Two-celled teliospores were infrequently observed. The mature teliospores, either one-celled or two-celled, were uninucleate with a few exceptions.

On germination, the teliospores gave rise to a metabasidium, into which the nucleus migrated. The nucleus divided twice, and the four daughter nuclei were separated subsequently by septa to form a four-celled metabasidium (Fig. 17). Each of four metabasidium cells gave rise to a sterigma, on which a uninucleate basidiospore was formed (Figs. 17, 18). The nucleus in the metabasidium cell migrated into a basidiospore where the nucleus further divided so that the basidiospore became binucleate. Either one or two nuclei migrated into a germ tube arisen from the basidiospore (Fig. 19).

Single basidiospore inoculations were successful at a very low rate (less than 5%), resulting in the telium production. The infection process was not observed. The teliospores formed by the single basidiospore inoculations were unicellular.

Puccinia patriniae

Intercellular hyphae were uninucleate throughout the host mesophyll (Fig. 20). A telial primordium that was composed of an intercellular hyphal aggregate was uninucleate (Fig. 21). A layer of cells beneath the host epidermis gave rise to teliospore initials, which were also uninucleate (Fig. 22). The teliospore initial divided into a distal teliospore mother cell and a proximal cell. The teliospore mother cell further divided to become a two-celled teliospore and a subtending pedicel cell. Both immature and mature teliospores were uninucleate (Fig. 23). Very rarely, a few telial primordium cells appeared to be binucleate (Fig. 24).

The teliospore germinated into either a short or elongated metabasidium (Figs. 25, 26). The nucleus migrated into the metabasidium from the teliospore cell. The nucleus divided twice to become four daughter nuclei. The four nuclei were separated by septa concomitantly laid down with the nuclear division, the metabasidium becoming four celled with a single nucleus in each cell.

Each of four cells of the metabasidium gave rise to a peg- or a whiplike germ tube instead of producing a normal basidiospore. The nucleus in the metabasidium cell migrated into the peg- or whiplike projection (Fig. 27). No additional nuclear division took place in the germ tube. No further observation was made as to the infection process of the basidial germ tube.

