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REVIEW

Molecular and Genetic Differentiation of Small-Spored Species of the Yeast Genus *Metschnikowia* Kamienski

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Abstract—Information concerning molecular reidentification of the species of the rejected genus *Chlamydozyma* is generalized. The yeasts *Chl. pulcherrima* Wickerham (1964) and *Chl. reukaufii* Wickerham (1964) are shown to be sibling species of *Metschnikowia pulcherrima* Pitt et Miller (1968) and *M. reukaufii* Pitt et Miller (1968), respectively. Restoration of the species *M. zygota* (Wickerham) Fell at Hunter (1968) is proposed. The parasexual cycle and the prospects of its application for investigation of the *Metschnikowia* yeasts are discussed.

Keywords: yeasts, *Chlamydozyma, Metschnikowia*, sibling species, DNA–DNA hybridization, molecular phylogeny, mating types, mitotic haploidization.

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Taxonomy of the genus *Metschnikowia*, which had been originally described by Metschnokoff [1] as *Monospora*, parasites of daphnia, is presently an actively developing field due to the application of modern molecular technique, ecological and biogeographic approaches. Within the genus *Metschnikowia*, forming needle-shaped spores, three groups of species are differentiated. The first group comprises aquatic species *M. bicuspidata* (Metschnikoff) Kamienski (var. *bicuspidata*, var. *californica* Pitt et Miller, and var. *chathamia* Fell et Pitt), *M. australis* (Fell et Hunter) Mendonça-Hagler et al., *M. zobellii* (van Uden et Castelo-Branco) van Uden, and *M. krissii* (van Uden et Castelo-Branco) van Uden. The second group comprises terrestrial species, mostly isolated from flowers, fruits, and insects, usually in regions of temperate climate. To this group belong well-known species *M. pulcherrima* Pitt et Miller, *M. reukaufii* Pitt et Miller and species that are to some extent their phenotypic twins: *M. anduensis* Molnár et Prillinger, *M. chrysoperlae* Suh et al., *M. fructicola* Kurtzman et Droby, *M. shanxiensis* Giménes-Jurado, *M. koreensis* Hong et al., *M. lachancei* Giménes-Jurado et al., *M. noctiluminum* Nguyen et al., *M. vanudenii* Giménes-Jurado et al., and *M. viticula* Peter et al. *M. pulcherrima* and its sibling species are able to synthesize pulcherrimin and possess spherical chlamydospores (initials of asci), while the species related to *M. reukaufii* do not synthesize pulcherrimin and have oval or cylindrical chlamydospores. The first and second groups include small-spored species (*Metschnikowia* sensu stricto in our interpretation). They are related to someextent to small-spored species *M. corniflorae* Nguyen et al., *M. kunwiensis* Brysch-Herzberg, and *M. lunata* Golubev. The third group comprises large-spored, mainly tropical yeasts (*Metschnikowia* sensu lato) of a dozen and a half species; their number is constantly increasing. We do not list the species of the partially heterogeneous third group, since it includes the species which are only loosely related to the yeasts of the first two groups [2].

The subject of the present research are species of the previously rejected genus *Chlamydozyma* (*Metschnikowia*) (Table 1).

THE *CHLAHYDOZYMA* YEASTS

Wickerham [3, 4] described the "protosexual" genus *Chlamydozyma*, based on their specific sexual cycle without formation of ascospores or any other sexual spores. The so-called bisexual *Chlamydozyma* strains can form three types of cells—active bisexual, inactive bisexual, and haploid monosexual cells of two mating types. The author explained that the active bisexual stage (interpreted as a dikaryon) was difficult to maintain without selection under special conditions. Monosexual cells of the opposite mating types are able to form zygotes, which may in turn produce active bisexual cells by budding. Inactive bisexual cells were believed to be diploid monokaryons. We are omitting some curious concepts of Wickerham, which have been subsequently demonstrated to be unfounded.

