



Review

Phylogenetic classification and physiological and ecological traits of *Metarhizium* spp.

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ABSTRACT

The genus *Metarhizium* (Hypocreales: Clavicipitaceae) is mostly composed of entomopathogenic fungal species. Many of these species are anamorphic and difficult to distinguish morphologically. Furthermore, most isolates of this genus have a broad host range, making classification based on host-insect species uncertain. Molecular phylogenetic analysis based on DNA sequence information distinguishes these species well and revises the taxonomy of *Metarhizium*. However, in the revisions, the major groups within the genus, such as *M. anisopliae* complex, were classified regardless of their phenotypic differentiation. Therefore, the characteristics of the individual species remain unclear. To explore the species-specific characteristics of *Metarhizium* spp., the author performed a phylogenetic analysis and characterization of *Metarhizium* spp. in Japan. The results showed that strains of the *M. brunneum* and *M. pempighi* clades exhibited cold-active growth characteristics and preferred forested environments over *M. pingshaense*. In the *M. majus* clade, a specialist of scarab beetles, isolates from different Scarabaeidae species, including the coconut rhinoceros beetle (*Oryctes* spp.) and flower chafer beetle (*Protaetia orientalis*), formed separate subclades and showed strong virulence against their original hosts. This review describes the current state of understanding of the taxonomy and species-specific characteristics of the genus *Metarhizium*, and includes the author's own previous study.

Keywords: entomopathogenic fungi, habitat, phylogenetic analysis, temperature growth, virulence

Article history: Received 17 April 2024, Revised 4 July 2024, Accepted 18 July 2024, Available online 1 August 2024.

1. Introduction

Metarhizium (Hypocreales: Clavicipitaceae) consists mostly of entomopathogenic species that produce green conidia on their host insect's corpus (Kepler et al., 2014). It is commonly referred to as green muscardine fungus because of its color (Fig. 1). The fungus can infect various insect species and has a diverse host range (Zimmerman, 2007). For example, *M. anisopliae* (Metchn.) Sorokin sensu lato is well-known for its broad host range, infecting more than 200 insect species (Zimmerman, 2007). In contrast, *M. acridum* (Driver & Milner) J.F. Bisch., Rehner & Humber and *M. album* Petch are specific to locusts and small plant hoppers, respectively (Bischoff et al., 2009; Rombach et al., 1987).

This genus has been extensively studied for its use in the biological control of various agricultural and sanitary insects, and some strains have been used as commercial biopesticides. Formulations using *M. anisopliae* sensu lato account for 33.9% of all entomopathogenic fungus-based formulations, with *Beauveria bassiana* Bals.-Criv. sensu lato (33.9%) being the most commonly used (Faria

& Wright, 2007). One of the most studied strains of this genus is *M. brunneum* Petch F52 strain, which was first developed as a formulation against black vine weevils; however, its applicable target has been expanded to various greenhouse pests, such as whiteflies, thrips, and aphids (EFSA et al., 2020). *Metarhizium anisopliae* strain RNO31, a mutant strain with enhanced virulence induced by UV irradiation, has been commercialized as a soil conditioner in Japan to reduce damage to grass and crop roots caused by white grubs (Yokoyama, 2005).

The genus has been reported to be diverse not only in its entomopathogenic characteristics, but also in temperature characteristics, UV tolerance, and habitat preference, making it a promising biopesticide for a wide variety of pests (Bidochka et al., 2001; Driver et al., 2000; Fargues et al., 1996; Rangel et al., 2005; Welling et al., 1994). However, because of the paucity of morphological characteristics of anamorphic fungi, traditional classification based on the morphology of the conidia and conidiophores fails to adequately reflect the diversity of the genus (Rombach et al., 1986, 1987; Tull-och, 1976). Host-based classification was also largely unreliable because *M. anisopliae* sensu lato, which has a wide host range, dominated the majority. Since the 1990s, this genus has undergone strain identification and taxonomic revision based on DNA sequencing information (Curran et al., 1994; Driver et al., 2000). In the revisions, *M. anisopliae* and *M. flavoviride* W. Gams & Rozsypal

