Metschnikowia chrysoperlae sp. nov., *Candida picachoensis* sp. nov. and *Candida pimensis* sp. nov., isolated from the green lacewings *Chrysoperla comanche* and *Chrysoperla carnea* (Neuroptera: Chrysopidae)

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Fourteen yeast isolates comprising three taxa were cultured from digestive tracts of adult *Chrysoperla* species (Neuroptera: Chrysopidae) and their eggs. The yeast taxa were distinguished based on an estimated molecular phylogeny, DNA sequences and traditional taxonomic criteria. The new yeasts are closely related to *Metschnikowia pulcherrima* but are sufficiently distinguished by sequence comparison of rRNA gene sequences to consider them as novel species. Here, three novel species are described and their relationships with other taxa in the Saccharomycetes are discussed. *Metschnikowia chrysoperlae* sp. nov. (type strain, NRRL Y-27615^T = CBS 9803^T) produced needle-shaped ascospores and was the only teleomorph found. Large numbers of chlamydospores similar to those observed in *M. pulcherrima* were also produced. The other two novel species are asexual yeasts, *Candida picachoensis* sp. nov. (type strain, NRRL Y-27607^T = CBS 9804^T) and *Candida pimensis* sp. nov. (type strain, NRRL Y-27619^T = CBS 9805^T), sister taxa of *M. chrysoperlae* and *M. pulcherrima*. A specialized relationship between yeasts and lacewing hosts may exist, because the yeasts were isolated consistently from lacewings only. Although *M. chrysoperlae* was isolated from eggs and adult lacewings, suggesting the possibility of vertical transmission, no yeast was isolated from larvae.

INTRODUCTION

Symbiotic yeasts have been reported from a variety of insects, including planthoppers, aphids and beetles (for examples, see van der Walt, 1961; Nardon & Grenier, 1989; Noda & Omura, 1992; Suh *et al.*, 2003). Many of the yeasts have been cultured easily on simple agar media; others, including some endosymbionts, do not grow readily in culture and are known only from DNA sequences or microscopic observations (Zhang *et al.*, 2003; Noda & Omura, 1992). The majority of these symbionts have been identified as true yeasts (Ascomycetes: Saccharomycetes), but a few have arisen from at least two independent filamentous ascomycete clades (Jones & Blackwell, 1996; Jones *et al.*,

1999; Noda *et al.*, 1995; Noda & Kodama, 1996; Suh *et al.*, 2001, 2003).

Yeasts have also been reported to be associated with green lacewings, *Chrysoperla* species (Neuroptera: Chrysopidae), generalist predators that are often used in the biological control of other insects. Hagen *et al.* (1970) and Hagen & Tassan (1966, 1972) observed budding yeasts in adult lacewings and reported them to be *Torulopsis* sp. More recently, *Metschnikowia pulcherrima* was reported from *Chrysoperla rufilabris* (Woolfolk & Inglis, 2003); however, the taxonomy of the yeasts has not been studied fully. In this study, we describe three novel species of true yeasts (Ascomycota: Saccharomycetes) isolated from *Chrysoperla comanche* and *Chrysoperla carnea*, and discuss the biology and phylogenetic relationships of the yeasts.

METHODS

Yeast isolation and identification. Lacewings were frozen after collection, allowed to thaw for approximately 15 min, then rinsed with a solution containing 10% bleach, 70% ethanol and 20% distilled water. The insects were dissected in fresh insect Ringer's solution. The diverticulum was excised, placed on a glass slide, and yeast

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Abbreviations: ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AY452039–AY452052 for the LSU rRNA gene, AY452053–AY452055 for the SSU rRNA gene and AY494780–AY494785 for the ITS region and 5-8S rRNA gene.

presence or absence was determined. In a laminar-flow cabinet, the coverslip was removed, one drop of sterile distilled water was added, and the diverticulum was lacerated into a slurry with flame-sterilized minuten pins. One drop of the slurry was applied to an acidified YM agar plate (Difco YM broth, 2% plain agar, adjusted to pH 3.5–4.0 with HCl) with a pipette. Yeasts were purified by single-colony isolation at least twice, and were deposited at the NRRL and CBS (Table 1).

