CHAPTER 1
General Introduction

1.1 INTRODUCTION

The kingdom Fungi is undoubtedly conspicuous for its species diversity owing to the natures of minute, simple, and plastic growth forms and various nutritional modes. However, the diversity is apparently far from being fully understood (Hawksworth 1991, 2001). In an estimate (Hawksworth 2001), the known species occupy less than 3.8% of the total fungal species in spite of the fact that 56,360 species have already been described (Hawksworth et al. 1995).

Among the four phyla of the Fungi, the Zygomycota is numerically smallest (Hawksworth et al. 1995) but remarkably diverse and less studied than other three phyla (Benny et al. 2001). Therefore, the phylogenetic relationships between the Zygomycota and other phyla and those between zygomycetous taxa have attracted much interest. Recent investigations using molecular techniques revealed a part of the relationships. That is, (1) The Zygomycota is a paraphyletic group and it cannot be separated from the phylum Chytridiomycota (Van de Peer & De Watchter 1997). (2) Several clades exist in the Zygomycota-Chytridiomycota cluster (Nagahama et al. 1995, Benny & O' Donnell 2000, O' Donnell et al. 2001). (3) The division of two classes in the Zygomycota, the Zygomycetes and the Trichomycetes has no phylogenetic basis (O' Donnell et al. 1998, Tanabe et al. 2000).

One of the clades included in the Zygomycota-Chytridiomycota cluster consists of the Kickxellales Kreisel ex R. K. Benj. 1979 (Zygomycetes), the Dimargaritales R. K. Benj. 1979 (Zygomycetes), and the Harpellales Lichtwardt & Manier 1978 (Trichomycetes), which is phylogenetically much varied than other clades (Tanabe et al. 2000). The divergence in the nutrition mode of the clade is also remarkable compared with any other clades; the members live as saprobes (most of the Kickxellales), mycoparasites (Dimargaritales and a few kickxellaleans), or intact parasites (guests) of arthropods (Harpellales) (Alexopoulos et al. 1996). The clade would serve as an ideal model for studying fungal evolution because nutrition modes are the conclusive results of natural selections of respective physiological features (Cooke & Whipps 1993). Because nowadays the Kickxellales is much more diverse than other two orders of the clade in nutrition and morphology, investigations on the order would promote a better understanding of the evolution in the clade and in the Zygomycota.

The importance was fully known, however, no comprehensive taxonomic studies

of the Kickxellales have been conducted for more than 40 years after the admirable works of Benjamin (1958, 1959, 1961, 1963). This retardation arises from by the limitation of fungal materials available for the study. Furthermore, the study of the order is accompanied with many difficulties described as follows.

The principal difficulty is that kickxellaleans are rarely encountered fungi in the field (Kwaśna et al. 1999a, Benny et al. 2001). Many kickxellalean species appear to be extremely rare since more than a half of known species have never been rediscovered after the original descriptions. Even a few species of *Coemansia* Tiegh. et G. Le Monn. 1873, which relatively often occur on soil (Hendrix et al. 1971; Tokumasu et al. 1976; Miura 1978; Udagawa 1978a, b; Benny et al. 2001), are not listed in a generally accepted compendium of common soil fungi (Domsch et al. 1980a, b).

Another difficulty occurs in finding kickxellaleans from samples incubated in the laboratory. Kickxellaleans are frequently overlooked since they grow slowly and tend to be overgrown or covered by other fungi that thrive in the sample (Benny & Benjamin in O'Donnell et al. 1998, Benny et al. 2001) . Further, arthropods or nematodes included in the samples often physically destroy the mycelia of kickxellaleans and prevent them from sporulating (unpublished). Thus, the view that kickxellaleans can be easily found owing to their relatively large and peculiar asexual reproductive apparatus (Kirk 1993) appears to be too optimistic.

Even though we could find out the objective fungi in samples, the establishment of pure cultures of them is not easy in general. In fact, Torrey (1921) and Kwaśna et al. (1999b) stated that they had failed in establishing pure cultures of several kickxellalean species because the spores had never germinated on ordinary media for saprobes.