The Tenth International Botanical Congress (1964) rejected Wickerham's proposal of the above protosexuality as a perfect stage in yeasts [5]. Pitt and Miller [6, 7] and Fell and Hunter [8] revealed ascus

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Table 1. The analyzed related yeast of the genera *Chlamydozyma, Metschnikowia*, and *Candida*

Original name of the species	nos. in collections				Origin
	NRRL	UCD	CBS	VKM	
Chl. pulcherrima	YB-2272	–	–	–	Aphis on willow (*Salix*), Peoria, IL
	YB-2272-6e	64-9	–	–	Haploid derivate of strain NRRL YB-2272
	YB-2272-7	64-10	–	–	Ibid
	Y-6148	–	5536	Y-1490	Flowers of *Trifolium pratense*
	Y-6149	–	5537	Y-1468	Ibid
	Y-6259 (T)	–	–	–	*Trifolium repens* clover, Corvallis, OR
Chl. reukaufii	Y-5941 (T)	–	–	–	*Trifolium pratense* clover, Peoria, IL
	Y-5941-53	64-13	5534	Y-1467	Haploid derivate of strain NRRL Y-5941
	Y-5941-62	64-14	5535	Y-1479	Ibid
Chl. zygota	Y-4714 (T)	68-18	–	–	*Rubus strigosus* berries, Quebec, Canada
	YB-4719	64-11	5553	Y-1466	Ibid
	YB-4720	64-12	5554	Y-1542	Ibid
M. pulcherrima	Y-7111 (T)	214	5833	–	*Vitis labrusca* berries, California
	–	214H3	–	–	Haploid derivate of strain NRRL Y-7111
	–	67-1036A	–	–	Monosporic culture of strain UCD 67-10
M. reukaufii	Y-7112 (T)	62-311	5834	–	Flowers of *Epilobium angustifolium*, Canada
	–	62-311H10	–	–	Haploid derivate of strain UCD 62-311
	–	62-311H11	–	–	Ibid
M. bicuspidata var. *bicuspidata*	YB-4993 (T)	63-49	5575	–	Larva of the trematode *Dislostomum flexicaudum*, United States
	YB-4993F27	–	–	–	Haploid derivate of strain NRRL YB-4993
	YB-4993A-1	–	–	–	Ibid
M. bicuspidata var. *chathamia*	Y-17917	67-2	5980	–	Pond on Chatam Island, New Zealand
	–	67-2A	–	–	Monosporic culture of strain NRRL Y-17917
	–	67-2B	–	–	Ibid
M. gruessii	Y-17809	–	7657	–	Flowers of *Hebe salicifolia*, Portugal
C. pulcherrima	Y-6344 (T)	67-14	610	Y-64	Fruit of *Phoenix dactylifera*, Egypt
C. reukaufii	Y-6346 (T)	67-15	1903	–	–

Note: Abbreviations for collection names: NRRL = Northern Region Research Center, Peoria, IL, United States (http://web-uni-corvinus.hu:8089/NCAIM/index.jsp); UCD = Herman J. Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, United States (http://phaffcollection.org); CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands (http://222.cbs.knaw.nl); VKM = All-Russian Collection of Microorganisms, Moscow, Russia (http://www.vkm.ru). T stands for type strain.

formation in *Chlamydozyma* and transferred them to the genus *Metschnikowia*. The first authors [6, 7] considered *Chl. pulcherrima* Wickerham and *Chl. reukaufii* Wickerham as the synonyms to *M. pulcherrima*, and *Chl. zygota* Wickerham as a synonym to *M. reukaufii*. Fell and Hunter [8] suggested a new combination *M. zygota* (Wickerham) Fell et Hunter. For certain reasons, neither Wickerham [3] for *Chl. reukaufii* and

Chl. pulcherrima, nor Pitt and Miller [6, 7] for *M. reukaufii* and *M. pulcherrima* used the type cultures of imperfect yeasts *Candida reukaufii* (Grüss) Diddens et Lodder (NRRL Y-6346) and *Candida pulcherrima* (Lindner) Windisch (NRRL Y-6344). Each laboratory cited different type cultures for these perfect fungi.