Discussion

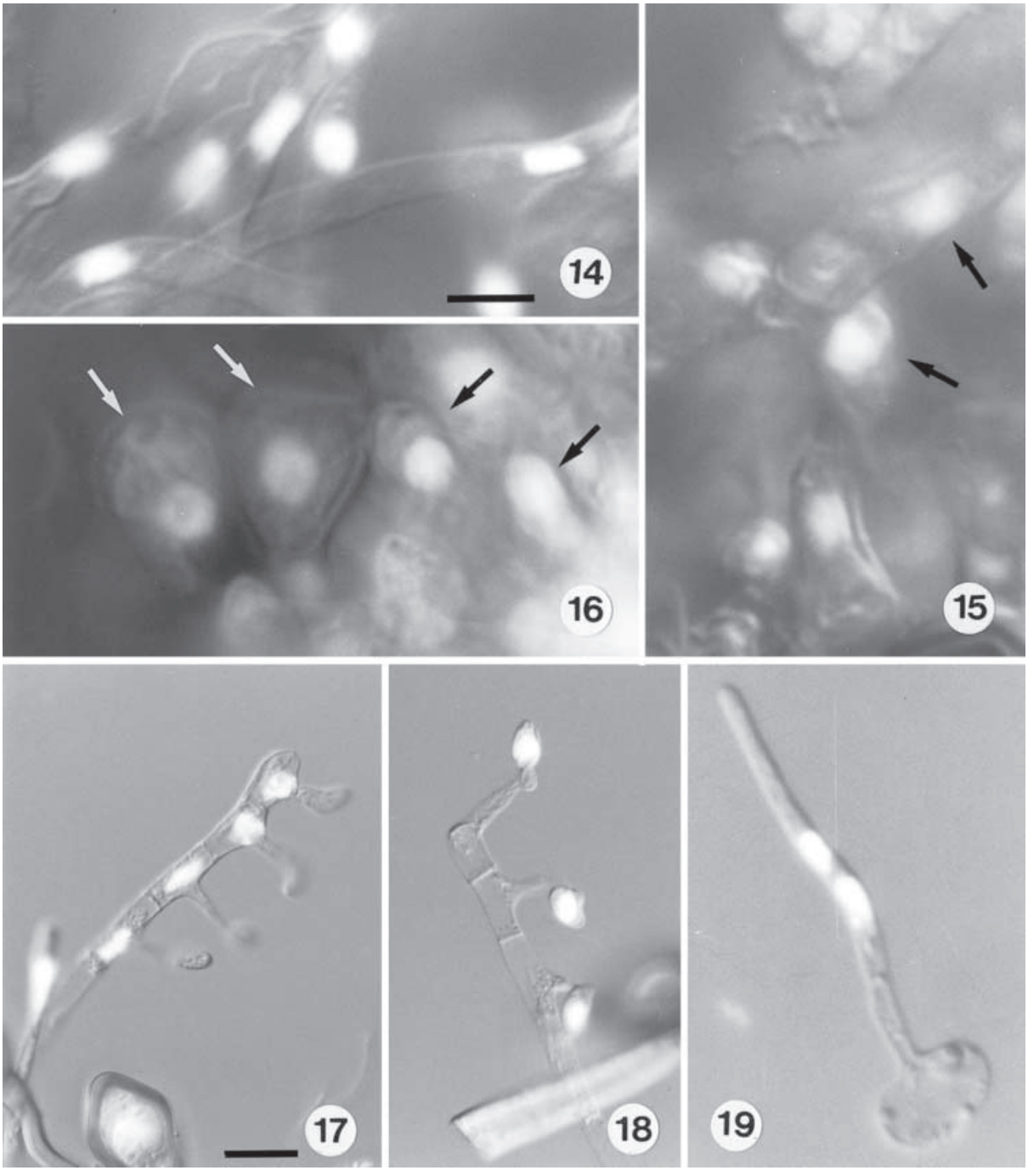
In the rust species with a macrocyclic life cycle, either autoecious or heteroecious, a regular alternation of generation is commonly observed, i.e., karyogamy and meiosis take place at the end of a sporophytic generation (teliospores), and dikaryotization at the initiation of sporophytic generation (aeciospores and urediniospores), and the gametophytic generation (spermogonium and aecial primordium) intercalates between the two phases of the sporophytic generation. Examples of this nuclear behavior observed in the microcyclic life cycle rusts include *Puccinia malvacearum* Bertero (Blackman and Fraser 1906; Olive 1911; Werth and Ludwig 1912; Moreau 1914; Lindfors 1924; Allen 1933), *Endophyllum sempervivi* (Alb. & Schw.) de Bary (Maire 1900; Hoffman 1912; Moreau and Moreau 1918b; Ashworth 1935), *P. grindeliae* Peck (Brown 1940), *P. xanthii* Schw. (Brown 1940), *P. ruelliae* (Berk. & Br.) Lagerh. (Singh 1979), *Cystopsora oleae* Butler, pro parte (Thirumalachar 1945), *Uromyces rayssiae* Anikst. & Wahl (Anikister et al. 1980), and *Puccinia pampeana* Speg. (Hennen et al. 1984). *Puccinia pampeana* possesses the *Endophyllum*-type teliospore in addition to the *Puccinia*-type teliospore, both of which are formed only after the cross-spermatization on the basidiospore-infected plant.

On the other hand, in the microcyclic rust species, i.e., those with secondarily reduced life cycle, a variety of nuclear behaviors have been observed (Jackson 1931; Petersen 1974).

Kuehneola japonica

In this study, *K. japonica* was shown, as has previously been reported by Kohno et al. (1975, 1977), to exhibit the regular nuclear cycle commonly observed in macrocyclic rust species.

