Phylogenetic analysis of the angiosperm-floricolous insect–yeast association: Have yeast and angiosperm lineages co-diversified?

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ABSTRACT

Metschnikowia (Saccharomycetales, Metschnikowiaceae/ *Metschnikowia* clade) is an ascomycetous yeast genus whose species are associated mostly with angiosperms and their insect pollinators over all continents. The wide distribution of the genus, its association with angiosperm flowers, and the fact that it includes some of the best-studied yeasts in terms of biogeography and ecology make *Metschnikowia* an excellent group to investigate a possible co-radiation with angiosperm lineages. We performed phylogenetic analyses implementing Bayesian inference and likelihood methods, using a concatenated matrix (≈ 2.6 Kbp) of nuclear DNA (*ACT1*, 1st and 2nd codon positions of *EF2*, *Mcm7*, and *RPB2*) sequences. We included 77 species representing approximately 90% of the species in the family. Bayesian and parsimony methods were used to perform ancestral character reconstructions within *Metschnikowia* in three key morphological characters. Patterns of evolution of yeast habitats and divergence times were explored in the *Metschnikowia* clade lineages with the purpose of inferring the time of origin of angiosperm-associated habitats within Metschnikowiaceae. This paper presents the first phylogenetic hypothesis to include nearly all known species in the family. The polyphyletic nature of *Clavispora* was confirmed and *Metschnikowia* species (and their anamorphs) were shown to form two groups: one that includes mostly floricolous, insect-associated species distributed in mostly tropical areas (the large-spored *Metschnikowia* clade and relatives) and another that comprises more heterogeneous species in terms of habitat and geographical distribution. Reconstruction of character evolution suggests that sexual characters (ascospore length, number of ascospores, and ascus formation) evolved multiple times within *Metschnikowia*. Complex and dynamic habitat transitions seem to have punctuated the course of evolution of the Metschnikowiaceae with repeated and independent origins of angiosperm-associated habitats. The origin of the family is placed in the Late Cretaceous (71.7 Ma) with most extant species arising from the Early Eocene. Therefore, the Metschnikowiaceae likely radiated long after the Mid-Cretaceous radiations of angiosperms and their diversification seem to be driven by repeated radiation on a pre-existing diverse resource.

Keywords:

Ascomycetous yeasts

Candida

Clavispora

Diversification

Metschnikowia

Metschnikowiaceae

1. Introduction

Metschnikowia is a genus of ascomycetous yeasts primarily characterized by the presence of multilateral budding of spheroidal to ellipsoidal (sometimes pyriform, cylindrical or lunate) vegetative cells and the formation of needle-shaped ascospores in elongate or morphologically distinct asci which develop directly from vegetative cells (by elongation or conjugation) or from chlamydospores (Lachance, 2011b). The genus consists of 47 species that are mostly homogeneous with respect to nutrient utilisation and other physiological characteristics (Giménez-Jurado et al., 1995; Lachance, 2011b) but with morphological variation (Tables 1 and 2)

in ascus shape and size, ascospore size, number of ascospores per ascus, mode of ascus development, production of mycelial structures, and production of pulcherrimin pigment, which have been traditionally used in the delineation of taxa. Initially named *Monospora* by Metschnikoff (1884), the genus *Metschnikowia* was later proposed by Kamienski (1899) and placed within the order Endomycetales (Spermothoraceae, Kreger-van Rij, 1984; Metschnikowiaceae, Von Arx and Van der Walt, 1987). The incorporation of DNA data has helped to stabilize fungal taxonomy and to develop a phylogenetic classification to the level of order (Hibbett et al., 2007). In this context, molecular analyses based on nuclear sequences have confirmed the inclusion of the genus *Metschnikowia* within the order Saccharomycetales, where it forms a well-supported clade (*Metschnikowia* clade/Metschnikowiaceae) together with the genus *Clavispora* (three species) and some asexual forms (37 species) assigned to the genus *Candida* (Suh et al., 2006). Close

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Table 1

Geographical distribution, habitat and morphological characters. Data was taken from Lachance (2011a, b), Lachance et al. (2011) and original species descriptions.

Taxon	Distribution	Habitat	Asexual cell shape	Pseudomycelium/true mycelium
<i>Candida</i>				
<i>C. aechmeae</i>	Brazil	Leaves of bromeliads	Ellipsoidal to ovoid	+/+
<i>C. akabanensis</i>	Japan, Australia	Frass insect and plant exudate	Spherical to ovoid	+/-
<i>C. asparagi</i>	China	Fruit	Ellipsoidal to elongate	+/-
<i>C. blattae</i>	Panama, USA	Roach, gut of dobson flies	Globose to ellipsoidal	+/+
<i>C. bromeliacearum</i>	Brazil	Water tank of bromeliads	Ellipsoidal to elongate	-/-
<i>C. chrysomelidarum</i>	Panama	Chrysomelid beetles	Ellipsoidal	-/-
<i>C. dosseyi</i>	USA	Gut of dobsonflies	Globose to ellipsoidal	+/-
<i>C. flosculorum</i>	Brazil	Flower bract of heliconias	Ovoid to ellipsoidal	-/-
<i>C. fructus</i>	Japan	Banana	Subglobose to ovoid	+/-
<i>C. gelsemii</i>	USA	Floral nectar	Ovoid	-/-
<i>C. golubevii</i>	Thailand, Brazil	Insect frass, flower of morning glories	Ovoid to lunate	-/-
<i>C. haemulonii</i> type I	Portugal, USA, Surinam, France, Hawaii	Sea-water, dolphin skin, gut of fish, clinical specimens, furniture polish	Ovoid, ellipsoidal	v/-
<i>C. haemulonii</i> type II	USA	Clinical specimens	-	
<i>C. hawaiiiana</i>	Hawaii, Costa Rica	Flower of morning glory, beetles	Spherical to ovoid	-/-
<i>C. intermedia</i>	Africa, Brazil, Mexico, Costa Rica, Sweden, South Africa, Germany	Clinical specimens, grapes, fermenting stem of banana, soil, beer, sea-water, etc.	Ovoid	+/-
<i>C. ipomoeae</i>	Hawaii, Costa Rica, Brazil, USA	Flowers of morning glory, fruit flies, nitidulid beetles	Ovoidal to cylindrical	+/-
<i>C. kipukae</i>	Hawaii, USA, Panama, South America	Nitidulid beetles	Spherical to ovoid	-/-
<i>C. kofuensis</i>	Japan	Wild grape	Globose to ellipsoidal	+/-
<i>C. melibiosica</i>	Japan, Spain, USA, South Africa	Clinical specimens, film on wine, soil, tunnel of <i>Xylopsocus</i> sp.	Globose to ovoid	+/-
<i>C. mogii</i>	Japan, Slovakia	Miso fermentations, clinical specimens	Ovoid	+/-
<i>C. oregonensis</i>	USA	Insect frass, alpechin	Ovoid	+/-
<i>C. picachoensis</i>	USA	Lacewings	Globose	-/-
<i>C. pinguabensis</i>	Brazil	Water accumulated in flower bracts	Spherical	-/-
<i>C. pimensis</i>	USA	Lacewings	Globose to subglobose	-/-
<i>C. pseudointermedia</i>	Japan, French Guyana, Brazil	Fish paste, flowers	Subglobose to short ovoid	+/-
<i>C. rancensis</i>	Hawaii, USA, Chile	Nitidulid beetles, floral nectar, decayed wood	Subglobose, ovoid or ellipsoidal	-/-
<i>C. saopaulonensis</i>	Brazil	Water in flower bract of heliconias	Short ellipsoidal	-/-
<i>C. thailandica</i>	Thailand	Insect frass	Ovoidal to cylindrical	-/-
<i>C. tolerans</i>	Australia, Cook islands	Fruit fly, flowers, nitidulid beetles	Ovoid to cylindrical	+/-
<i>C. torresii</i>	Australia	Sea-water	Ovoid to long-ovoid	-/-
<i>C. tsuchiyaе</i>	Japan	Moss	Globose to ovoid	-/-
<i>C. ubatubensis</i>	Brazil	Water tanks of bromeliads	Spheroidal to ovoid	-/-
<i>Clavispora</i>				
<i>Cl. lusitaniae</i>	Europe, Israel, New Zealand, India, Mexico, USA, Venezuela, Cayman islands, Canada, Bahamas	Broad array of substrates of plant and animal origin, as well as industrial wastes and clinical specimens	Subglobose, ovoid to elongate	+/-
<i>Cl. opuntiae</i>	Australia, Peru, USA, Brazil, Bahamas, The Antilles, Caribbean, Mexico, Hawaii	Rots of prickly pears	Subglobose, ovoid to elongate	+/-
<i>Cl. reshetovae</i>	Germany	Soil	Spheroid to ovoid	-/-