GENETIC INVESTIGATION OF RELATIONS BETWEEN *CHLAMYDOZYMA* AND *METSCHNIKOWIA* SPECIES

Wickerham [3] demonstrated that all three *Chlamydozyma* species (*Chl. reukaufii, Chl. pulcherrima*, and *Chl. zygota*) and *M. bicuspidata* var. *bicuspidata* possess a common system of mating types enabling them to cross. A close genetic relationship between the three terrestrial species and the aquatic *M. bicuspidata* was thus demonstrated. Strains forming haploid vegetative cells of the opposite mating types were used as type cultures: *Chl. pulcherrima* NRRL Y-6259, *Chl. reukaufii* NRRL Y-5941, *Chl. zygota* NRRL Y-4714, and *M. bicuspidata* var. *bicuspidata* NRRL YB-4993 (Table 1). The strain *Chl. pulcherrima* NRRL YB-2272 probably behaved in a similar manner. Wickerham was known to provide both Fell and Hunter [8] and Pitt and Miller [6, 7] with haploid derivates of *Chl. pulcherrima* NRRL YB-2272-6e and NRRL YB-2272-7, *Chl. reukaufii* NRRL Y-6941-53 and NRRL Y-5941-62, *M. bicuspidata* var. *bicuspidata* NRRL YB-4993 F27 and NRRL YB-4993 A-1, as well as haploid strains *Chl. zygota* NRRL Y-4719 and NRRL Y-4720 having mating types.

Based on crossing results, Fell and Hunter [8] confirmed the relationship between *Chlamydozyma* and *M. bicuspidata* var. *bicuspidata*. They also found out that these yeasts, as well as other aquatic taxa, *M. australis* and *M. bicuspidata* var. *chathamia*, possess a common system of mating types. (The *M. bicuspidata* var. *chathamia* strains used in [6, 7] were originally misidentified as *M. zobellii*/*M. bicuspidata* var. *zobellii* [7, 9]). Analysis of the hybrids of *M. australis* with *Chl. pulcherrima, Chl. reukaufii, M. zygota*, and *M. bicuspidata* var. *bicuspidata* revealed an important pattern: interspecific hybrids were to some extent sterile, either not forming asci, or producing asci with infrequent or abortive spores and without spores. However, the viability of infrequent spores of the interspecific hybrids was not determined.

Pitt and Miller [6] investigated 16 strains of *C. pulcherrima* and *C. reukaufii* and determined that most of them formed ascospores; the type of their spore-bearing asci places them within the perfect genus *Metschnikowia*. Teleomorphs of *M. pulcherrima* (type NRRL Y-7111) and *M. reukaufii* (type NRRL Y-7112) were described for sporulating strains. Pitt and Miller [6, 7], as well as Fell and Hunter [8] determined that three *Chlamydozyma* species have a mating type system common with *M. bicuspidata* var. *chathamia*, although interspecific hybrids do not sporulate. Ability of the type haploid culture of *C. reukaufii* NRRL Y-6346 to cross with haploid *Chlamydozyma* strains, found by Wickerham [3] was confirmed. Haploid cultures of all three *Chlamydozyma* species were found to cross not only with strain NRRL Y-6346, but also with haploid tester strains *M. bicuspidata* var. *chathamia* UCD 67-2A and UCD 67-2B. For *M. pulcherrima* and *M. reukaufii*, Pitt and Miller [6, 7], independently from Fell and Hunter [8], have demonstrated fertility of the intraspecific hybrids and sterility of the interspecific ones. This approach was used for reidentification of *Chlamydozyma* species [6, 7]. The results of analysis of the hybrids of *Chlamydozyma* and *Metschnikowia* species are summarized in Table 2. As was already mentioned, based on formation of chlamydospores and asci with spores typical of the corresponding species, the *Chl. zygota* strains were assigned to *M. reukaufii*, while *Chl. reukaufii* and *Chl. pulcherrima* strains to *M. pulcherrima*.. The control interspecific hybrids were sterile. The shortcomings of the experiments by Pitt and Miller [7] will be discussed below.