The morphological observations and metabolic tests for standard yeast identification were performed on at least two isolates from each species according to established methods (Yarrow, 1998; Barnett *et al.*, 2000). Photomicrographs were made using a Nikon Microphot FXA microscope equipped with a Spot RT Slider digital camera. To observe ascospores in cultures, the yeasts were incubated at 15 °C for up to 2 months on diluted V8 agar (1 : 2, 1 : 9, 1 : 19; Yarrow, 1998). *Candida picachoensis* sp. nov. and *Candida pimensis* sp. nov. isolates (Table 1) were crossed in all possible combinations to determine if the yeasts were heterothallic.

Molecular phylogeny. Nucleic acids were extracted and purified following the procedures of Lee & Taylor (1990). The primer sets NS1–NS8, LS1–LR5 and ITS5–ITS4 were used for PCR amplification of the small subunit (SSU) and large subunit (LSU) rRNA genes, and the internal transcribed spacer (ITS) region, respectively (White *et al.*, 1990; Hausner *et al.*, 1993). The purified double-stranded PCR products were used as templates for sequencing with an ABI PRISM BigDye Terminator Cycle sequencing kit. The complete sequence of the SSU rRNA gene and the D1/D2 region of the LSU rRNA gene were sequenced with the primers NS1, NS2, 18H, NS5, NS8, LS1, LR3, ITS1 and ITS4 by using an ABI PRISM 377 Automated DNA sequencer. DNA sequences were initially aligned with the multialignment program CLUSTAL_X (Thompson *et al.*, 1997). The alignments were optimized visually, and ambiguous

regions were excluded from the analyses. The sequences from newly isolated yeasts were compared with LSU and SSU rRNA gene sequences of other yeasts and fungi obtained from GenBank. Maximum-parsimony analyses were performed using PAUP 4.0b10 (Swofford, 2002). Heuristic tree searches were executed using the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. Bootstrap values of the most parsimonious tree were obtained from 1000 replications. Bayesian Markov Chain Monte Carlo (B-MCMC) analysis was performed with MrBayes version 3.0b4 (Huelsenbeck, 2000) to estimate the probability of nodes. The analysis consisted of 500 000 generations of four chains sampling every 10 generations, and the first 50 000 generations were discarded as burn in. The remaining trees were imported into PAUP to estimate the posterior probability. Base pair differences in a gene were counted using BLAST 2 sequences (Tatusova & Madden, 1999) or from a manually aligned sequence database.

RESULTS AND DISCUSSION

Novel yeast species from lacewings

Yeasts were isolated from 14 individuals of either *Chrysoperla comanche* or *Chrysoperla carnea*, collected in AZ, USA, although the insect species were not distinguished in the early part of the study (Table 1). Most of the yeasts were isolated from adult lacewings, but two isolates (NRRL Y-27615^T and NRRL Y-27616) were from eggs.

We sequenced about 600 bp of variable D1/D2 loop sequence of the LSU rRNA gene for all isolates for rapid identification and compared the sequences with other data in GenBank. The 14 yeast isolates consisted of three

Table 1. Strains of *Metschnikowia chrysoperlae*, *Candida picachoensis* and *Candida pimensis* isolated from lacewings

For all three novel species, no strains belonging to a specific species showed differences in their D1/D2 regions when compared to the type strain of the species.

Species	Strain designation*			Source
	CBS	NRRL	LSU	
<i>Metschnikowia chrysoperlae</i>	9803 ^T	Y-27615 ^T	Gibson-A ^T	Eggs from adult <i>Chrysoperla</i> sp., Tucson, AZ, USA
		Y-27616	Gibson-C	Eggs from adult <i>Chrysoperla</i> sp., Tucson, AZ, USA
		Y-27613	Gibson-3	Adult <i>Chrysoperla</i> sp., Tucson, AZ, USA
		Y-27614	Gibson-4	Adult <i>Chrysoperla</i> sp., Tucson, AZ, USA
		Y-27617	Gibson-5.1	<i>Chrysoperla comanche</i> (female), Tucson, AZ, USA
		Y-27618	Gibson-5.2	<i>Chrysoperla comanche</i> (female), Tucson, AZ, USA
<i>Candida picachoensis</i>	9804 ^T	Y-27607 ^T	Gibson-1 ^T	Adult <i>Chrysoperla</i> sp., Picacho Peak, AZ, USA
		Y-27608	Gibson-2	Adult <i>Chrysoperla</i> sp., Tucson, AZ, USA
		Y-27609	Gibson-5	Adult <i>Chrysoperla</i> sp., Picacho Peak, AZ, USA
		Y-27610	Gibson-6	Adult <i>Chrysoperla</i> sp., Picacho Peak, AZ, USA
		Y-27611	Gibson-10.4	<i>Chrysoperla comanche</i> (male), Tucson, AZ, USA
		Y-27612	Gibson-11.1	<i>Chrysoperla comanche</i> (male), Tucson, AZ, USA
<i>Candida pimensis</i>	9805 ^T	Y-27619 ^T	Gibson-1.1 ^T	<i>Chrysoperla carnea</i> (female), Tucson, AZ, USA
		Y-27620	Gibson-8.2	<i>Chrysoperla carnea</i> (male), Tucson, AZ, USA