<i>Metschnikowia</i>				
<i>M. aberdeeniae</i>	Kenya, Tanzania	Beetles and fruit flies associated with morning glory flowers	Spheroid to ovoid	–/–
<i>M. agaves</i>	Mexico	Basal rots of leaves of blue agave plants	Ovoid to ellipsoidal	–/–
<i>M. andauensis</i>	Austria	Frass insects	Globose to ovoid	–/–
<i>M. arizonensis</i>	USA	Flowers, floricolous insects	Spheroidal to ovoid	+/–
<i>M. australis</i>	Antarctic	Sea-water	Spheroid to ovoid	–/–
<i>M. bic. var. bicuspidata</i>	USA	Trematodes, <i>Artemia salina</i> , white pond lily	Elipsoidal to cylindrical	+/–
<i>M. bic. var. californica</i>	USA	Sea-water, kelp	Elipsoidal to cylindrical	+/–
<i>M. bic. var. chathamia</i>	New Zealand	Fresh water pond	Elipsoidal to cylindrical	+/–
<i>M. borealis</i>	Canada, USA	Flowers, nitidulid beetles	Ovoid to ellipsoidal	–/+
<i>M. cerradonensis</i>	Brazil	Nitidulid beetles	Spherical to ovoid	v/–
Taxon	Distribution	Habitat	Asexual cell shape	Pseudomycelium/True mycelium
<i>M. chrysoperlae</i>	USA	Lacewings	Globose to ovoid	–/–
<i>M. colocasiae</i>	Costa Rica, Brazil	Nitidulid beetles, flowers	Spherical	+/–
<i>M. continentalis</i>	Brazil, Germany	Nitidulid beetles, flowers, floral nectar	Ovoid to ellipsoidal	–/+
<i>M. corniflorae</i>	USA	Soldier beetles	Subglobose to ovoid	–/–
<i>M. cubensis</i>	Cuba	Flowers, nitidulid beetles	–	+/–
<i>M. dekortorum</i>	Costa Rica	Nitidulid beetles	Ovoid to elongate	–/–
<i>M. drosophilae</i>	Cayman islands	Fruit flies, flowers	Ovoid to bacilliform	–/–
<i>M. fructicola</i>	Israel	Table grapes	Spherical to ovoid	–/–
<i>M. gruessii</i>	Europe	Floral nectar	Ovoid to ellipsoidal (cylindrical)	–/–
<i>M. hamakuensis</i>	Hawaii	Nitidulid beetles, flowers	Spherical to ovoid	+/–
<i>M. hawaiiensis</i>	Hawaii	Flowers, fruit flies, nitidulid beetles	Ovoid to ellipsoidal	–/+
<i>M. hibisci</i>	Australia	Fruit flies, flowers, nitidulid beetles	Ovoid to ellipsoidal	–/+
<i>M. kamakouana</i>	Hawaii	Nitidulid beetles, fruit flies	Spherical to ovoid	+/–
<i>M. koreensis</i>	Korea, French Guiana, Antarctica	Flower, floral nectar	Ellipsoidal to short cylindrical	–/–
<i>M. krissii</i>	USA	Sea-water	Spherical to ellipsoidal	+/–
<i>M. kunwiensis</i>	Germany, Korea	Bumble-bees, flowers, floral nectar	Globose to subglobose	–/–
<i>M. lachancei</i>	USA	Floral nectar	Elipsoidal to cylindrical	+/–
<i>M. lochheadii</i>	Costa Rica, Hawaii	Nitidulid beetles, fruit flies	Ovoid to ellipsoidal	+/+
<i>M. lunata</i>	Russia, Japan	Flower, plant exudate	Lunate (ovoid)	+/–
<i>M. mauiuiana</i>	Hawaii	Nitidulid beetles	Spherical to ovoid	+/–
<i>M. noctiluminum</i>	USA	Lacewings	Subglobose to ellipsoidal	–/–
<i>M. orientalis</i>	Cook islands, Malaysia	Nitidulid beetles	Ovoid to elongate	–/–
<i>M. pulcherrima</i>	Canada, Cayman islands, USA, Japan	Grapes, fruit flies, plant exudates, flowers	Globose to ellipsoidal	+/–
<i>M. reukaufii</i>	USA, Canada, Germany	Flowers, floral nectar	Elipsoidal to cylindrical	v/–
<i>M. santaceciliae</i>	Costa Rica	Nitidulid beetles, fruit flies, halictid bees	Ovoid to ellipsoidal	+/–
<i>M. shanxiensis</i>	China	Fruit	Globose to ovoid	–/–
<i>M. shivogae</i>	Tanzania, Kenya	Floricolous insects	Ovoid to elongate	–/–
<i>M. similis</i>	Costa Rica	Nitidulid beetles	Elongate	+/–
<i>M. sinensis</i>	China	Fruit	Globose to ovoid	–/–
<i>M. vanudenii</i>	Canada	Musoid flies, floral nectar	Ovoid to cylindrical	w/–
<i>M. viticola</i>	Hungary	Berries of grape	Globose to ellipsoidal	+/–
<i>M. zizyphicola</i>	China	Fruit	Globose to ovoid	–/–
<i>M. zobellii</i>	USA, Scotland	Sea-water, gut content of fish, kelp	Spherical to ellipsoid	+/–

Table 2

Character states of sexual characters with presumptive diagnostic value in the genus

Metschnikowia. Data was taken from Lachance (2011b) and original species descriptions.

Taxon	Ascus development	No. Ascospores	Ascospore shape	Ascospore/Ascus length (µm)	Sexuality	Ploidy
<i>M. aberdeeniae</i>	Conjugation	2	Needle-shaped	35–50	Heterothallic	1N
<i>M. agaves</i>	Conjugation	2	Needle-shaped	15–20	Heterothallic	1N
<i>M. andauensis</i>	Chlamydospores	1–2	Needle-shaped	24–34	Homothallic	2N
<i>M. arizonensis</i>	Conjugation	2	Needle-shaped	60–100	Heterothallic	1N
<i>M. australis</i>	Conjugation	2	Needle-shaped	25–40	Heterothallic	1N
<i>M. bicuspidata</i> var. <i>bicuspidata</i>	Elongation	2	Needle-shaped	15–50	Homothallic	2N
<i>M. bicuspidata</i> var. <i>californica</i>	Elongation	2	Needle-shaped	15–50	Heterothallic	2N
<i>M. bicuspidata</i> var. <i>chathamia</i>	Elongation	2	Needle-shaped	15–50	Heterothallic	2N
<i>M. borealis</i>	Conjugation	2	Needle-shaped	100–200	Heterothallic	1N
<i>M. cerradonensis</i>	Conjugation	2	Needle-shaped	40–150	Heterothallic	1N
<i>M. chrysoperlae</i>	Chlamydospores	1–2	Needle-shaped	20–40	Homothallic	2N
<i>M. colocasiae</i>	Conjugation	2	Needle-shaped	70–100	Heterothallic	1N
<i>M. continentalis</i>	Conjugation	2	Needle-shaped	200	Heterothallic	1N
<i>M. corniflorae</i>	Chlamydospores	1–2	Needle-shaped	10–20	Homothallic	2N
<i>M. cubensis</i>	Conjugation	2	Needle-shaped	80–120	Heterothallic	1N
<i>M. dekortorum</i>	Conjugation	2	Needle-shaped	40–50	Heterothallic	1N
<i>M. drosophilae</i>	Conjugation	2	Needle-shaped	20–25	Heterothallic	1N
<i>M. fructicola</i>	Chlamydospores	2	Needle-shaped	20–27	Homothallic	2N
<i>M. gruessii</i>	Chlamydospores	1–2	Needle-shaped	31–60	Homothallic	2N
<i>M. hamakuensis</i>	Conjugation	2	Needle-shaped	40–60	Heterothallic	1N
<i>M. hawaiiensis</i>	Conjugation	2	Needle-shaped	140–180	Heterothallic	1N
<i>M. hibisci</i>	Conjugation	2	Needle-shaped	20–30	Heterothallic	1N
<i>M. kamakouana</i>	Conjugation	2	Needle-shaped	90–110	Heterothallic	1N
<i>M. koreensis</i>	Chlamydospores	2	Needle-shaped	13–19	Homothallic	2N
<i>M. krissii</i>	Elongation	1	Needle-shaped	18–26	Homothallic	2N
<i>M. kunwiensis</i>	Chlamydospores	1–2	Needle-shaped	8–12	Homothallic	2N
<i>M. lachancei</i>	Elongation	2	Spindle-shaped	14–23	Homothallic	2N
<i>M. lochheadii</i>	Conjugation	2	Needle-shaped	100–150	Heterothallic	1N
<i>M. lunata</i>	Chlamydospores	1–2	Needle-shaped	13–40	Homothallic	2N
<i>M. mauinuiana</i>	Conjugation	2	Needle-shaped	70–80	Heterothallic	1N
<i>M. noctiluminum</i>	Chlamydospores	1(2)	Needle-shaped	20–40	Homothallic	2N
<i>M. orientalis</i>	Conjugation	2	Needle-shaped	30–60	Heterothallic	1N
<i>M. pulcherrima</i>	Chlamydospores	2(1)	Needle-shaped to filiform	9–27	Homothallic	2N
<i>M. reukauffii</i>	Chlamydospores	2(1)	Needle-shaped	7–30	Homothallic	2N
<i>M. santaceiliae</i>	Conjugation	2	Needle-shaped	100–150	Heterothallic	1N
<i>M. shanxiensis</i>	Chlamydospores	1–2	Needle-shaped	22–35	Homothallic	2N
<i>M. shivogae</i>	Conjugation	2	Needle-shaped	45–60	Heterothallic	1N
<i>M. similis</i>	Conjugation	2	Needle-shaped	50–70	Heterothallic	1N
<i>M. sinensis</i>	Chlamydospores	1–2	Needle-shaped	19–33	Homothallic	2N
<i>M. vanudenii</i>	Chlamydospores	2	Needle-shaped to filiform	25–45	Homothallic	1N
<i>M. viticola</i>	Chlamydospores	2(1)	Needle-shaped	18–48	Homothallic	2N
<i>M. zizyphicola</i>	Chlamydospores	1–2	Needle-shaped	20–34	Homothallic	2N
<i>M. zobellii</i>	Elongation	1	Needle-shaped	18–24	Homothallic	2N