MOLECULAR INVESTIGATION OF RELATIONS BETWEEN *CHLAMYDOZYMA* AND *METSCHNIKOWIA* SPECIES

The data on DNA G + C base content were significant to suggest that *Chl. reukaufii* NRRL Y-5941-53 and *Chl. pulcherrima* NRRL YB-2272 could not belong to the species *M. pulcherrima*, since the values were 42.2, 44.6, and 48.3 mol %, respectively. For both *M. reukaufii* (T) and *Candida reukaufii* (T), the G + C content was 44.1% [10–12]. Meyer and Phaff [12] obtained even more convincing evidence against classification of these *Chl. reukaufii* and *Chl. pulcherrima* strains as *M. pulcherrima* by DNA–DNA hybridization. DNA homology between the *M. pulcherrima* type strain and the strains of *Chl. reukaufii, Chl. pulcherrima*, and *Candida reukaufii* did not exceed 28.6%. Giménez-Jurado [13] subsequently demonstrated low DNA–DNA hybridization (5%) between *Chl. zygota* NRRL Y-4719 (IGC 3684) and the type culture of *M. reukaufii*. This *Chl. zygota* strain also exhibited 10% homology with two *M. gruessii* strains, including the type culture CBS 7657. The latter yeast is known as a sibling species of *M. reukaufii*. In spite of these data, in the yeast manuals published in 1970 and 1984, Miller and van Uden [14] and Miller and Phaff [15], respectively, interpreted *Chl. pulcherrima* and *Chl. reukaufii* as synonyms of *M. pulcherrima*. In the 1998 yeast manual [16], Miller and Phaff did not provide explanations for their classification of *Chl. pulcherrima* as *M. pulcherrima* and of *Chl reukaufii* and *Chl. zygota* as *M. reukaufii*. The strains of *Chl. reukaufii* and *Chl. pulcherrima* were used in construction of the phylogenetic tree of the genus *Metschnikowia* [2, 17–19]. These data demonstrate that *Chl. reukaufii* NRRL Y-5941 (T) and *Chl. pulcherrima* NRRL Y-6148 are geneti-

Table 2. Results of hybridization of *Chlamydozyma* and *Metschnikowia* [6, 7]

Hybrid	Chlamydospore formation	Formation of asci with spores
Intraspecific crossbreeding		
Chl. zygota × *M. reukaufii*		
NRRL YB-4720 × UCD 62-311H10	Typical of *M. reukaufii*	+
NRRL YB-4719 × UCD 62-311H12	Ibid	+
NRRL YB-4720 × NRRL Y-6346	Ibid	+
Chl. reukaufii × *M. pulcherrima*		
NRRL Y-5941-53 × UCD 67-1036A	Typical of *M. pulcherrima*	–
NRRL Y-5941-62 × UCD 214H3	Ibid	+
Chl. pulcherrima × *M. pulcherrima*		
YB-2272-6e × UCD 214H3	Typical of *M. pulcherrima*	–
YB-2272-7 × UCD 67-1036A	Ibid	+
Interspecific crossbreeding;		
Chl. zygota × *M. pulcherrima*		
NRRL Y-4719 × UCD 67-1036A	Atypical	–
NRRL Y-4720 × UCD 214H3	Ibid	–
Chl. reukaufii × *M. reukaufii*		
NRRL Y-5941-53 × NRRL Y-6346	Atypical	–
*Chl. pulcherrim*a × *M. reukaufii*		
NRRL Y-2272-7 × NRRL Y-6346	Atypical	–
Chl. zygota × *Chl. reukaufii*		
NRRL YB-4720 × NRRL Y-5941-62	Atypical	–

cally close, but not conspecific, to respective type strains of *M. reukaufii* and *M. pulcherrima*. Moreover, the recently described species *M. koreensis* is the closest relative of *Chl. reukaufii* NRRL Y-5941, while *Chl. pulcherrima* (NRRL Y-6148) is closely related to other new species, *M. chrysoperlae* and *M. shanxiensis* [2, 18–20]. Phylogeny of small-spored terrestrial and aquatic species of *Metschnikowia* sensu stricto is presented in Fig. 1. Unfortunately, the type culture *Chl. pulcherrima* NRRL Y-6259 was not used in [2, 17–19]. The type culture *Chl. zygota* NRRL Y-4714 and two other strains of this taxon (NRRL Y-4719 and NRRL Y-4720) were also not studied.