Because of these difficulties, no comprehensive taxonomic studies of the order have been carried out for a long time, while several new taxa were added to the order (Kurihara et al. 2000, Ogawa et al. 2001). Due to the inclusion of these newly added taxa, the current classification system lacks the consistency. Therefore, the present study aimed to construct a more consistent taxonomic system of the Kickxellales. To accomplish this purpose, the following strategy was employed. At the beginning, the attempts to isolate kickxellalean fungi were made, and the results are described in chapter 2. In chapter 3, by using the isolates obtained, morphological characteristics of kickxellaleans are assessed at the optical microscopic level. In chapter 4, based on the results of the assessments,

all kickxellalean genera are divided into groups. The groups are evaluated by observations of septal ultrastructure (in chapter 5) and ribosomal RNA (rRNA) gene sequence analyses (in chapter 6). And in chapter 7, taxonomical conclusions of the Kickxellales are drawn from these results.

1.2 OUTLINES OF THE KICKXELLALES AND ITS RELATED TAXA

In this section, the Kickxellales and its related taxa are outlined briefly. In the following parts, the Kickxellales (Zygomycetes, Zygomycota) and its probable close relatives, the Dimargaritales (Zygomycetes) and the Harpellales (Trichomycetes), are referred to as 'kickxellids' in general.

1.2.1 Kickxellales and its higher taxa

In this part, the phylum Zygomycota, the class Zygomycetes, and the order Kickxellales are introduced.

(1) The phylum Zygomycota

The Zygomycota is one of the phyla that constitute the kingdom Fungi. The phylum is principally defined by producing zygospores as a consequence of sexual reproduction. Production of sporangiospores as asexual spores, the presence of chitin or chitosan in cell walls, and the absence of flagellate cells and centrioles are other important characters of the phylum (Benny et al. 2001). Having these characteristics is the reason for the accommodation of the species to the phylum of which zygospores are not discovered (O'Donnell 1979).

The Zygomycota is divided into two classes, Zygomycetes and Trichomycetes according to their nutrition modes (Table 1-1). Fungi that live inside the gut of arthropods are classified into the Trichomycetes, and others are included in the Zygomycetes.

(2) The class Zygomycetes

Most members of the class Zygomycetes are saprobes or parasites of other organisms, particularly fungi that belong to the same class (Benny et al. 2001). Morphologically, the class is characterized by producing sporangiospores as a result of asexual reproduction and hyphae without regular septa (Alexopoulos et al. 1996, Benny et al. 2001). Within the Zygomycetes, 7 orders are recognized (Hawksworth et al. 1995; Table 1-1) based on nutrition mode, vegetative and

reproductive morphology, and the presence or absence of septa (Benjamin 1959, 1979; Hawksworth et al. 1995; Humber 1989; Morton & Benny 1990; Benny et al. 2001). Lately, Cavalier-Smith (1998) proposed 3 new orders based on the molecular analyses. And more recently, 2 orders (Glomerales Morton & Benny 1990 and Geosiphonales Cavalier-Smith 1998) were removed from the class to make a new phylum (Schüßler et al. 2001). Among these orders of the class, the order Mucorales *sensu* Benjamin (1979) is the numerically largest and studied most sufficiently, while little is known about other orders.

(3) The order Kickxellales

The Kickxellales Kreisel ex R. K. Benj. 1979 is a small and unique order of the Zygomycetes (Table 1-1). It is composed of 1 family, 10 genera, 28 species, and 1 variety (Table 1-2). Most members of the order are saprobes except for three species presumed to be mycoparasitic (Benjamin 1959, 1979), and usually isolated from feces or soil. Infra-ordinal taxonomy of the Kickxellales is essentially based on asexual reproductive morphology at microscopic level (Benjamin 1959, 1979). Moss & Young (1978) named the total asexual reproductive morphology 'coemansioid pattern' and pointed out its commonality with that of the Harpellales.