*CBS, Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection, Utrecht, The Netherlands; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA; LSU, Mycology Laboratory, Dept of Biological Sciences, Louisiana State University, Baton Rouge, LA, USA.

genotypes, each with identical D1/D2 sequences, and each genotype was distinguished from the others by more than 5 bp difference. Based on BLAST searches the closest known species to the lacewing isolates was *M. pulcherrima*, which varied by six substitutions from the closest lacewing yeast genotype. The yeasts were distinct from one another and from any known *Metschnikowia* species, not only on the basis of genotype, but also on the basis of morphological and physiological data (Table 2; Barnett *et al.*, 2000; Miller & Phaff, 1998). Of the three lacewing yeast genotypes, only one group was observed to produce needle-shaped ascospores in elongated asci, a unique trait of some *Metschnikowia* species. No ascospores were observed in yeasts in the other two groups. Here we describe three novel yeast species: *Metschnikowia chrysoperlae* sp. nov. and two closely related anamorphs, *Candida picachoensis* sp. nov. and *Candida pimensis* sp. nov.

Latin diagnosis of *Metschnikowia chrysoperlae* Suh, Gibson et Blackwell sp. nov.

Fig. 1(a–c). *In medio liquido dextrosus et peptonum et extractum levidinis continente post 7 dies ad 25 °C cellulae vegetative globosae aut ovoidae (4–10 × 4–10 µm), singulae vel binae; pseudohyphae non fiunt. Cultura in agar extramalti et faecis continente post 7 dies ad 25 °C, candida et teres. In agar farina Zeae maydis confecto post 10 dies ad 25 °C, pseudohyphae et hyphae verae non fiunt. Ascospores fiunt in agar V8 cremor post 10–14 dies ad 15 °C. Glucosum et galactosum (variabile) fermentantur. Maltosum, methyl α-D-glucosidum, sucrosum, trehalosum, melibiosum, lactosum, cellobiosum, melezitum, raffinum, inulinum, amyllum solubile et D-xylosum non fermentantur. Assimilantur glucosum, galactosum, L-sorbozum, D-ribosum (infirme, variabile), sucrosum, maltosum, trehalosum, methyl α-D-glucosidum, cellobiosum, salicinum, arbutinum, melezitum, glycerolum, ribitolum, xylitolum, D-glucitolum, D-mannitolum, gluconolactonum, 2-keto-D-gluconatum, D-gluconatum, acidum succinicum (lente), acidum citricum (lente, infirme), ethanolum et propane-1,2-diolum (lente). Non assimilantur D-glucosaminum, D-xylosum, L-arabiosum, D-arabiosum, L-rhamnosum, melibiosum, lactosum, raffinum, inulinum, amyllum solubile, erythritolum, L-arabinitolum, galactitolum, inositolum, D-glucuronatum, DL-acidum lacticum, methanolum, butano-2,3-diolum, acidum quinicum, D-glucaratum et D-galactonatum. Assimilantur ethylaminum, L-lysinum, cadaverinum et glucosaminum (infirme). Non assimilantur kali nitratum, sodii nitritum, creatinum, creatininum, imidazolium et D-tryptophanum (infirme). Amyllum non formatur. Biotinum externum ad crescentiam necessarium est. Augmentum non fiunt in temperatura 37 °C. Non crescit in medio 10 µg ml⁻¹ cycloheximido addito. Holotypus: NRRL Y-27615^T (= CBS 9803^T), designat stirpem typicum. Isolata a ile *Chrysoperla* sp., Tucson, AZ, USA, depositata in Collectione Culturalum ARS (NRRL), Peoria, IL, USA.*

Description of *Metschnikowia chrysoperlae* Suh, Gibson & Blackwell sp. nov.