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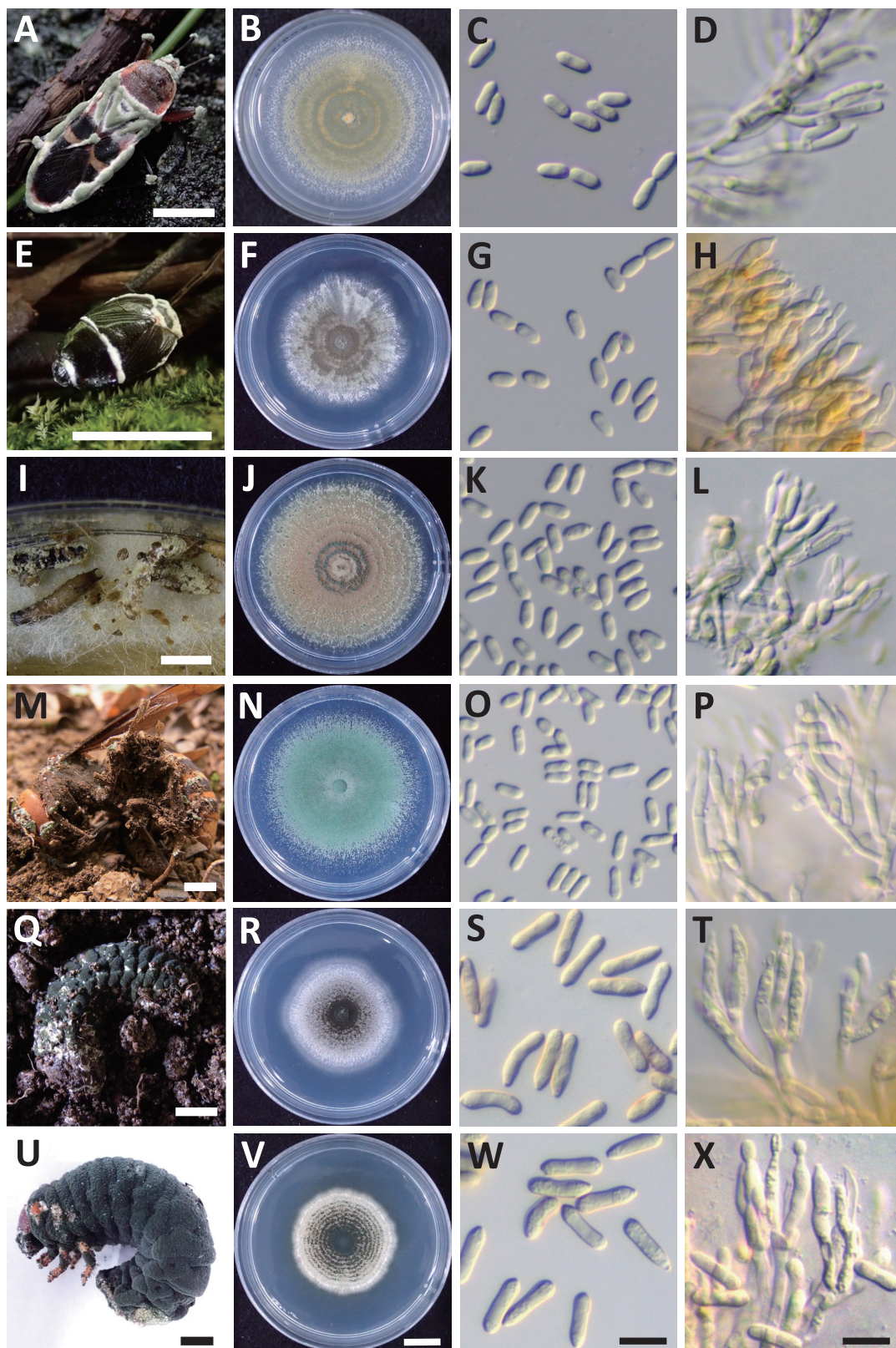


Fig. 1. Macro- and micrographs of *Metarhizium* spp. in Japan (A–D: *M. pingshaense* OMNS180916-7 isolated from *Physopelta gutta* (Homoptera: Largidae); E–H: *M. brunneum* OMNS190909-2 isolated from a soil stinkbug (Homoptera: Cydnidae); I–L: *M. humeri* OMNS110531-1 isolated from a coconut hispine beetle *Brontispa longissima* (Coleoptera: Chrysomelidae), a kind gift from Prof. Keiji Takasu (Kyushu University); M–P: *M. pemphigi* MAFF 245744 isolated from a wasp (Hymenoptera: Vesidae); Q–T: *M. majus* MAFF 243306 isolated from a flower chafer beetle *Protaetia orientalis* (Coleoptera: Scarabaeidae); U–X: *M. majus* OR10 isolated from a coconut rhinoceros beetle *Oryctes rhinoceros* (Coleoptera: Scarabaeidae), a kind gift from Prof. Satoshi Kamitani (Kyushu University); A, E, I, M, Q, U: Host insects (Bars: 5 mm); B, F, J, N, R, V: Colony (10 or 14-day-old on PDA, Bars: 10 mm); C, G, K, O, S, W: Conidia (Bars: 10 μ m); D, H, L, P, T, X: Conidiophore (Bars: 10 μ m)).

were recognized as species complexes because many cryptic species were recognized in their closely related lineages based on Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Bischoff et al., 2006, 2009; Luz et al., 2019). In the revisions, the major groups within the genus, such as *M. anisopliae* complex, were classified regardless of their phenotypic differentiation. Therefore, the unique phenotype of each species remains unclear. In addition, each species clade contained strains derived from diverse origins, not associated with host insect species or geographic origins. The association of species clades with thermal characteristics, UV tolerance, and habitat preferences is largely unknown.