relationships between these three genera are supported by their relatively uniform nutritional profile (Lachance, 2011a, b; Suh et al., 2006).

Some *Metschnikowia* species (and anamorphs) have broad geographic ranges encompassing all the continents (i.e. Lachance, 2011b; Lachance et al., 1998a) (Table 1). Others are confined to small areas, as for example four endemic species that are found only in the Hawaiian Islands (Lachance et al., 2005; Lachance et al., 1990) and one that seems to be restricted to the Cerrado ecosystem of the Jalapão area in Brazil (Rosa et al., 2007). Some species are versatile and able to tolerate many different environments, whereas others are highly specialized and can only survive in certain specific natural habitats, including extreme environments (Table 1). *Metschnikowia bicuspidata* var. *bicuspidata* is part of the biota of hypersaline waters (Butinar et al., 2005) and seven other species are able to grow in an environment with high concentration of sugars, namely the floral nectar of angiosperms (Brysch-Herzberg, 2004; Giménez-Jurado et al., 2003; Peay et al., 2012). *Metschnikowia* species (and anamorphs) may be terrestrial or aquatic (as free-living forms or as invertebrate parasites). Most species are terrestrial and form diverse mutualistic symbiotic associations that include a preponderance of associations with angiosperms and their associated insects (Lachance, 2011b; Lachance et al., 2011). Interactions with angiosperms have often been cited as important driving factors underlying diversification in other

lineages. After the Jurassic appearance of crown-group angiosperms (Bell et al., 2010; Brenner, 1996) evidence for an increase in the diversity of major insect groups [major herbivorous hemipterans, coleopterans, and ants (Farrell, 1998; Grimaldi and Engel, 2005; McKenna et al., 2009; Moreau et al., 2006)], amphibians (Roelant et al., 2007), ferns (Schneider et al., 2004), and primates (Bloch et al., 2007) appears to begin during the Mid Cretaceous, concurrent with the rise of angiosperms to widespread floristic dominance (Soltis et al., 2008). Although over 75% of the *Metschnikowiaceae* are now present in angiosperm-associated habitats, the possible impact of the rise of flowering plants on its diversification has not yet been studied.

Despite the fact that *Metschnikowia* includes some of the best-studied yeasts in terms of biogeography and ecology (Lachance et al., 2008; Wardlaw et al., 2009) a proper phylogeny of the genus has not been proposed to date. Previous molecular phylogenetic analyses have been based on relatively short rDNA sequences (generally of few hundred nucleotides) (Mendoza-Hagler et al., 1993; Yamada et al., 1994) or the sampling has been limited to a group of species (i.e. Lachance et al., 2001; Marinoni and Lachance, 2004; Naumov, 2011) and did not attempt to address the phylogenetic diversity of the genus. Studies of *Metschnikowia* systematics have so far been largely restricted to the description of new species. Previous phylogenetic hypotheses may, therefore, not represent the evolutionary history of a genus with considerable rDNA

sequence divergence among species despite their relative phenotypic homogeneity (Mendoça-Hagler et al., 1993). Here we present the results of phylogenetic analyses of the *Metschnikowia* clade based on data from four protein-coding genes (*ACT1*, 1st and 2nd codon positions of *EF2*, *Mcm7* and *RPB2*) from most of its known diversity (77 out of approximately 87 valid species). Our study aims to address the following objectives: (1) to infer sister-group relationships within the *Metschnikowia* clade, (2) to interpret evolution of traditional diagnostic characters, and (3) to gain insight into the degree and nature of temporal correlation in floricolous insect–yeasts and angiosperm diversification.

2. Material and methods

2.1. Taxon sampling and DNA sequencing

A total of 84 strains (mostly type strains) representing most currently described species of *Metschnikowia* (41 of ca. 47 taxonomically recognized), all of *C. lavispora* (three spp.) and 32 spp. of the asexual forms assigned to the genus *Candida* (37 recognized) were sampled for nuclear DNA sequencing (Table 1; Table S1). Four ascomycetous (*Debaryomyces hansenii*, *Lodderomyces elongisporus*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*) yeast species were chosen as outgroup. Additionally one basidiomycetous (*Cryptococcus neoformans* or *Cryptococcus gattii*) yeast was included in the outgroup to calibrate the divergence between Ascomycota and Basidiomycota in the molecular dating analysis.

Total genomic DNA was extracted from approximately 20 mg of yeast cells with the DNeasy Blood & Tissue kit (QIAGEN Laboratories) according to the manufacturer's recommendations with slight modifications (Flahaut et al., 1998) and amplified using the PCR (Polymerase Chain Reaction) in a MJ Research (Massachusetts) thermal cycler. Three commonly used protein-coding genes in fungal phylogenetics (Hibbett et al., 2007; James et al., 2006; Lutzoni et al., 2004): actin (*ACT1*), elongation factor 2 (*EF2*) and the second largest subunit of RNA polymerase II (*RPB2*) as well as the novel DNA replication licensing factor *Mcm7* gene (Schmitt et al., 2009) were amplified and sequenced. Standard primers and PCR protocols used in this study are shown in Supplementary information Table S2. PCRs were performed in 20 µl containing 0.4 µl of Biotaq DNA polymerase (Bioline), 2 µl of NH₄-based Reaction Buffer (10 ×), 0.8 µl (*ACT1*) or 0.6 µl (*EF2*, *Mcm7*, *RPB2*) of 50 mM MgCl₂, 0.2 µl (*ACT1*, *Mcm7*, *RPB2*) or 0.1 µl (*EF2*) of 25 mM deoxynucleoside triphosphates, 1.5 µl of each forward and reverse primer (10 µM) and 1 µl of genomic DNA. Additionally, a volume of 1 µl of Bovine Serum Albumin (BSA, 0.4%) was included in *Mcm7* reactions. When required, the *RPB2* gene fragment was amplified in two overlapping segments using the 1F-1014R and 980F-7R primer combinations.

To remove superfluous dNTPs and primers, PCR products were purified using ExoSAP-IT (GE Healthcare) according to the manufacturer's protocols except *Mcm7* amplicons which were purified using the Isolate PCR and Gel kit (Bioline) after excision from the gel of the expected length band (85% of the samples). Using the same primers as for amplification sequencing PCRs were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The protocol included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 96 °C for 30 s, primer annealing at 50–58 °C for 15 s and primer extension at 60 °C for 4 min. Sequencing PCR products were purified and sequenced at the Estación Biológica de Doñana, CSIC facilities (LEM, Laboratorio de Ecología Molecular) using an ABI 3700 (Applied Biosystem) automated sequencer. *RPB2* gene fragments, whether amplified in two overlapping segments or in a single one, were sequenced using two internal primers (980F and 1014R) due to the length of the fragment.