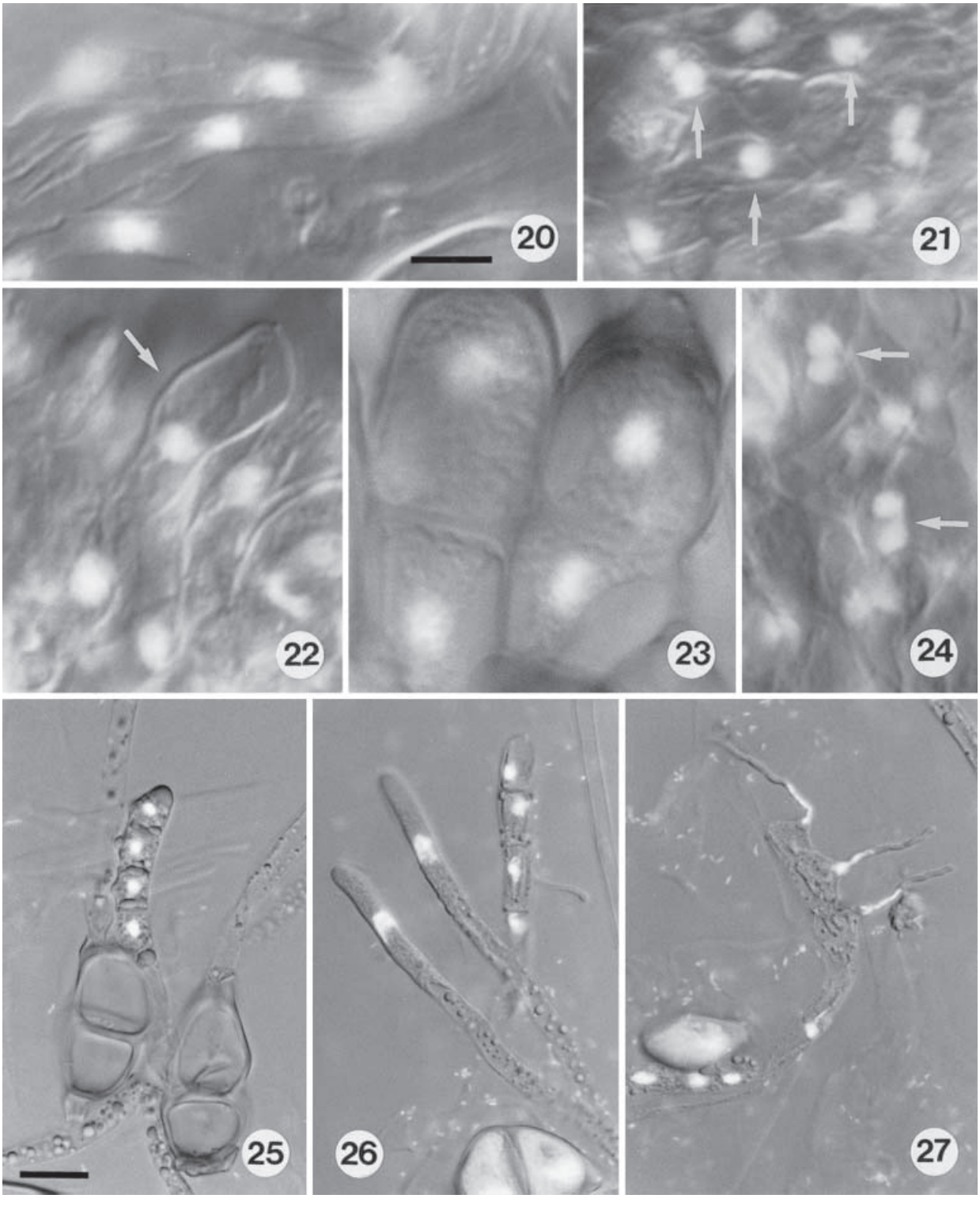
Successful single basidiospore inoculation proved that this fungus is homothallic and that the uninucleate infection