In a series of previous studies, the author revealed differences in growth temperature, habitat preference, and virulence among the phylogenetic species of each clade in *M. anisopliae* and *M. flavoviride* complexes in Japan. This review focuses on the two-species complex and examines their taxonomy and physiological and ecological characteristics.

2. Taxonomy

The genus *Metarhizium* consists of many morphologically indistinguishable species, which are currently classified and identified based on DNA sequence information (Bischoff et al., 2006, 2009; Driver et al., 2000). The range of the genus was defined by Kepler et al. (2014) through phylogenetic analysis and was further revised by Mongkolsamrit et al. (2020). Mongkolsamrit et al. (2020) determined the phylogenetic relationships of 63 species that covered almost all accurately described species. The phylogenetic relationships based on their study are shown in Fig. 2. Within this genus, there are four large monophyletic groups: the *M. anisopliae* complex (19 species), *M. flavoviride* complex (13 species), small planthopper-parasite clade (six species, including *M. album*), and cicada-parasite clade (eight species, including *M. cylindrosporum* Q.T. Chen & H.L. Guo). The outer smaller clades that diverged at the ancestral stage include the caterpillar-specific *M. rileyi* (Farl.) Kepler, S.A. Rehner & Humber, the chameleon pathogens, *M. viri-*

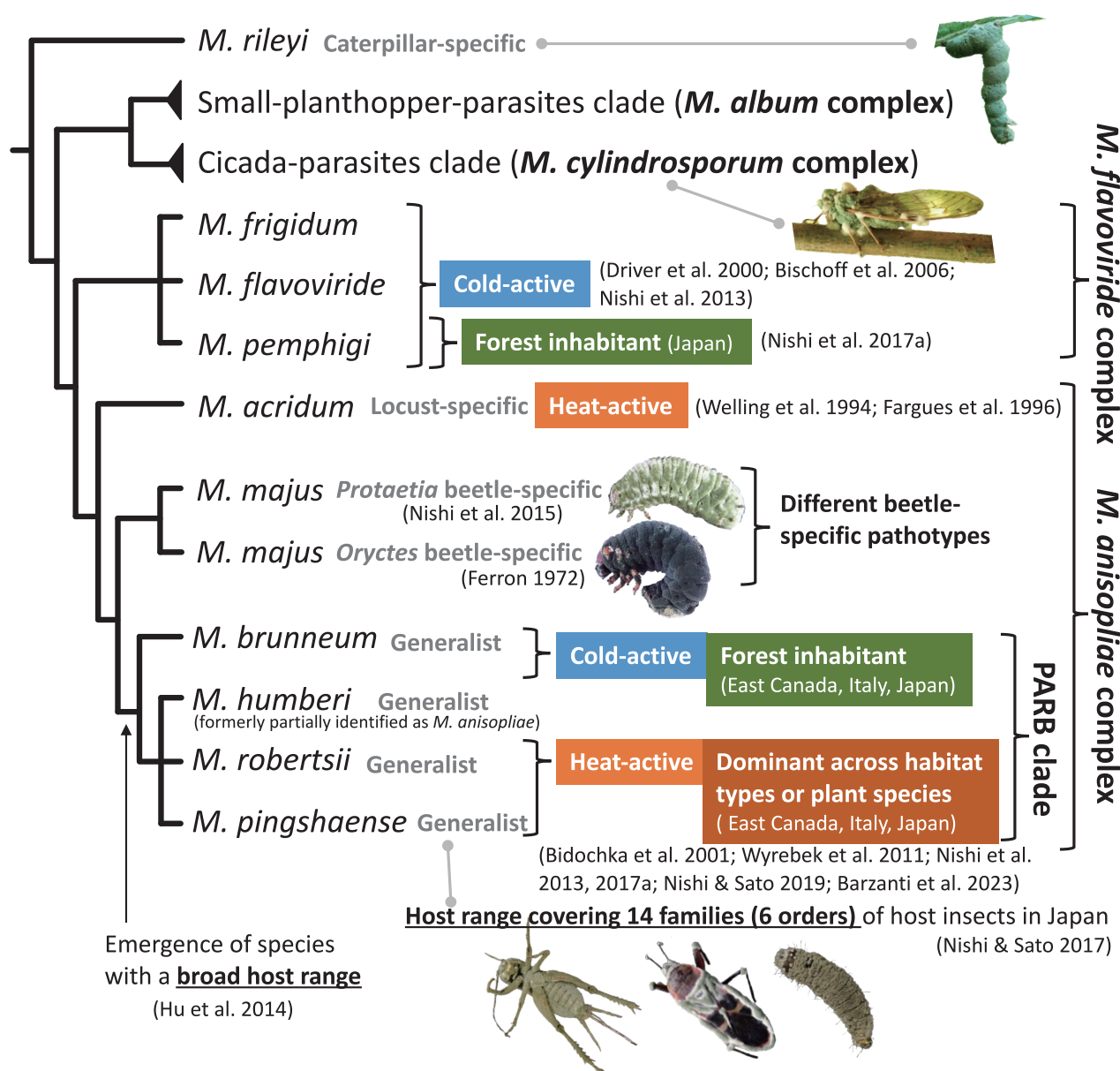


Fig. 2. Schematic diagram for phylogenetic relationships of *Metarhizium* spp. All photos were taken by the author.

de (Segretain, Fromentin, Destombes, Brygoo & Dodin ex Samson) Kepler, S.A. Rehner & Humber and *M. granulomatis* (Sigler) Kepler, S.A. Rehner & Humber, and teleomorph species infecting beetle larvae in rotten logs, such as *M. atrovirens* (Kobayasi & Shimizu) Kepler, S.A. Rehner & Humber. In addition, there are other described species, such as *M. viridicolumnare* (Matsush.) Matsush. (Matsushima 1993), whose phylogenetic positions remain unknown.

Metarhizium anisopliae, originally known to have a wide host range (over 200 species), is a member of the *M. anisopliae* complex. Most isolates originally identified as *M. anisopliae* belonged to the PARB clade in this species complex (Bischoff et al., 2009; Luz et al., 2019), which consists of *M. brunneum*, *M. humeri* Luz, Rocha & Delalibera, *M. pingshaense* Q.T. Chen & H.L. Guo, and *M. robertsii* J.F. Bisch., Rehner & Humber. *M. anisopliae* sensu stricto was originally a member of this clade but was transferred outside by Mongkolsamrit et al. (2020). "PARB" is supposedly acronyms for the constituent species, however, Bischoff et al. (2009) did not specify the etymology. Members of the PARB clade are distributed worldwide and are the most frequently found species in the soil in many field studies (Fig. 3). Among the insect-derived isolates of *M. anisopliae* sensu lato from Japan, *M. pingshaense* and *M. brunneum* were the two most frequently isolated species (Nishi & Sato, 2017). According to Bischoff et al. (2009), the four species are morphologically indistinguishable because their colony color, conidial size, and shape closely overlap.

Most species, other than those in the PARB clade, have few known host insect species, suggesting that their host ranges are narrow. The low occurrence of species outside the PARB clade in soil microflora surveys using bait insects of mealworms and wax moth larvae suggests that they are unlikely to infect insects other than their original hosts because of the narrow host range. However, there were some exceptions. For example, *M. pemphigi* (Driver & R.J. Milner) Kepler, S.A. Rehner & Humber belonging to the *M. flavoviride* complex, has been isolated from insects of three orders (Coleoptera, Hemiptera, and Hymenoptera) and can also be isolated from soil using the bait method with mealworms (Fisher et al., 2011; Nishi & Sato, 2017).

As described above, the correspondence between large phylogenetic groups in *Metarhizium* and host ranges is partially recognized; however, reliable diagnostic features remain undetermined for many species. For example, *M. bibionidarum* O. Nishi & H. Sato was distinguished from its sister species *M. pemphigi*, by its consistently larger conidia, but could not be distinguished from some non-sister species (Nishi et al., 2017b). *Metarhizium purpureogenum* O. Nishi & H. Sato, on the other hand, can be identified by the dimensions of conidia and its unique property of turning the agar medium red (Nishi et al., 2017b). As Bischoff et al. (2009) noted, the ability to separate cryptic species using objective phylogenetic criteria has enabled systematic efforts to identify the physiological and ecological features that further distinguish these phylogenetic species.