2.2. Molecular analyses

2.2.1. DNA sequence alignment and phylogeny reconstruction

Sequences were edited in Sequencer 4.9 (Gene codes) and aligned using M-Coffee (Wallace et al., 2006), a meta-method for assembling results from different alignment algorithms, accessible from a public server (Moretti et al., 2007). Data partitions were translated to amino acids in MEGA5 (Tamura et al., 2011) to confirm conservation of the amino acid reading frame, ensure proper alignment, and to check for premature stop codons. Subsequently, Gblocks (Talavera and Castresana, 2007) was applied to eliminate ambiguously aligned regions of individual amino acid gene data sets using relaxed settings as follows: allow gap positions=all; minimum length of a block=5 and default settings for all other options.

Optimal models of nucleotide substitution were determined for each sequenced data set and partition (see below) according to the Akaike Information Criterion (AIC) using jModelTest 0.1 (Posada, 2008). Potential substitutional saturation in the four nucleic genes, as well as in the three codon positions separately, was evaluated by performing a test of substitutional saturation based on the ISS statistic as proposed by Xia et al. (2003) and implemented in DAMBE (Xia and Xie, 2001). The proportion of invariant sites used in the saturation test was obtained from the jModelTest output.

Since the partition homogeneity test results have been shown to be misleading (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002) preliminary phylogenetic analyses of data from each gene were performed to check for topological congruence among data partitions. Conflict among clades was considered as significant if a well-supported clade (bootstrap support values (BS) ≥ 80% and/or posterior probabilities (PP) ≥ 0.95) for one marker was contradicted with significant support by another. Maximum likelihood (ML) bootstrap analyses and the inference of the optimal tree were performed with the program RAXML (Stamatakis, 2006; Stamatakis et al., 2008) under the general time reversible (GTR) nucleotide substitution model with among-site rate variation modeled with a gamma distribution. Five hundred independent searches starting from different maximum parsimony (MP) initial trees were performed using RAXML version 7.2.7 on the CIPRES portal teragrid (www.phylo.org; Miller et al., 2010). Clade support was assessed with 5000 replicates of a rapid bootstrapping. Bayesian Inference (BI) analyses were conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) on the CIPRES portal teragrid (www.phylo.org; Miller et al., 2010), using the best-fit models of nucleotide substitution detected with jModelTest. Two independent but parallel Metropolis-coupled MCMC analyses were performed with default settings (except nswaps=5 for *RPB2*). Each search was run for 10 (*EF2*, *Mcm7*), 60 (*ACT1*) and 50 (*RPB2*) million generations, sampled every 1000th generations. The initial 10% (25% for *ACT1* and *EF2* datasets) of samples of each Metropolis-coupled MCMC run were discarded as burn-in, and the remaining samples were summarized as 50% majority rule consensus phylograms with nodal support expressed as posterior probabilities. The standard deviation of split frequencies between runs was evaluated to establish that concurrent runs had converged (<0.01). Tracer v1.5 (Rambaut and Drummond, 2007) was used to determine whether the MCMC parameter samples were drawn from a stationary, unimodal distribution, and whether adequate effective sample sizes for each parameter (ESS > 100) were reached.

The Bayesian tree topology obtained from the *EF2* data set resulted in strongly supported conflicts with respect to tree topologies obtained from the other three single gene data sets. However, when the analysis was restricted to first and second codon positions of *EF2*, the resulting phylogeny was congruent with those obtained with the other three single gene datasets. Accordingly, the third codon position of the *EF2* gene of the *Metschnikowia*

clade and outgroup taxa was excluded in subsequent analyses. We did not succeed in sequencing the *Mcm7* gene in 38 *Metschnikowiaceae* species or in the outgroup taxa and for this reason, a data set consisting of three molecular markers (*ACT1*, 1st and 2nd codon positions of *EF2* and *RPB2*) was also analysed to investigate the potential problems associated with missing data. Phylogenetic relationships were inferred as described above except for Bayesian analyses parameters (four markers data set: generations = 46.56×10^6 , burnin = 10%; three markers data set: generations = 50×10^6 , nchains = 6, temp = 0.1, nswaps = 5, burnin = 25%).

Bayesian criteria (Bayes factors; Kass and Raftery, 1995) were employed to determine the best partitioning strategy for the combined data set, comparing between partitioned by (1) gene, (2) gene and codon positions 1 and 2 vs. 3 and (3) gene and codon positions (Brown and Lemmon, 2007). Harmonic means of post-burnin of tree likelihood values for both combined data sets and each partition strategy were obtained using the *sump* command in MrBayes.

2.2.2. Character evolution

Patterns of evolution of three morphological characters (ascus formation, ascospore length, and ascospore number) were explored. These morphological characters scored are inapplicable outside the Ascomycota or in asexual species. All *Candida* species within the *Metschnikowia* clade are asexual and so we restricted the reconstruction of these morphological characters to *Metschnikowia* species (and *Clavisporalutitaneae* as outgroup) using the best-scoring tree from the RAxML likelihood search. Optimizations were performed in Mesquite 2.75 (Maddison and Maddison, 2011) using a parsimony model of unordered states. In addition, to account for values of phylogenetic mapping uncertainty, probabilities of ancestral states for the three characters were estimated on 10,564 Bayesian trees using the BayesMultiState program contained in the BayesTraits 1.0 package (Pagel and Meade, 2007), under the Monte Carlo Markov Chain (MCMC) method and allowing transitions between character states in both directions. To reduce the autocorrelation of successive samples, 5282 trees were drawn from each of the two Bayesian distributions of 52,816 trees, by sampling every 10th generation of each chain used in the phylogenetic analysis. As suggested in the BayesMultiState manual, to reduce some of the uncertainty and arbitrariness of choosing the prior probability in MCMC studies, we used the hyperprior approach, specifically the reversible-jump (RJ) hyperprior with a gamma prior (mean and variance seeded from uniform distributions from 0 to 10 for ascospore length and from 0 to 5 for the number of ascospores and ascus formation). Preliminary analyses were run to adjust the *ratedev* parameter until the acceptance rates of proposed changes was around 20–40%. Using the *ratedev* parameter set to 40 (ascospore length), 15 (number of ascospores) and 10 (ascus formation), we ran the RJ MCMC analyses three times independently for 40×10^6 iterations, sampling every 1000th iteration and discarding the first 10×10^6 iterations. All runs gave similar results and we report one of them here. We used the “Addnode” command to find the proportion of the likelihood associated with each of the possible states at selected nodes.

Patterns of evolution of habitat type were also explored in the *Metschnikowia* clade using a data set limited to one specimen per sampled species. Because parsimony character state reconstructions are extremely sensitive to outgroup choice ancestral state reconstruction of habitat was only implemented under a Bayesian approach in BayesTraits using the same parameters as above (except for *rjhp* gamma 0 10 0 10 and *ratedev* = 0.09). Information on habitat type and morphological character states was obtained from Lachance (2011a,b), Lachance et al. (2011) and original species descriptions.

2.2.3. Molecular dating

A likelihood ratio test (LRT) was used to test the molecular clock hypothesis, according to Huelsenbeck and Crandall (1997). The LRT results ($\chi^2 = 4667.1$, *df* = 82) suggested a heterogeneous rate of variation for each gene and therefore a clocklike evolution of sequences could not be assumed. Consequently, dates of divergence were inferred using a relaxed molecular clock, following the uncorrelated relaxed lognormal clock as implemented in BEAST (v. 1.5.4, Drummond et al., 2006; Drummond and Rambaut, 2007). This analysis was limited to one specimen per sampled species and carried out on the four protein-coding genes concatenated matrix partitioned by genes. Excessive run times prevented us from using the best partitioning strategy (genes and codon). A Yule prior process was applied to model speciation. The time to the most recent common ancestor (MRCA) between each clade was estimated under the models selected by jModeltest (Posada, 2008) for each gene. Nodes obtained in the Bayesian and ML analyses of the combined sequences were constrained to ensure that topology remained identical in the new analysis. We did four independent runs with BEAST, each one for 120 million steps sampled every 4000th. Convergence to stationarity and effective sample size (ESS) of model parameters were assessed using TRACER 1.5 (Rambaut and Drummond, 2007). Samples from the four independent runs were pooled after removing a 10% burnin using LogCombiner 1.5.4 (Drummond and Rambaut, 2007).