Figs. 14–19. *Puccinia lantanae*. **14** Uninucleate vegetative mycelium. **15** Uninucleate telial primordium cells (*arrows*). **16** Uninucleate teliospore mother cells (*thin black arrows*) and young teliospores (*thick white arrows*). **17** Uninucleate metabasidium cells, on which a basidiospore is being produced. **18** Uninucleate basidiospores on a sterigma. **19** Binucleate germ tube from a basidiospore. *Bars* **14–16, 19** 5 μm; **17, 18** 10 μm

hypha gives rise to the binucleate mycelium. The dikaryotization seems to take place by mitotic nuclear division that is not followed by septum formation (Maire 1899; Walker 1928; Dodge 1929) at an early point of the infection. The results, that well-isolated single basidiospore inoculation resulted in the teliospore production and that dikaryotization took place at an early stage of infection, may rule out the two possiblities of dikaryotization by nuclear migration from an adjacent mycelial cell (Blackman 1904) or by anastomosis of uninucleate mycelial cells (Christman 1905; Kursanov 1917).

In the course of teliospore differentiation, two nuclei in a teliospore mother cell fused while those in a pedicel cell remained separated. Therefore, nuclear division observed in the metabasidium developing upon germination of the teliospore is believed to be meiosis.



Figs. 20–27. *Puccinia patriniae*. **20** Uninucleate vegetative mycelium. **21** Uninucleate telial primordium cells (*arrows*). **22** Uninucleate teliospore mother cell (*arrow*). **23** Two-celled, uninucleate teliospores. **24** Binucleate vegetative mycelium cells (*arrows*) were rarely observed. **25** Short, four-celled metabasidium with a single nucleus in each cell. **26** Long, four-celled metabasidium with a single nucleus in each cell (*right*). Two developing metabasia with a single nucleus (*left*). **27** Whip-like hyphae from metabasidium cells. A single nucleus is moving into each hypha. *Bars* **20–24** 5 μm; **25–27** 10 μm

The uninucleate basidiospores often became binucleate by an additional mitotic division as commonly observed in other rust species (Anikister 1983, 1984). Either one of the two or both nuclei migrated into the germ tube. However, only one of them seems to take part in the infection, another being degenerated. Consequently, upon infection of a uninucleate infection hypha, a uninucleate intracellular hypha resulted from the basidiospore infection.

Nuclei involved in the dikaryotization, karyogamy, and meiosis in the alternation of generation possess the same genetic constituent. Thus, this fungus is considered to reproduce genetically homogeneous progenies in the homothallic sexual process, which is highly contrasted to the macrocyclic species with regular alternation of generation such as *P. helianthi* Schw., *P. graminis* Pers., *P. recondita* Desm., *P. coronata* Corda, *P. sorghi* Schw., *P. phragmitis* (Schm.) Koern., *Melampsora lini* (Ehrenb.) Lév., *Uromyces appendiculatus* (Pers.) Unger, *U. vignae Barcl.*, *Gymnosporangium asiaticum* Yamada, *G. juniperi-virginianae* Schw., *G. globosum* Farl., and *Cronartium ribicola* J. C. Fischer. These species are proven or assumed to be heterothallic (Cragie 1927a,b; Allen 1933; Brown 1940), and sexual recombination is an essential part of the life cycle in these macrocyclic species.

Puccinia lantanae

The results, showing that the fungus was uninucleate throughout the life cycle and single basidiospore inoculations resulted in telium production, suggest that the fungus reproduces apomictically. Although four uninucleate basidiospores are formed on the four-celled metabasidium as normally observed in sexually reproducing species, the nuclear division during the development of the metabasidium and the basidiospore is considered to be mitosis.