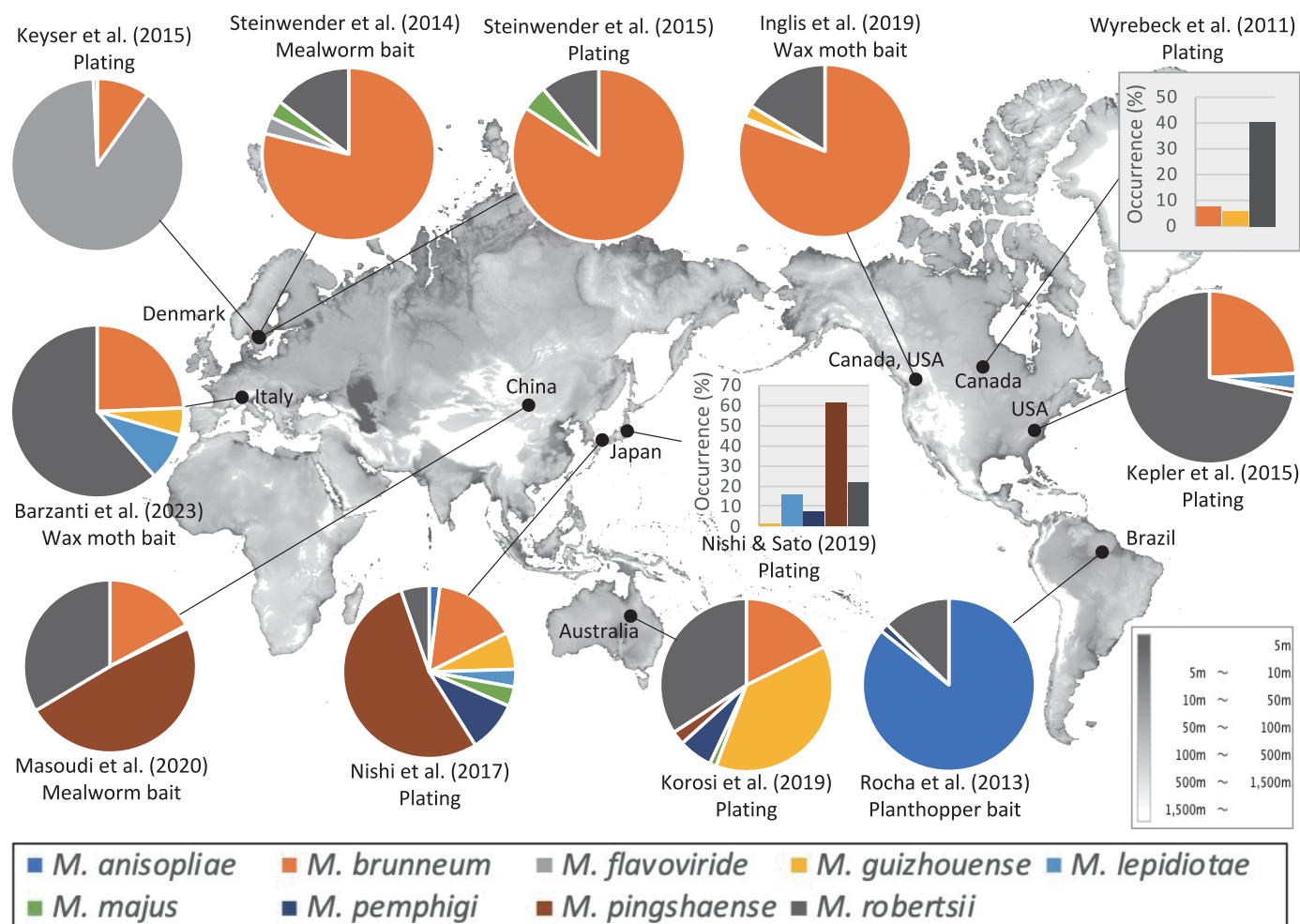


Fig. 3. Summary of species composition of *Metarhizium* spp. isolated from soils worldwide. The world map is based on GSI's map (<https://maps.gsi.go.jp>).

3. Regional differences in species composition

As described above, taxonomy based on molecular phylogenetic analysis has subdivided the species delimitation of *Metarhizium* (e.g., Bischoff et al., 2006, 2009). Taxonomic revisions have revealed significant regional differences in species composition. Fig. 3 shows 12 studies on the species composition of *Metarhizium* spp. isolated from soils in different countries. It should be noted that comparisons of these studies do not reflect purely regional differences because the isolation methods (i.e., plating or insect bait methods) and environmental types of the collection sites differ among these studies (i.e., cultural fields or forests). In Japan, Nishi et al. (2011, 2017a) found that *M. pingshaense* was the most common species in 302 soil samples collected from various environments at latitudes between 28° and 45°. *Metarhizium pingshaense* is also the most common species found in forest soils in China (Masoudi et al., 2020). However, it has been detected in soil at low frequencies in field studies in the USA and Australia and was not detected in Brazil, eastern and western Canada, Denmark, and Italy (Barzanti et al., 2023; Inglis et al., 2019; Kepler et al., 2015; Keyser et al., 2015; Korosi et al., 2019; Rocha et al., 2013; Steinwender et al., 2014, 2015; Wyrebek et al., 2011). The most frequent species were *M. robertsii* in eastern Canada, Italy, and the USA; *M. brunneum* in Denmark and Western Canada; and *M. anisopliae* (currently *M. humeri* according to Mongkolsamrit et al., 2020) in Brazil. The extremely high frequency of *M. anisopliae* in the survey in Brazil may be partly due to the use of Hemiptera species as insect bait. All PARB clade members are considered a group of species with a broad host range and may be ecologically equivalent species occupying biologically similar niches in different regions. Among the PARB clade members, only *M. brunneum* clearly showed cold-active characteristics regardless of its diverse isolation sources (Nishi et al., 2013), which may reflect the high occurrence of this species in cold regions such as Denmark. This may explain why *M. flavoviride* was predominant in agricultural field soils in Denmark (Keyser et al., 2015) since this species also displays cold-active characteristics (Driver et al., 2000).