The fossil record for fungi is poor and for many reasons dating of fungal divergences has yielded highly inconsistent results (i.e. Berbee and Taylor, 2007; Padovan et al., 2005; Taylor and Berbee, 2006) making difficult the use of previous estimated dates. *Paleopyrenomycites devonicus* (Taylor et al., 1999) is the oldest fossil evidence of Ascomycota but morphological features preserved in it do not permit a phylogenetic placement of the taxon without ambiguities (Berbee and Taylor, 2007; Eriksson, 2005; Padovan et al., 2005; Taylor and Berbee, 2006; Taylor et al., 2004, 2005). Lücking et al. (2009) reassessed the systematic placement of this important fungal fossil and recalibrated published molecular clock trees (Hughes and Eastwood, 2006; Kendall, 1949; Moran, 2000) obtaining dates that are much more consistent than previous estimates. Accordingly, one calibration point consisted of the estimated Basidiomycota–Ascomycota divergence age (Lücking et al., 2009) modelled as a normal distribution of 575 ± 70 Ma. Another geological calibration point was based on radiometric ages of lava from the Hawaiian Islands (Price and Clague, 2002), as four *Metschnikowia* species are endemic to this archipelago. Using the formation of these oceanic islands as a divergence time, we constrained the node common to the Hawaiian lineage to a normal distribution of 5.2 ± 1 Ma.

A Lineages-through-time plot (LTT) for the *Metschnikowia* clade was calculated using Beast estimated dates and the APE package (Paradis et al., 2004) in R software v2.14.1 (R Development Core Team, 2011) to visualize the number of lineages present at sequential time points.

2.2.4. Lineage diversification in the *Metschnikowia* clade

In order to identify potential diversification rate shifts within the *Metschnikowia* clade we used MEDUSA (Alfaro et al., 2009), a stepwise birth–death model fitting approach based on the Akaike Information Criterion (AIC). Applied to the diversity tree of *Metschnikowiaceae* (MCC tree produced by BEAST where each tip has been assigned a species richness value) MEDUSA analysis was implemented using R statistical software (<http://www.r-project.org/>) with the “geiger v1.3–1” (Harmon et al., 2008) package. The estimate Extinction parameter was set to “True” and a moderate corrected AIC threshold (cut-off = 4 units) was selected for the improvement in AIC score required to accept a more complex model. In previous studies (e.g. Alfaro et al., 2009), lineages are

collapsed to branches representing orders or other higher-level groupings to account for missing species. Since we are working with a species-level phylogeny we assigned to tips a diversity of one. Outgroup taxa and two subspecies of *M. bicuspidata* were pruned from the MCC tree.

3. Results

3.1. Characteristics of ACT1, EF2, RPB2 and Mcm7 sequences

A total of 283 (ACT1: 83; EF2: 77; RPB2: 79; Mcm7: 44) sequences from four protein-coding genes were newly obtained in the present study. Descriptive statistics for the individual datasets are given in Table 3 and Supplementary Tables S3 and S4. The Index of Substitution Saturation (Iss, Xia et al., 2003) revealed significant levels of saturation in the third codon position of both Mcm7 and RPB2 (Table 3) under the assumption of a symmetrical tree when excluding outgroup taxa. Since both tree topologies do not

conflict with the rest of marker sets the effect of saturation on the phylogenetic signal of these two markers is likely to be minimal.

3.2. Phylogeny reconstruction

Exclusion (three markers data set) or inclusion (four markers dataset) of the Mcm7 dataset did not alter the topology for major relationships, although its inclusion provided a more slightly robust phylogenetic estimate. In addition, Bayes factors favoured gene plus three-codon position partition over the other strategies. The subsequent presentation of the results of the Bayesian and ML analyses will be limited to the trees derived from this partitioned strategy on the four markers dataset.

The best-scoring ML tree (result not shown) was identical to the Bayesian tree (Fig. 1) with minor exceptions at terminal nodes. The two analyses were consistent at different places: (1) the *Clavispora* species were not monophyletic; (2) *Metschnikowia* species and some anamorphs were divided into two groups, one that included species isolated from floricolous insects and the aquatic *C. torresii*

Table 3

Characteristics, summary of models and model parameters for alignments of Metschnikowiaceae sequences from the different loci used in this study.

	ACT1 (total)	ACT1 (1st position)	ACT1 (2nd position)	ACT1 (3rd position)	EF2 (Total)	EF2 (1st position)	EF2 (2nd position)	EF2 (3rd position)
No. of sites in alignment	582	194	194	194	627	209	209	209
No. of excluded sites ^a	0	0	0	0	0	0	0	0
No. of characters analysed	582	194	194	194	627	209	209	209
Variable sites (%)	25.60	15.98	7.22	81.44	33.49	16.74	9.57	74.16
%GC	46.91	43.30	39.87	57.56	48.91	53.07	39.88	53.85
%A	24.55	30.58	30.92	12.14	25.38	28.68	31.47	15.95
%C	22.07	11.17	25.44	29.57	22.98	13.52	22.49	32.98
%G	24.84	32.14	14.43	27.99	25.93	39.55	17.39	20.87
%T	28.54	26.11	29.21	30.30	25.71	18.25	28.65	30.20
Maximum sequence divergence (K-2-p)	18.01%	–	–	–	17.64%	–	–	–
Model estimated ^b	GTR+G	F81+G	F81+G	GTR+I+G	GTR+I+G	HKY+I+G	GTR+I+G	SYM+G
Among-site rate variation: I/G	–/0.18	–/0.15	–/0.07	0.09/1.12	0.57/0.71	0.80/1.32	0.83/0.63	–/0.58
ISS statistic (ISS/ISS.cSym for 32 taxa data subsets) ^c	0.11/ 0.71**	0.03/0.70**	0.02/0.70**	0.32/0.70**	0.35/ 0.71**	0.41/0.68**	0.31/0.68**	0.31/0.68**
ISS statistic (ISS/ISS.cAsym for 32 taxa data subsets) ^c	0.11/ 0.38**	0.03/0.38**	0.02/0.38**	0.32/0.38**	0.35/0.38	0.41/0.37	0.32/0.37	0.31/0.37
	RPB2 (Total)	RPB2 (1st position)	RPB2 (2nd position)	RPB2 (3rd position)	Mcm7 (Total)	Mcm7 (1st position)	Mcm7 (2nd position)	Mcm7 (3rd position)
No. of sites in alignment	1057	353	352	352	537	179	179	179
No. of excluded sites ^a	42	14	14	14	21	7	7	7
No. of characters analysed	1039	338	337	337	516	172	172	172
Variable sites (%)	48.56	33.14	17.24	95.74	60.4	50.00	66.86	98.25
%GC	46.81	47.24	39.98	53.29	53.61	54.27	34.45	72.09
%A	27.55	28.84	33.84	19.90	22.60	24.28	32.35	11.20
%C	21.64	14.69	24.06	26.32	25.52	23.05	20.85	32.66
%G	25.17	32.55	15.92	26.47	28.09	31.22	13.60	39.43
%T	25.64	23.92	26.18	26.81	23.79	21.45	33.20	16.71
Maximum sequence divergence (K-2-p) (%)	34.30%	–	–	–	52.32%	–	–	–
Model estimated ^b	GTR+I+G	GTR+G	GTR+I+G	GTR+I+G	SYM+I+G	GTR+G	GTR+I+G	GTR+I+G
Among-site rate variation: I/G	0.43/ 0.55)	–/0.23	0.47/0.35	0.04/1.44	0.36/1.05	–/0.41	0.53/0.76	0.02/2.46
ISS statistic (ISS/ISS.cSym for 32 taxa data subsets) ^c	0.50/ 0.75**	0.18/0.68**	0.27/0.68**	0.65/0.68	0.49/ 0.70**	0.20/0.70**	0.28/0.70**	0.69/0.70
ISS statistic (ISS/ISS.cAsym for 32 taxa data subsets) ^c	0.50/ 0.45**	0.18/0.35**	0.27/0.35**	0.65/0.35**	0.49/ 0.38**	0.20/0.40**	0.28/0.40*	0.69/0.40**

^a Excluded sites comprise ambiguously aligned regions identified by Gblocks (Talavera and Castresana, 2007).

^b Estimated by the Akaike Information Criterion (AIC) implemented in jModeltest (Posada, 2008).

^c Proportion of Invariable sites (I) and Gamma distribution shape parameter (G) as estimated by jModeltest (Posada, 2008).

* $p \leq 0.05$.

** $p \leq 0.01$.

Fig.1. Phylogeny of the *Metschnikowia* clade based on nuclear *ACT1*, 1st and 2nd codon positions of *EF2*, *Mcm7* and *RPB2* sequences, using Bayesian Inference (BI). Numbers above branches show posterior probabilities. Numbers below branches show bootstrap support for clades recovered by maximum likelihood analysis and in agreement with the BI.