These data demonstrate the necessity for renewal of *Chl. reukaufii* and *Chl pulcherrima* as new species, or rather combinations, within the genus *Metschnikowia*, probably under different species epithets. DNA–DNA hybridization has demonstrated [13] that *Chl. zygota* Wickerham is not a synonym for *M. reukaufii* and the species *M. zygota* (Wickerham) Fell et Hunter should therefore be restored. Mistaken reidentification of the species *Chl. reukaufii, Chl. pulcherrima,* and *Chl. zygota* by Pitt and Miller [6, 7] probably results from the fact that survival of the ascospores of the hybrids *M. reukaufii* × *Chl. zygota, M. pulcherrima* × *Chl. pulcherrima*, and *M. pulcherrima* × *Chl. reukaufii*, which we believe to be sterile, has not been analyzed. For the genera *Saccharomyces, Kluyveromyces* (syn. *Zygofabospora*), *Arthroascus, Williopsis*, and *Zygowilliopsis*, interspecific hybrids are known to produce ascospores, albeit not viable ones [21–27]. Moreover, mistaken reidentification of *Chl. reukaufii* [6, 7] may be due to the impreciseness of one of the diagnostic characteristics of *M. pulcherrima*, namely, the shape of chlamydospores. Instead of spherical or slightly oval, only spherical chlamydospores of this species should be considered.

Final revision of *Chlamydozyma* species requires sequencing of the D1/D2 domain of the 26S rRNA of the type cultures *Chl. pulcherrima* NRRL Y-6259 and *Chl. zygota* NRRL Y-4714. The morphological differences between their chlamydospores and asci suggest a low probability of conspecificity of these species.

Molecular reidentification of *Chl. reukaufii, Chl. pulcherrima*, and *M. zygota*, as well as discovery of other sibling species of *M. reukaufii* and *M. pulcherrima*, suggest that comparative molecular analysis of the genome is required for yeasts synonymous to *M. reukaufii* and *M. pulcherrima*, primarily *Torulopsis dattila* (Kluyver) Lodder var. *rohrbachense* von Szilvi-