4. Habitat preference and growth temperature

Metarhizium spp. are distributed in various environments worldwide (reviewed by Zimmermann, 2007). As expected, isolates of *Metarhizium* from different climatic zones and habitat types exhibit physiological characteristics that are adapted to their respective environments (Rangel et al., 2005). These physiological variations in *Metarhizium* have been considered intraspecific; however, in the current taxonomy, they are recognized as interspecific differences. For example, *M. flavoviride* sensu lato, isolated from locusts in the tropics and subtropics, shows high heat and UV tolerance (Fargues et al., 1996; Welling et al., 1994), whereas *M. flavoviride* from Europe shows cold-active growth characteristics (Driver et al., 2000). In the current taxonomy, the former has been transferred to *M. acridum* (Bischoff et al., 2009). Its high heat and UV tolerance is considered an adaptation to the sunburn behavior of desert locusts (Elliot et al., 2002). Another example of this is *M. anisopliae* sensu lato population in eastern Canada, where a genetic group dominant in forests tended to grow faster at low temperatures and slower at high temperatures, had lower UV-resilience than that dominant in cultural fields, indicating that the two were differentiated in habitat types and physiological characteristics (Bidochka et al., 2001). In the current taxonomy, the cold-active and forest-dominant group has been identified as *M. brunneum*, while the heat-active and cultural field-dominant group has been identified as *M.*

robertsii (Bischoff et al., 2009). Wyrebek et al. (2011) reported similar results for the distribution of *M. brunneum* in a survey of *Metarhizium* spp. in wild flower rhizospheres in the same region. Nishi et al. (2017a) reported similar interspecific differences in temperature and habitat preferences in Japan. *M. brunneum* and *M. pemphigi* were restricted to forest environments and were cold-active, whereas *M. pingshaense* was distributed in a variety of environments and was heat-active (Fig. 4). Therefore, *M. brunneum* in Eastern Canada and Japan exhibit similar habitat and temperature preferences. Regarding habitat preference, Barzanti et al. (2023) reported that *M. brunneum* was restricted to woodlands, whereas *M. robertsii* was predominant in both, woodlands and open fields in Italy. The case of *M. robertsii* in Barzanti et al. (2023) is similar to *M. pingshaense* in Japan, in that both are dominant species in a wide range of environments. On the other hand, in Denmark, *M. brunneum* was reported to be a dominant species in a cultural field (Steinwender et al., 2014, 2015). In addition, field studies in Chile, Turkey, and western Canada (British Columbia) reported no association between genetic groups and habitat type or location (Inglis et al., 2008; Sevim et al., 2012; Velasquez et al., 2007). These differences in habitat preferences are possibly caused by differences in temperatures due to climatic conditions because open fields in cold climates are sometimes cooler than forests in hot climates. It is also possible that environmental factors that were not considered in these studies may have strongly influenced their distribution. A simple habitat type classification of forest versus arable land does not adequately explain the diversity of habitats for each *Metarhizium* species.

5. Host insects and virulence

Until taxonomic revision based on molecular phylogenetic analysis, generalists and specialists of the genus *Metarhizium* were identified as the same species. For example, *M. majus* (J.R. Johnst.) J.F. Bisch., Rehner & Humber, which is specific to white grubs, was originally identified as the generalist species *M. anisopliae* (i.e., *M. anisopliae* var. *majus*, Ferron et al., 1972; Raid & Cherry, 1992). Additionally, as mentioned above, locust-specific *M. acridum* was identified as *M. flavoviride*. Molecular phylogenetic analyses not only distinguished between specialists and generalists but also revealed their phylogenetic relationships. Hu et al. (2014) revealed that specialists are more ancestrally divergent, and that generalist species in the PARB clade are later derived from lineage groups (Fig. 2).

However, much remains unclear regarding the associations between phylogenetic positions and host insect groups. For example, *Metarhizium* spp., with a narrow host range, can be further differentiated into different pathotypes. As for *M. majus* derived from several species of scarabaeid larvae, Ferron et al. (1972) showed that isolates from coconut rhinoceros beetles *Oryctes* spp., flower chafer beetles, and other Scarabaeidae species were pathogenic only to their respective host species. The two pathotypes identified in *M. anisopliae* var. *majus*, which originated from a rhinoceros beetle (*O. rhinoceros*) and flower chafer beetle larva (*Cetonia aurata*), respectively, showed clear differences in pathogenicity, germination, appressorium formation in the cuticle, and immunological characteristics (Fargues et al., 1981; Fargues & Robert 1983; Vey et al., 1982;). Nishi et al. (2015) attempted to organize the molecular phylogeny of this intraspecific variation and found that isolates from the fruit beetle *Protaetia orientalis* were phylogenetically independent of isolates from coconut rhinoceros beetles and other scarabaeid species and exhibited unique pathogenicity specific to its original host. Phylogenetic analysis in this study suggested that