(99 PP, 78% BS) and another consisting of species isolated from diverse habitats and geographical areas (100 PP, 97% BS); (3) a sister-group relationship existed between the *Metschnikowia drosophilae*-*C. torresii* group (100 PP, 100% BS) and the remaining species isolated from floricolous insect species (100 PP, 100% BS); and (4) the five Hawaiian endemic accessions (100 PP, 99% BS) formed a natural group.

Both BI and ML indicated monophyly of the respective conspecific accessions. Similarly, *C. musae*–*C. fructus* accessions as well as the teleomorph/anamorph pair of accessions were respectively resolved as well-defined (100 PP, 100% BS) monophyletic groups. The number of nucleotide differences between accessions is shown in Table S5.

3.3. Character evolution

A summary of historical character state reconstruction, as applied to three morphological characters and habitat, follows:

1. Ascospore length (Fig. 2A). The reconstruction yielded some conflicts between parsimony and Bayesian analyses. According to parsimony reconstruction small ascospores are common within a basal grade of *Metschnikowia* however the Bayesian approach estimated a higher probability for large (0.51) and giant (0.49) ascospores to be ancestral. Both parsimony and Bayesian reconstructions inferred a dynamic evolution of this character within group 3. Ancestral states at the basal grade

of group 3 were reconstructed as small ascospores in the MP analysis, but large and giant ascospores displayed the highest posterior probability. Character optimisation suggests two independent origins of small ascospores (reversals to plesiomorphic state according to parsimony reconstruction) within the LSM clade. Large and giant ascospores could have evolved at least three and two independent times, respectively, within the LSM clade.

2. Number of ascospores per ascus (not figured). MP optimization and Bayesian approach inferred the ancestral number of ascospores in *Metschnikowia* to be two. Both analyses showed five independent reductions to one ascospore within group 4 although in three of the five lineages species have also retained the ancestral state (two ascospores).
3. Ascus formation (Fig. 2B). Parsimony reconstruction traced ascus formation from heterothallic conjugants as the ancestral state in *Metschnikowia*. Withingroup 4 chlamydoconidia as precursors of ascus evolved once, the development of asci by elongation appeared two independent times, and a reversal to the ancestral state occurred in *M. australis*.
4. Habitat. The habitat character appeared highly homoplastic within the *Metschnikowia* clade (Fig. 3). Despite the reconstruction being equivocal in inferring the state at some nodes (including the ancestral node), shifts between different habitats appeared dynamic (i.e. at least five independent origins of floricolous insect habitat, at least three of fruit and associated insect habitat and two of aquatic habitats).

Fig. 2. Results of the ancestral state reconstruction analysis for ascospore length (A) and ascus formation (B) mapped onto the best-scoring tree obtained in the ML analysis of *ACT1*, 1st and 2nd codon positions of *EF2*, *Mcm7* and *RPB2* sequences combined. Parsimony reconstruction is shown as branch colour. Pie charts at nodes represent the posterior probabilities of Bayesian inference character state evolution. The character states found in the terminal taxa are indicated as rectangles of the respective colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Divergence times

Molecular dating analysis estimated a Cretaceous (Fig. 3; Supplementary Fig. S1) divergence of Metschnikowiaceae and *Debaryomyces*. However it is not until the Upper Cretaceous (71.7 Ma, CI 48.2–97.6) that the ancestor of the *Metschnikowia* clade begins to diverge (Fig. 3, Supplementary Fig. S1), with the bulk of speciation

taking place during the middle Palaeogene and the Neogene. The ancestor of group 3 split about 58.03 Ma (CI 37.9–79.0), whereas group 4 divergence took place about 52.5 Ma (CI 34.5–72.0) (Fig. 3, Supplementary Fig. S1).

The LTT plot of Metschnikowiaceae (inset of Fig. 3) reflected a period of little diversification between 71.7 and 48.8 Ma followed by a phase during which lineages arose rapidly and steadily and

Fig. 3. Maximum clade credibility chronogram of the *Metschnikowia* clade lineages based on the combined dataset of *ACT1*, 1st and 2nd codon positions of *EF2*, *Mcm7* and *RPB2* sequences. Branch lengths represent million of years before present (Ma). The geological timescale (Ma) is provided below the topology. Outgroup taxa (*Cryptococcus* sp., *Debaryomyces etchellsii*, *Lodderomyces elongisporus*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) have been removed for legibility. Pie charts at selected nodes represent the posterior probabilities of Bayesian inference character state evolution. The character states found in the terminal taxa are indicated as rectangles of the respective colours. Species distribution plotted on the right side of the chronogram: ○ Afrotropical, ⚡ Antarctic, ⌘ Australasian, ⚡ Hawaii (in brackets non -native Hawaiians), ▼ Indomalayan, ■ Nearctic, ● Neotropical, ◆ Oceanian, □ Palearctic. Lineages-through-time plot of the Metschnikowiaceae lineages are also shown (inset). Changes in rates of diversification (r) and relative extinction (ϵ) are indicated at the appropriate node. (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4

Summary of MEDUSA results (cut-off=4) based on Δ AIC score for Metschnikowiaceae lineages indicating number of rates shifts, clade involved, likelihood, number of parameters (p) for AIC calculation, estimated net diversification (r), relative extinction (ε), speciation (b) and extinction (d) rates and model selection criteria (AIC) for each model and improvement in AIC scores (Δ AIC) after the addition of a turnover in diversification rates.

Shifts	Clade	Likelihood	p	r	ε	b	d	AIC	Δ AIC
0		-309.66	2	0.035	1.6×10^{-6}	0.035	<0.000001	623.32	-
1	<i>M. pulcherrima</i> ^a	-299.47	5	0.578	1.08×10^{-8}	0.578	<0.000001	608.94	14.38
2	<i>M. arizonensis</i> ^b	-291.39	8	0.156	0.195	0.194	0.038	598.78	10.16

AIC, Akaike Information Criterion.

^a Including *C. rancensis*, *C. picachoensis*, *M. fructicola*, *M. pulcherrima*, *M. shanxiensis*, *M. sinensis* and *M. zizyphicola*.

^b Including *C. ipomoeae*, *M. arizonensis*, *M. borealis*, *M. cerradonensis*, *M. cubensis*, *M. hamakuensis*, *M. hawaiiensis*, *M. kamakouana*, *M. lochheadii*, *M. mauinuiana*, and *M. santaceciliae*.

a sharp rise in the number of lineages when we approach the present.

3.5. Lineage diversification in the Metschnikowiaceae clade

The diversification analysis suggests that the current diversity of Metschnikowiaceae is best explained by two shifts in the rates of diversification during their evolutionary history (Fig. 3). The diversification analysis found the net rate of diversification (r) of Metschnikowiaceae to be 0.035 lineages per million years (Table 4). The lineage leading to a group containing seven species (hereafter *M. pulcherrima* group) (*C. rancensis*, *M. shanxiensis*, *M. sinensis*, *M. pulcherrima*, *M. fructicola*, *M. zizyphicola*, *C. picachoensis*) (Fig. 3) mostly isolated from fruits was identified as having experienced a 16-fold increase in its rate of diversification ($r=0.58$; Δ AIC=14.38) and a decrease in extinction ($\varepsilon=1.08 \times 10^{-8}$) (Table 4). A strikingly increase in extinction is inferred to have happened in the lineage leading to a group containing 11 species (hereafter *M. arizonensis* group) (*C. ipomoeae*, *M. arizonensis*, *M. borealis*, *M. cerradonensis*, *M. cubensis*, *M. hamakuensis*, *M. hawaiiensis*, *M. kamakouana*, *M. lochheadii*, *M. mauinuiana*, and *M. santaceciliae*) ($\varepsilon=0.19$; AIC=24.54) (Table 4). Models with more than two shifts did not improve AIC values (results not shown).

4. Discussion

4.1. Phylogenetic relationships in the Metschnikowiaceae clade

Ribosomal genes, specially the D2 domain of the 26S rRNA gene, are extremely divergent in Metschnikowiaceae despite the relative phenotypic homogeneity of these taxa (Mendoça-Hagler et al., 1993). The level of sequence divergence observed between members of the *Metschnikowia* clade can exceed intergeneric rRNA divergence levels reported for Saccharomycetales (Yamada et al., 1994). In contrast none of the four protein-coding genes (*ACT1*, 1st and 2nd codon positions of *EF2*, *Mcm7*, and *RPB2*) sampled from the *Metschnikowia* clade was found to be particularly divergent. The phylogenetic distance between members of the clade was not greater than the distance between the Metschnikowiaceae and the Saccharomycetaceae outgroup taxa. These results reveal that Metschnikowiaceae genomes have experienced a dramatic acceleration of evolutionary rate in rRNA genes, although a high rate of divergence has been noted also for the DNA topoisomerase II gene of *Clavispora lusitaniae* compared to other yeasts species with similar ecologies (Kato et al., 2001).