Taxonomically, since Linder (1943) established the family Kickxellaceae Linder 1943 (Zygomycetes) for *Kickxella* Coem. 1862, *Martensella* Coem. 1863, and *Coemansia*, seven genera have been added to the family: *Linderina* Raper et Fennell 1952, *Martensiomyces* Meyer 1957, *Spirodactylon* R. K. Benj. 1959, *Dipsacomyces* R. K. Benj. 1961, *Spiromyces* R. K. Benj. 1963, *Ramicandelaber* Ogawa et al. 2001, and *Myconymphaea* Kurihara et al. 2001. Among these genera, *Coemansia* is the largest genus that comprises 16 species and 1 variety. All the rest are small genera composed of one or two species.

The Kickxellales is characterized by producing septa (cell walls that delimit cells of fungi) in hyphae with a central pore filled up with a plug persistent in acidic stains and 2-3% aqueous solution of potassium hydroxide (KOH), convoluted asexual reproductive structures, sporangiospores in monosporic sporangiola, and usually smooth zygospores (Benjamin 1979).

The mycoparasitism of the order is unique among the Zygomycota since the known mycoparasites of the order select a hyphomycete (Fungi Imperfecti) or a polypore (Basidiomycota) as their hosts (Benjamin 1979). This stands in contrast to other zygomycetous mycoparasites including dimargaritalean species that

utilize the Mucorales (Zygomycota) as their hosts (Benjamin 1979).

1.2.2 Presumptive close relatives of the Kickxellales (kickxellids)

In this part, presumptive close relatives of the Kickxellales (kickxellids) are briefly described.

(1) The order Dimargaritales

The order Dimargaritales R. K. Benj. 1979 is one of the presumptive lineages of the Kickxellales. The order contains 1 family, 4 genera, and 14 species (Kirk et al. 2001; Table 1-1), and most members of the order are haustorial and obligate parasites of the Mucorales (Benjamin 1979). Among the genera hitherto described, *Dimargaris* Tiegh. 1875, *Dispira* Tiegh. 1875, and *Tieghemiomyces* R. K. Benj. 1959 are generally accepted. The affinity of the remaining genus, *Spinalia* Vuill. 1904 with the order is doubted (Hawksworth et al. 1995, Kirk et al. 2001), and the genus is not discussed in the present work.

The Dimargaritales produces plugged septa (Fig. 1-4) and the sexual and asexual reproductive structures (Figs 1-2, 1-3) that resemble those of the Kickxellales. These resemblances in morphology between the two orders are the basis of the hypothesis that they are close relatives (Figs 1-1, 1-2, 1-3, 1-4). Ultrastructural (Saikawa 1977), karyological (Benjamin 1959, 1966), and histochemical studies (Benny 1972) also supported their close affinity.

Historically, based on these support, Kreisel (1969) accommodated the Dimargaritaceae R. K. Benj. 1959 and the Kickxellaceae to the Kickxellales *sensu* Kreisel, *nomen nudum* that was invalid because of the lack of a Latin diagnosis (in accordance with Art. 36.1 of the International Code of Botanical Nomenclature; Greuter et al. 2000). However, when Benjamin (1979) described the Kickxellales Kreisel ex R. K. Benj. 1979, he excluded the Dimargaritaceae from the order and established a new order Dimargaritales with emphasizing the differences between these two families. Differing from the Kickxellales, the Dimargaritales produces two-spored sporangiola on sporiferous branchlets, septal plugs that are associated with protuberances and soluble in the acidic stains and 2-3% KOH (Benjamin 1959, 1965, 1966, 1979; Brain et al. 1982), and zygospores with surface ornamentation (Figs 1-1, 1-2, 1-3, 1-4).

The comparisons of 18S ribosomal DNA (rDNA) sequences of the two orders (Tanabe et al. 2000, Benny et al. 2001) present uncertain phylogenetic relationships between them. This ambiguity would be caused by the extensive base

substitution in the 18S rRNA gene of the Dimargaritales (Tanabe et al. 2000).