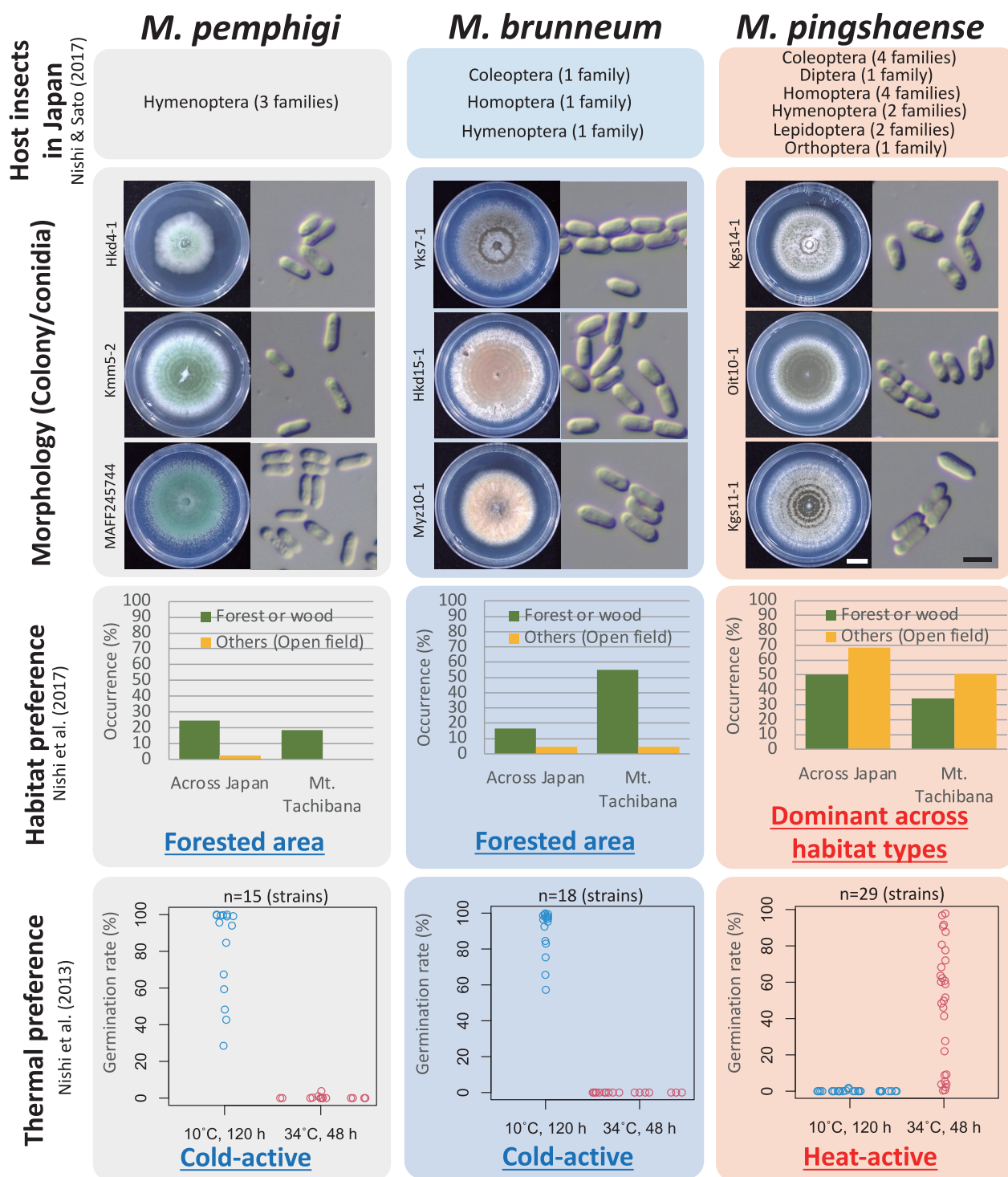


Fig. 4. Comparisons of natural host insects, morphology, habitat, and temperature growth characteristics of *Metarhizium brunneum*, *M. pemphigi*, and *M. pingshaense*. Bars: Colony 10 mm; Conidia = 5 μ m.

the DNA sequence information of the IGS region of rDNA included sufficient variation to classify the basal groups associated with host species and pathotypes. Phylogenetic analysis also revealed that an isolate from the flower beetle *Cetonia aurata* (CBS 648.67) originally identified as *M. anisopliae* var. *majus*, actually belongs to *M. bibionidarum* clade, which is a member of *M. flavoviride* complex (Nishi et al., 2017b). A similar subdivision has been suggested for *M. rileyi*, which is specific to lepidopteran larvae, and inoculation tests have confirmed that *M. rileyi* isolates prefer their respec-

tive host species or their close relatives (Ignoffo et al., 1985). However, the molecular phylogenetic status of host-preference differentiation remains unclear.