In the present study, we conducted molecular phylogenetic analyses of 77 species of ascomycetous yeasts from the *Metschnikowia* clade. As far as we know, this is the first comprehensive phylogenetic study on this fungal group encompassing much of Metschnikowiaceae diversity. Only ascospore morphology and Co-Q system (Co-Q₈ in *Clavispora* and Co-Q₉ in *Metschnikowia*; La-

chance, 2011a,b; Suzuki and Nakase, 1999) served to separate *Clavispora* and *Metschnikowia* species. However, contrary to what was found in previous studies (Lachance, 2011a, b), *Clavispora* was not, strictly speaking, sister to *Metschnikowia* (Fig. 1) but to some asexual forms of the *Metschnikowia* clade. A relationship between *Clavispora* and certain *Candida* species of the *Metschnikowia* clade was previously suggested based on assimilation and fermentation patterns (Phaff and Carmo-Sousa, 1962) and D1/D2 LSU (Kurtzman and Robnett, 1998; Yurkov et al., 2009) and SSU (Sugita and Nakase, 1999) rRNA gene sequencing. In addition, *Clavispora arehetovae* was recovered as a distinct lineage from *Cl. lusitaniae* and *Cl. opuntiae* (Fig. 1). This apparent paraphyly cannot be simply explained away by assuming that intervening *Candida* species will eventually be reassigned to *Clavispora* but could indicate that the claviform ascospore morphology of this genus is in fact a symplesiomorphy retained from the common ancestor of all Metschnikowiaceae.

Phylogenetic analysis of the D1/D2 LSU rRNA gene region (Rui-vo et al., 2006) placed *C. pinguabensis* as the sister species of *C. saopaulonensis* (group 2). An exact placement of both species within the clade could not be inferred based on previous studies. Our results suggest a basal position with respect to two large groups containing *Metschnikowia* species and several anamorphs (Fig. 1).

The grouping of *Metschnikowia* species into two different lineages (groups 3 and 4; Fig. 1) agree to a large extent with a previous phylogeny based on D1/D2 LSU rRNA gene sequences (Lachance, 2011b), although in the latter, bootstrap support for either group was low. Some major discrepancies were found, however, between the phylogenetic hypothesis presented here and those from other studies. For example, the present phylogeny placed the sister species pairs *Metschnikowia drosophilae* - *C. torresii* and *M. orientalis* - *C. hawaiiensis* within group 3 whereas the D1/D2 LSU rRNA gene sequence phylogeny (Lachance, 2011b) inferred a close relationship with members of group 4. Indeed, here we reported for the first time a basal position of *M. drosophilae* and *C. torresii* within group 3 (Fig. 1). Another point of disagreement is the derived position of *M. corniflorae* (group 4) within *Metschnikowia* found in the present study whereas a basal position with respect to the LSM clade is inferred when analyzing the D1/D2 LSU rRNA gene (Nguyen et al., 2006). In contrast, the LSM clade is mostly recovered as a strongly supported monophyletic group regardless of genetic marker and analytical method of phylogenetic inference (Lachance, 2011b; Lachance et al., 2001). The clade is ecologically highly homogeneous as all the species are associated with beetles and have restricted geographical distributions. In contrast, phylogenetic relationships within the more diverse (in terms of habitat and geographical distribution) group 4 are less well supported.

Candida musae has been regarded by some to be a synonym of *C. fructus* based on D1/D2 LSU rRNA gene (Kurtzman and Robnett, 1997) and ITS/5.8S rDNA (Lu et al., 2004) sequences. However, the lack of DNA-DNA hybridization experiments plus a 0.9% base sequence dissimilarity in the SSU rRNA gene (Suzuki and Nakase,

1999) has prevented their recognition as synonyms (Lachance, 2011b). In our phylogenetic tree, both species formed a coherent, highly supported group (100 PP, 100% BS; Fig. 1, Table S5), indicating that these two accessions have a close genealogical relationship. Similarly, the undescribed species *C. magnifica* has been suggested to be a synonym of *M. reukaufii* based on D1/D2 LSU rRNA gene (Kurtzman and Robnett, 1997) sequences. The 100% (ACT1) and 99.99% (EF2, RPB2) base sequence similarity found in the present study (Table S5, Fig. 1) supports the treatment of *C. magnifica* as a synonym of *M. reukaufii*. Several phylogenetic associations between *Metschnikowia* species and possible anamorphs were apparent. In most cases, however, an anamorph–teleomorph connection cannot be inferred as the amount of sequence identity (Table S5) between pairs of accessions is low. *Candida pimensis* is identical to *M. chrysoperlae* for ACT1 and EF2 sequences (Table S5), which could be considered as a suggestion of a possible anamorph/teleomorph relationship, but growth responses and D1/D2 LSU rRNA gene sequences (Suh et al., 2004) do not support taxonomic equivalence. The possibility that *Metschnikowia viticola* is the teleomorph of *C. kofuensis*, based on D1/D2 LSU sequences, has been considered but rejected by Péter et al. (2005). Their almost identical ACT1 sequences support this connection, but the 54 divergent positions in their RPB2 sequences (Table S5) speak to a different conclusion.

4.2. Evolutionary trends of morphological key characters within *Metschnikowia*

Seven morphological characters have been traditionally considered in the diagnostic of *Metschnikowia* (Giménez-Jurado et al., 1995). Data for some of these characters were not available for all species included in the study (Table 1), others (i.e. mycelial structures) seem to be very plastic and show a great deal of variation in detectability, and others (i.e. pulcherrimin production) have been found to be unreliable diagnostic characters as the number of *Metschnikowia* species increased (Lachance, 2011b). We have mapped three characters on the multiple gene phylogeny, namely ascospore length, number of ascospores per ascus and mode of ascus formation. Evolution of sexual characters must be inferred with caution, given that relevant character states are absent in anamorph species. *Metschnikowia* ascospores may vary in length from 8–12 µm in *M. kunziensis* to 100–200 µm in *M. borealis* (Table 1). Our analysis (Fig. 2A) suggests that the three states of ascospore length (short, large and giant) did not evolve in a unidirectional manner in *Metschnikowia*. Since there is no evidence about the function of ascospore length, it is hard to interpret large and giant ascospores in the core LSM clade as an adaptation to the highly specific interaction between these *Metschnikowia* species and *Conotatus* beetles (Lachance et al., 1998b) since sister species with different ascospore length inhabit identical environments (flower beetles, Fig. 3). One or two ascospores per ascus are found across the *Metschnikowiaceae* (results not shown). Presumably, the progenitor of the family produced up to four ascospores, which is the archetype across ascomycetous yeasts. Marinoni et al. (2003) demonstrated that the two-spored asci of *Metschnikowia* species are the result of meiosis where ascospore production is coupled with the first meiotic division. This major evolutionary path would appear to be maintained throughout the family. Multiple shifts between two- and one-spored asci seemingly reflect the more plastic nature of this change, which in some cases is due to mating between closely related, but distinct species (Lachance and Bowles, 2004). The two-spored state is maintained within the LSM clade and relatives, except in hybrid asci. Whether this also applies to species outside that clade remains to be determined.