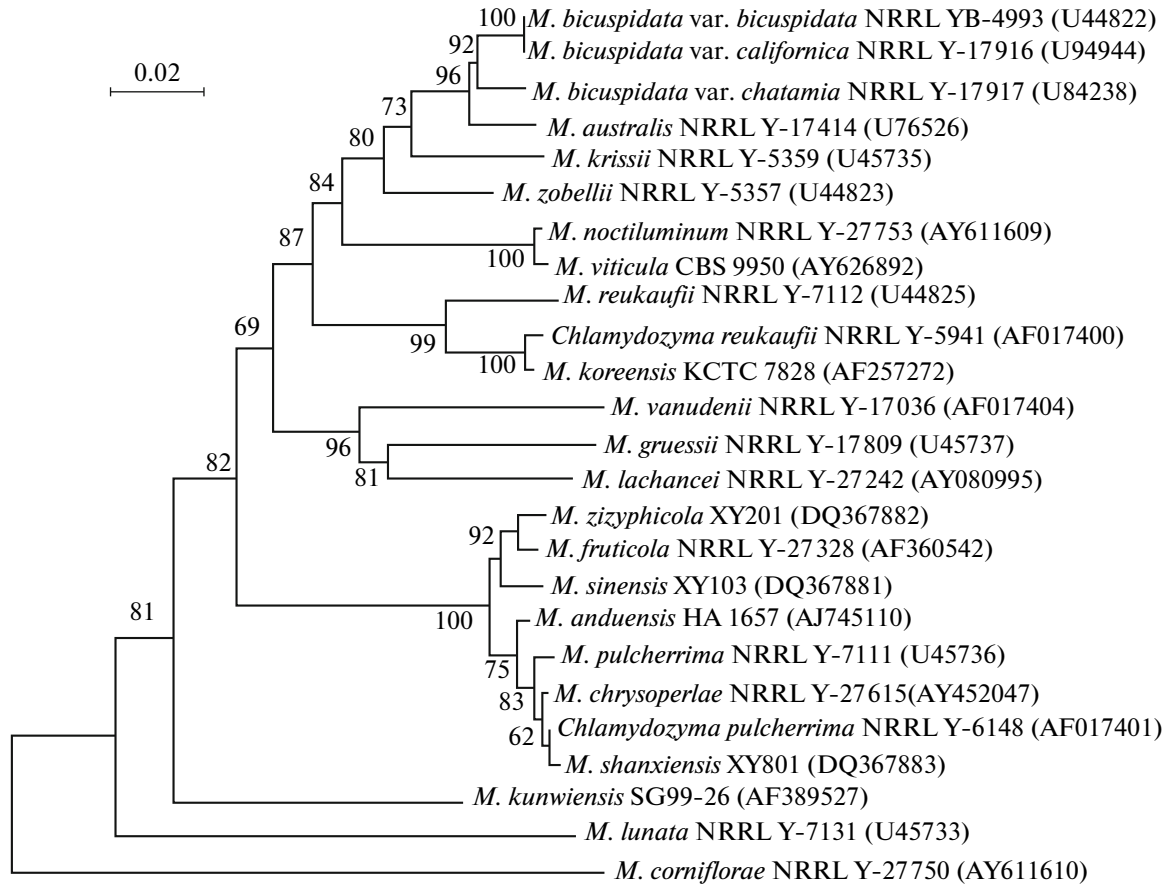

Fig. 1. Phylogenetic analysis of the nucleotide sequences of D1/D2 domains of 26S rRNA of *Metschnikowia* and *Chlamydozyma*. Bootstrap values >50% are shown. Scale bar corresponds to 20 replacements per 1000 nucleotides.

nyi et Kaulich, *Torulopsis burgeffiana* Benda, and *Candida rancensis* Ramírez et Gonzáles.

ECOLOGICAL AND PHENOTYPIC CHARACTERISTICS OF SOME *METSCHNIKOWIA* SPECIES

The phylogenetic tree of *Metschnikowia* species presented on Fig. 1 suggests some amendments to the ecological and phenotypic grouping discussed in the Introduction. First, *M. noctiluminum* and *M. viticula* are very closely related to aquatic species. Second, the species phenotypically close to *M. reukaufii* are more heterogeneous than the species complex related to *M. pulcherrima*. Finally, the species closely related to *M. lunata* and *M. kunwiensis* are not present in the tree; these species, especially *M. lunata*, are quite seldom isolated from environmental sources. We think that this is not accidental. The "species" characteristics of the type cultures, namely, lunate-shaped cells for *M. lunata* and location of the spores within the cell envelope (without the characteristic projection at the asci) for *M. kunwiensis*, may be the reason. The type strains of both species are probably infrequent mutants of the standard *Metschnikowia* phenotype: spherical or oval vegetative cells and spores in asci with projections. The regulatory effect of carbon and nitrogen sources on the cell shape of *M. lunata* [28, 29] is an indication of the possible natural genetic variability of this parameter. Species-specific molecular markers, rather than the so-called species phenotypic characteristics, should be therefore used for identification of the species *M. lunata* and *M. kunwiensis*.

THE PARASEXUAL CYCLE

Pitt and Miller [6, 30] did not completely succeed in the reproduction of the "protosexual" cycle in *Chlamydozyma* described by Wickerham [3, 4]. However, they demonstrated the possibility of infrequent mitotic haploidization in some diploid *Metschnikowia* species, viz. in *M. bicuspidata, M. pulcherrima*, and *M. reukaufii*. From our point of view, mitotic haploidization in *M. pulcherrima* is confirmed by Golubev [31]. Special media were used to demonstrate that diploid cells of this species, unlike haploid ones, do not produce pulcherrimin. Formation of red sectors in white diploid colonies was observed. The cells from the red sectors were haploid and possessed mating types.