Another example of taxonomically unorganized aspects of host-insect species associations is found in the PARB clade, which is considered to be a clade composed of generalist species. These species have been isolated from a variety of hosts, and some strains have been shown to infect multiple insects, such as *M. robertsii* ARSEF 2575, a model strain of a broad-host-range entomopatho-

genic fungus (Gao et al., 2011), and *M. brunneum* F52, which is used as a microbial pesticide against various agricultural pests (EFSA et al., 2020). However, some PARB lineages may have a narrower host range. For example, *M. anisopliae* sensu lato isolated from the Australian field cricket (*Teleogryllus commodus*) is identified as *M. pingshaense*, a member of the PARB clade, but has been confirmed to be specific to this cricket species and to possessed genomic characteristics unique to specialist species like *M. acridum* (Milner & Rowland, 1996; Wang et al., 2009). This clearly indicates the requirements for a more detailed classification of the PARB clades.

6. Future studies for further understanding the diversity of *Metarhizium* spp.

Metarhizium are largely classified using molecular phylogenetics. For example, phylogenetic analysis clarified that species with a wide host range and the parasites cicadas and leafhoppers are separately clustered into large clades (Fig. 2). In addition, independent monophyletic groups recognized by GCPSR, such as the *M. brunneum* clade and *M. pingshaense* clade, are associated with physiological characteristics and habitat type (Fig. 2, 4; Nishi et al., 2013, 2017a). However, the species-level classification of *Metarhizium* is still inadequate because there are cases of further diversification within species clades in relation to host insects, as suggested by the *M. majus* and *M. pingshaense* clades (Milner & Rowland, 1996; Nishi et al., 2015). Therefore, further detailed molecular phylogenetic analyses and detection of phenotypic differentiation should be conducted, considering the possibility that strains isolated from different hosts are different species. For in-depth phylogenetic analysis, whole-genome sequencing is a powerful tool to clarify strongly supported phylogenetic relationships, as suggested by *Beauveria* (Kobmoo et al., 2021). However, a method available for the phylogenetic identification of many strains is the phylogenetic analysis of highly polymorphic intergenic regions selected for *M. anisopliae* complex by Kepler and Rehner (2013) through genome analysis.

With respect to phenotypic differentiation, it is important to identify inter-strain variations in virulence in the natural host. This test could reveal the differentiation of pathogenicity within a broad host-range species group and potentially reveal confounded species boundaries. Since some natural host insects are difficult to rear in the laboratory, a large amount of effort is sometimes required for experimentation. In addition to phenotypes that are directly related to the natural host insect (i.e., virulence), phenotypes that are related to the host habitat can also be examined to understand the diversity of host adaptation. Although temperature characteristics and UV tolerance have been evaluated, it is necessary to focus on other factors such as host plants, predators (i.e., fungivores), and light conditions. As *Metarhizium* has been reported to colonize the plant rhizosphere (Hu & St. Leger, 2002; Nishi & Sato, 2019), its affinity for the plant rhizosphere may also be relevant to natural hosts. In soil, there are many fungivorous animals that possibly prey on entomopathogenic fungi, such as protozoans, nematodes, mites, and collembolans (Scheepmaker & Butt, 2010), therefore defense against these fungivores may also be associated with natural host habitats. As some secondary metabolites produced by fungi have been reported to function as defensive substances against fungivores (e.g., Rohlf & Churchill, 2011), the type of secondary metabolites produced by a *Metarhizium* strain may be related to its persistence in the natural host habitat through defense against fungivores. In addition, a recently reported phenotype for *M. robertsii*, creeper/sleeper (Angelone et al., 2018), which is a phenotypic

polymorphism related to the light-dependent control of conidiation and mycelial growth, may also be associated with natural host habitats because light conditions are different in the ground and above ground.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study complied with the current laws of the countries in which they were performed.

Acknowledgements

I thank Prof. Susumu Shimizu, Prof. Chisa Yasunaga-Aoki, and Prof. Kazuhiro Iiyama for their guidance during my studies as a student at Kyushu University. I thank Dr. Hiroki Sato and Dr. Jun Takatsuka for their assistance with my study as a postdoctoral fellow at the FFPRI. I thank the members of the Mycological Society of Japan committee and my nominators for giving me the opportunity to write this review. I would also like to thank everyone who provided advice and fungal samples for this study. This work was supported by a Grant-in-Aid for JSPS Fellows [JSPS KAKENHI, Grant Numbers 11J00275 and 14J09097].

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