Morphological characters have been traditionally used not only in the definition of taxa but also as a basis for speculation about

taxonomic affinities within the genus. Giménez-Jurado et al. (1995) suggested that the type of ascus precursor (vegetative cells vs. chlamydospores) could serve to define two natural groups within *Metschnikowia* species. This hypothesis has not been rigorously tested in a phylogenetic context and sharing a character state with some relatives does not always imply a single origin from a common ancestor (homology). The historical reconstruction of the mode of ascus development (Fig. 2B) provided evidence that different ascus precursors did not constitute synapomorphic traits of monophyletic groups. This is in contrast to the hypothesis of Giménez-Jurado et al. (1995), which suffered from limited taxon sampling. Chlamydospores are produced by a broad assortment of fungi (Abou-Gabal and Fagerland, 1981; Gronvold et al., 1996; Kurtzman, 1973) but their biological functions remain enigmatic and whether the chlamydospores of certain *Metschnikowia* species are homologues of those in other families is questionable. The ability of terrestrial *Metschnikowia* species to produce chlamydospores was hypothesized to be derived from a hypothetical aquatic ancestor as a desiccation resistance mechanism (Pitt and Miller, 1970). According to our analysis (Fig. 2B) aquatic *Metschnikowia* species are derived rather than ancestral (Fig. 3). Additionally, chlamydospores do not appear to have been key to the colonization of terrestrial environments, given that other terrestrial species, in particular those in the LSM clade, lack chlamydospores and develop their ascus from vegetative cells. Different terrestrial environments surely have different water activities. However, the absence of chlamydospores in some terrestrial species cannot be explained by the relative water activity of their habitats (Giménez-Jurado et al., 1995). For instance, the sister species *M. gruessi* and *M. lachancei* share the same habitat (floral nectar) but not the ability to develop chlamydospores. The same is true for species that do not share sister relationships and that were isolated from floricolous insects (i.e., *M. noctiluminum*, LSM clade). Further analyses are needed to elucidate the functional role of chlamydospores in *Metschnikowia*. Resistance to *Pichia membranifaciens* mycocins (Golubev, 2011) and carbohydrate composition of cell walls (Lopandic et al., 1996) have been also suggested as differential characters between the two main ecological *Metschnikowia* groups (aquatic vs. terrestrial). Limited sampling (nine and 12 species respectively) in both studies prevents us from testing these hypotheses in a phylogenetic framework.

4.3. History of major habitat shifts

Ambiguity of ancestral habitat of the most basal nodes (Fig. 3) may reflect the complex and dynamic habitat transition that have punctuated the course of evolution of *Metschnikowiaceae*. However, the absence of clearly distinct trends may be also the consequence of the evidently large number of undescribed species (Lachance, 2006) and the fact that ecological generalizations may not be reliable for some species (i.e. *M. viticola*, *C. pinguabensis*, *C. tsuchiyae*) as their descriptions are based on one or two isolates. Repeated and independent origins of angiosperm-associated habitats have been inferred (Fig. 3). As the ancestral character state of most basal nodes of the *Metschnikowiaceae* could not be inferred unequivocally, the yeast–floricolous insect association in the *Metschnikowia* clade is thought to initiate at the stem group 3 which age was estimated at 58.03 Ma (CI 37.9–79.0). Subsequently, other transitions to floricolous insects (Fig. 3) have occurred within the *Metschnikowia* clade. Yeast species that have received attention appear to be preferentially associated with particular groups of insects. Nitidulid beetles (order Coleoptera) are the main host insect group within the large-spored *Metschnikowia* clade and relatives (group 3) (Table 1) while green lacewings (order Neuroptera) host species within group 4 and bees (order Hymenoptera) are vectors of a group of nectarivorous yeasts also placed with group

4. By contrast, species hosted by fruit flies (order Diptera) are scattered through the clade (Table 1). Overall, the phylogenetic evidence is suggestive of a relatively high level of lineage-specific association among insects and yeasts. These preferential associations may be linked to interactions whose nature remains unclear. Mutualistic associations with insects that feed on the yeasts and concurrently disperse them have been already suggested within cactophilic yeasts (Phaff and Starmer, 1987), yeasts associated with bark beetles (Leufven and Nehls, 1986), and slime flux yeasts (Batra, 1979). Additionally, studies of nectarivorous yeast are shedding light about the role of these microorganisms in the ecology and evolution of plant–pollinators interactions (Herrera et al., 2010; Herrera et al., 2008; Herrera and Pozo, 2010; Pozo et al., 2009). Further explicit experimental studies are needed to elucidate the level of interdependence among members of the insect–yeast–flower systems.

The poor support values within group 4 (Fig. 1) do not allow a detailed habitat reconstruction and comparison of most basal nodes. However, it is noteworthy that osmophilic (sugar-tolerant) and halophilic (salt-tolerant) species, with the exception of *C. torressi*, were closely related. Successful adaptation to environments such as floral nectar or seawater may include traits that allow species to manage the steep difference in osmotic pressure between the inside of the cell and the surrounding medium. In fungi, intracellular accumulation of compatible solutes (i.e., polyols, amino acids and small carbohydrates) seem to be the most important response to increases in osmotic pressure (Blomberg and Adler, 1992), where the type of osmoregulator differs as a function of external solute (Moran and Witter, 1979; Van Eck et al., 1989; Van Zyl and Prior, 1990). Further studies are needed to clarify the mechanism used by *Metschnikowia* species (and anamorphs) to respond to changes in osmotic gradients and whether convergence and independent acquisition rather than common ancestry (as suggest our results) best explains its origin in both aquatic and nectarivorous species.

4.4. Habitat shifts and diversification

Our analysis places the origin of crown group *Metschnikowia* to the upper Cretaceous (71.7 Ma with CI 48.2–97.6; Fig. 3 and Supplementary Fig. S1). A broader taxon sampling and the use of protein-coding gene sequences leads us to the younger estimate of the origin of the *Metschnikowia* clade than previously proposed based on divergence among SSU rRNA gene sequences (Lachance et al., 2003). However, the 71.7 Ma estimate could be the recent limit for the crown group *Metschnikowiaceae* as a consequence of the large number of undescribed species and therefore it must be taken with caution. Overwhelming evidence from both the fossil record (132–141 Ma, Brenner, 1996) and molecular estimations (167–199 Ma, Bell et al., 2010) place the origin of angiosperms in the Jurassic. Rapid mid-Cretaceous (Moore et al., 2007) radiations have however been inferred for major groups of angiosperms. The Early Eocene (Fig. 3 and inset) burst of diversification of the *Metschnikowia* clade coincides with a wide distribution of many potential yeast hosts such as rosids (Wang et al., 2009), asterids (Moore et al., 2010) and caryophyllales (Moore et al., 2010). Therefore diversification of the *Metschnikowia* clade did not take place in parallel with the diversification of angiosperms, or at least not during its early stages. Instead, it involved expansion into an existing, much older resource, a process that likely promoted specialization and speciation.

A yeast–insect co-speciation within the LSM clade and relatives has been suggested (Lachance et al., 2005). An Early Cretaceous (129.7 Ma, CI 117.3–142.0; Hunt et al., 2007) origin of the nitidulid beetles (subfam. Carpophilinae) associated with species within the

clade has been inferred. Although this date predates the beginning of the LSM diversification, the lack of information about the tempo and mode of diversification in this flower–beetle group prevents us from testing the co-radiation hypothesis.

None of the independent origins of angiosperm-associated habitats (Fig. 3) seem to be associated with enhanced diversity. On the contrary, the lineages within the *Metschnikowiaceae* appear to have evolved at a relatively constant rate of diversification ($r = 0.035$ sp/Ma, Table 4) with two abrupt changes in diversification rates in the Miocene and Pliocene (Fig. 3). The first group exhibiting acceleration in diversification ($r = 0.15$ sp/Ma, Fig. 3) is the clade that contains Hawaiian endemics and a sister group of mostly neotropical species. Given the restricted range of most of the lineages in that radiation (excluding *C. ipomoeae*, which encompasses almost the whole range of *Conotelus* nitidulid beetles; Lachance et al., 2001) such a burst of diversification suggests an important role for the biogeographic dispersal event to Hawaii and restricted areas of Cuba, Central America, and South America due to the ecological release from competitive and selective pressures and the abundance of ecological opportunities. The high relative extinction rate ($\epsilon = 0.19$ sp/Ma) implies high speciation and high turnover ($b = 0.19$ sp/Ma, $d = 0.03$ sp/Ma) that have synchronously dominated the evolution of this group. Additionally, the substantial acceleration in net diversification rate of the *M. pulcherrima* group ($r = 0.57$ sp/Ma, Fig. 3) may have resulted from the evolution of some as yet unknown physiological or allelopathic capability allowing the successful exploitation of fleshy fruits and associated insects.

5. Conclusions

The analyses presented in this study corroborate in a broader phylogenetic context some previous partial results, and provide further resolution of evolutionary relationships and tendencies within *Metschnikowiaceae*, in the context of a wider species representation, molecular sequence data from four protein-coding nuclear genes, and Bayesian inference and maximum likelihood phylogenetic analyses.

Divergence time estimates argue against the hypothesis of co-diversification of flowering plants lineages and the angiosperm–floricolous insect–yeast association. The relatively constant diversification rate inferred for the *Metschnikowia* clade does not parallel the repeated and independent habitat shifts occurred during its evolutionary history. In some lineages, geographic distribution of insect hosts, more than habitat shifts, may be responsible for speciation in the *Metschnikowia* clade.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jympev.2013.04.003>.

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