(2) The class Trichomycetes

The class Trichomycetes is defined as to be obligate parasites inhabiting the guts of living arthropods (Alexopoulos et al. 1996). The class is undoubtedly polyphyletic (Lichtwardt 1986), and even a protozoan was incorporated in it until quite recently (Benny & O' Donnell 2000). The Trichomycetes is consisted of 3 orders, Harpellales, Asellariales, and Eccrinales (Kirk et al. 2001; Table 1-1), and the former two orders are the presumptive relatives of the Kickxellales.

(3) The order Harpellales

The order Harpellales Lichtwardt & Manier 1978 is presumed to be phylogenetically close to the Kickxellales. The Harpellales is the largest order in the Trichomycetes, and 2 families, 33 genera, and 142 species are classified to the order (Kirk et al. 2001; Table 1-1). All members are obligate parasites (or endocommensals or guests) and live in the guts of larvae and nymphs of aquatic insects (Lichtwardt 1973). The two familles, Harpellaceae Léger & Duboscq 1929 and Legeriomycetaceae Pouzar 1972, are divided on the basis whether thalli are branched or unbranched and the adhesive place of the thalli along the gut (on the midgut or the hindgut) (Lichtwardt 1986).

In the Harpellales, gross asexual reproductive morphology both at the optical and electron microscopic levels (Young 1974, Benny & Aldrich 1975, Moss & Lichtwardt 1976, Moss & Young 1978) (Fig. 1-2) and the septal ultrastructure (Moss 1975, 1979, Moss & Young 1978, Saikawa et al. 1997; Fig. 1-4) show considerable resemblances to those of the Kickxellales and would be homologous with them. In addition to these facts, immunological (Sangar et al. 1972, Peterson & Lichtwardt 1987), karyological (Moss 1974), histochemical (Sangar & Dugan 1973, Whisler 1963), and molecular (O' Donnell et al. 1998, Tanabe et al. 2000) studies support their close phyletic affinity.

The major differences between the Harpellales and the Kickxellales is in the process of formation and morphology of zygospores (Moss & Lichtwardt 1977) (Fig. 1-3).

(4) The order Asellariales

The order Asellariales Lichtwardt & Manier 1978 is composed of 1 family, 3 genera, and 11 species (Kirk et al. 2001; Table 1-1). All species are obligate

parasites of isopods, collembolans, or dipterans, and inhabit the gut of these hosts (Alexopoulos et al. 1996). The Asellariales generates arthrospores as asexual spores, while the sexual reproduction has never been known apart from hyphal conjugations observed in *Asellaria ligiae* Tuzet & Manier ex Manier 1968 that was believed to be the initiation of the sexual reproduction (Lichtwardt 1986). The septal structures of the Asellariales resemble those of the Harpellales (Manier 1973, Moss 1975, Saikawa et al. 1997).

In this article, the Asellariales will not be discussed because of the scarcity of other information on the order (Benny 2001), although the order has been regarded as a close relative of the Harpellales based on the similarities in the septal structures (Moss & Young 1978) and the pattern of germination of asexual spores (Lichtwardt 1973).

(5) The genus Ballocephala

Ballocephala verrucospora M. J. Richardson 1970 (Meristacraceae, Entomophthorales, Zygomycetes) is a parasite of tardigrades. Saikawa (1989) proposed a hypothesis that the species and kickxellids are close relatives based on the similarities in their septal ultrastructure. However, ultrastructural observations of conidia (Saikawa & Oyama 1992) and zygospores (Saikawa & Sakuramata 1992) gave no positive evidence to support the affinity except that conidia slip out of its outermost layer of cell wall before infection to tardigrades (Saikawa & Oyama 1992), the same as that sporangiospores of some kickxellaleans slip sporangiola off before germination. I will not discuss the species since no other data on the fungus comparable with kickxellids are available. Further study may be necessary for the determination of the final taxonomic position of this species.