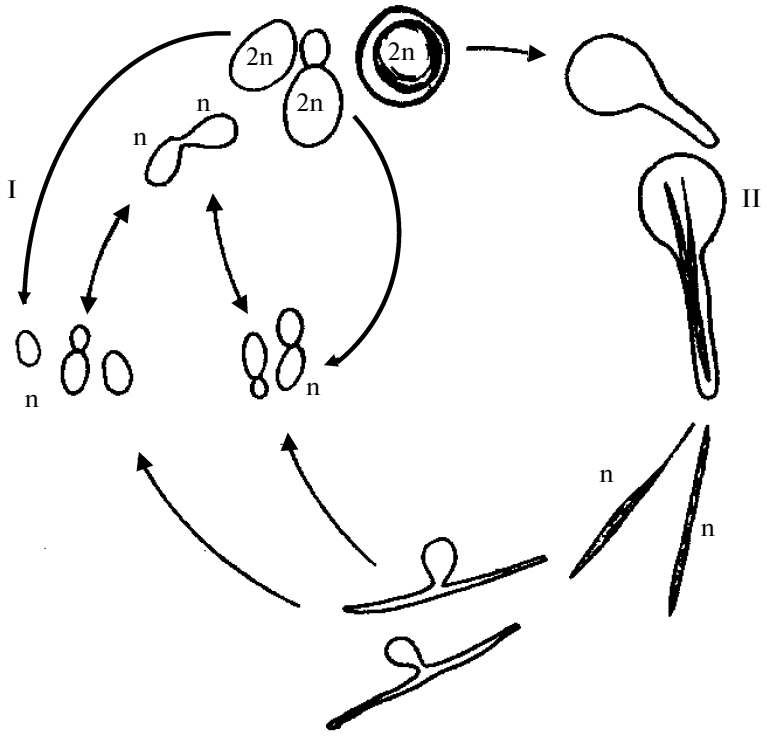

Fig. 2. Life cycle of *Metschnikowia* [35] in our modification. Mitotic haploidization (I), meiotic haploidization (II); n, cell ploidy.

Research on mitotic haploidization may be successfully continued by determination of the sexual activity of haploid cells on selective media. Auxotrophic mating testers, which are able to utilize the carbon sources not used by the analyzed strains, should be used for hybridization. Haploid cells of two mating types that occur seldom in diploid cultures may be thus visualized on the relevant minimal media. We think that such a genetic approach will make it possible to establish the biological relations between the perfect *Metschnikowia* species and the *Candida* species, which according to their ribosomal sequences, are arbitrarily assigned to the genus *Metschnikowia*: *C. chryzomelidarum* Nguyen et al., *C. ipomoeae* Lachance et al., *C. kofuensis* Nguyen et al., *C. magnifica, C. picachoensis, C. pimensis* Suh et al., and *C. rancensis* [2, 18]. Mitotic haploidization should be used also to investigate the so-called homothallic *Metschnikowia* strains, for which this life cycle was not confirmed by self-diploidization of the monosporic cultures isolated by micromanipulation. Determination of the mating types for all species of *Metschnikowia* sensu stricto will make it possible to establish the limits for interspecies crossbreeding and the degree of the relations between the species. It should be determined whether all species of *Metschnikowia* sensu stricto share a common system of mating types, as is typical of genetic genera, such as *Saccharomyces, Kluyveromyces, Arthroascus, Williopsis, Zygowilliopsis*, and *Galactomyces* [21–27, 32, 33].

Considering the data of Wickerham [3, 4], Pitt and Miller [6, 30], and Golubev [31], it is possible that *Metschnikowia* sensu stricto species possess the parasexual process established for mycelial fungi [34]. Formation of a dikaryon probably occurs after copulation of the cells. The heterokaryon stage may be of different duration, depending on the strains and cultivation conditions. A heterokaryon with haploid nuclei may generate both haploid cells of the opposite mating type or, after karyogamy, diploid cells. The latter may undergo mitotic haploidization with formation of haploid cells of the opposite mating type. The life cycle of *Metschnikowia* sensu stricto is presented in Fig. 2. According to Bab'eva and Gorin [36], alternation of haploid and diploid phases in the life cycle of terrestrial *Metschnikowia* species is associated with the change in their habitats (from sugary plant substrates into the visiting insects).

To conclude, we want to state that the yeasts *Metschnikowia* sensu stricto are a promising subject for evolutionary genetics, taxonomy, ecology, and biogeography of yeasts.

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