This type of nuclear behavior and metabasidium formation have been reported for a large uninucleate form of *E. euphorbiae-sylvaticae* (DC.) Wint. (=*E. euphorbiae* Plowr., *E. uninucleatum* Moreau) (Mme. Moreau 1911, 1914, 1915; Moreau and Moreau 1918a, 1919; Dodge 1929; Olive 1953), *E. centranthi-rubri* Poir. (Poirault 1915), and a uninucleate form of *Gymnoconia nitens* (Schw.) Kern & Thurst. (=*Caeoma nitens* Burrill) (Dodge 1929). This type has also been reported for a form of a demicyclic species *Puccinia podophylli* (Brumfield, Ined; cited from Dodge 1929).

Dodge (1929) considered that the nucleus observed in the vegetative mycelium and the teliospore of *E. euphorbiae-sylvaticae* is diploid because the spores of the uninucleate form are as large as those of the binucleate forms and that the nucleus is as large as two nuclei of the binucleate spore. He assumed that the uninucleate form of *E. euphorbiae-sylvaticae* arose from the parental form by fusion of two haploid nuclei in the vegetative mycelium, which does not normally occur in the parental form.

In contrast, the nucleus of a uninucleate form of *G. nitens* was considered as haploid because the spore is smaller in this form than the binucleate spore and because the nucleus is the same as that of the binucleate spore (Dodge 1929). The nucleus of *E. centranthi-rubri* was also considered as haploid without a stated reason (Dodge 1929). These two forms and species are different from the uninucleate form of *E. euphorbiae-sylvaticae* in producing a two-celled metabasidium.

It is unknown whether the nucleus in the *P. lantanae* life cycle is haploid or diploid. If haploid, *P. lantanae* is unique in being haploid, uninucleate throughout the life cycle, and reproducing apomictically with the production of the four-celled metabasidium.

Puccinia patriniae

The uninucleate life cycle and the apomictic reproduction observed in this species are the same as those in *P. lantanae*. Binucleate cells were rarely observed in the telial primordium. If this is not aberrant nuclear behavior, this may indicate that the haploid uninucleate vegetative mycelium becomes dikaryotized by an unknown method, immediately followed by karyogamy. However, no binucleate teliospores have been observed, and thus this fungus is better considered to be uninucleate, either diploid or haploid, throughout the life cycle. The nuclear division took place in the development of the four-celled metabasidium is considered to be mitosis.

Although the nuclear behavior in *P. patriniae* is the same as that of *P. lantanae*, the four metabasidium cells give rise to a peg- or a whiplike germ tube in the former species, instead of producing ordinary basidiospores as in the latter. The nucleus in the metabasidium cell migrated into the peg- or a whiplike projection. No additional nuclear division took place in the germ tube.

It is empirically known that, in water or under condition of oxygen deprivation, teliospores germinate into elongated germ tubes or metabasidia, which when formed give rise to narrow, whiplike germ tubes. However, in this study, teliospores were germinated on an agar film on a microscopic slide. Because ordinary metabasidia and basidiospores were formed in *K. japonica* and *P. lantanae* under the same condition and because whiplike germ tubes were exclusively observed in *P. patriniae*, the latter mode of germination is considered to be normal in this fungus, as has been reported in *Uromyce aloes* (Ck.) Magn. (Sato et al. 1980), *U. hobsoni* Vize (Payak 1953), *Endoraecium acaciae* Hodges & Gardner, and *E. hawaiiense* Hodges & Gardner (Hodges and Gardner 1984).

Finally, it is important to note that any type of nuclear behavior observed for one or a few populations from a microcyclic species may not represent the entire population of that species. Microcyclic derivatives might have arisen repeatedly from a parental macrocyclic species (Jackson 1931). Thus, different populations, even though originated from the same parental species, may have different types of nuclear behavior. Microcyclic derivatives may remain morphologically similar and thus are taxonomically conspecific; however, a reproductive isolation mechanism might have

developed among those populations because of their different nuclear behaviors.

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