Metschnikowia chrysoperlae (chry.so.per'lae. L. gen. plur. masc. n. *chrysoperlae* of *Chrysoperla* sp., referring to the green lacewing species with which this yeast was associated).

Growth in YM broth: cells globose to oval, mostly globose or subglobose after 7 days at 25 °C, 4–10 × 4–10 µm; occurring singly or in pairs (Fig. 1a); pseudohyphae not present; chlamydospores are abundant (Fig. 1a, b). Growth on YM agar: colonies white, viscous and smooth on surface after 7 days at 25 °C. Dalmau plate culture on corn meal agar: pseudohyphae or true hyphae not present after 10 days at 25 °C; aerobic growth white and smooth. Formation of ascospores: asci arising from chlamydospores on diluted V8 juice agar (1:19) after 10–14 days at 15 °C; asci sphaeropedunculate, usually 20–40 µm in length, containing 1–2 needle-shaped ascospores (Fig. 1b, c). See Table 2 for a summary of physiological and other characteristics.

The type strain is NRRL Y-27615^T (= CBS 9803^T).

Latin diagnosis of *Candida picachoensis* Suh, Gibson et Blackwell sp. nov.

Fig. 1(d, e). *In medio liquido dextrosus et peptonum et extractum levidinis continente post 7 dies ad 25 °C cellulae vegetative globosae (5–10 × 5–10 µm), singulae vel binae; pseudohyphae non fiunt. Cultura in agar extramalti et faecis continente post 7 dies ad 25 °C, candida et teres. In agar farina Zeae maydis confecto post 10 dies ad 25 °C, pseudohyphae et hyphae verae non fiunt. Ascospores non fiunt. Glucosum fermentantur. Galactosum, maltosum, methyl α-D-glucosidum, sucrosum, trehalosum, melibiosum, lactosum, cellobiosum, melezitum, raffinum, inulinum, amyllum solubile et D-xylosum non fermentantur. Assimilantur glucosum, galactosum, D-glucosaminum (lente, infirme), D-ribosum (lente, infirme), sucrosum, maltosum, trehalosum, methyl α-D-glucosidum, salicinum (infirme), arbutinum (infirme), melezitum, glycerolum (infirme), ribitolum (infirme), xylitolum (infirme), D-glucitolum, D-mannitolum (lente), gluconolactonum (infirme), D-gluconatum (infirme), acidum succinicum (infirme) et acidum citricum (variabile). Non assimilantur L-sorbozum, D-xylosum, L-arabiosum, D-arabiosum, L-rhamnosum, cellobiosum, melibiosum, lactosum, raffinum, inulinum, amyllum solubile, erythritolum, L-arabinitolum, galactitolum, inositolum, 2-keto-D-gluconatum, D-glucuronatum, DL-acidum lacticum, methanolum, ethanolum, propane-1,2-diolum, butano-2,3-diolum, acidum quinicum, D-glucaratum et D-galactonatum. Assimilantur ethylaminum (lente, infirme), cadaverinum et glucosaminum (variabile). Non assimilantur kali nitratum, sodii nitritum, L-lysinum, creatinum, creatininum, imidazolium et D-tryptophanum. Amyllum non formatur. Biotinum externum ad crescentiam necessarium est. Augmentum non fiunt in temperatura 35 °C. Non crescit in medio 10 µg ml⁻¹ cycloheximido addito. Holotypus: NRRL Y-27607^T (= CBS 9804^T), designat stirpem*

Table 2. Physiological characteristics of the three novel species and *Metschnikowia pulcherrima*

The following characteristics are invariable in all species compared. Fermentation of maltose (–), methyl α -D-glucoside (–), sucrose (–), α , α -trehalose (–), melibiose (–), lactose (–), cellobiose (–), melezitose (–), raffinose (–), inulin (–), starch (–), D-xylose (–); assimilation of D-glucose (+), L-arabinose (–), D-arabinose (–), L-rhamnose (–), sucrose (+), melibiose (–), lactose (–), raffinose (–), inulin (–), soluble starch (–), erythritol (–), L-arabinol (–), galactitol (–), *myo*-inositol (–), D-glucuronate (–), DL-lactate (–), methanol (–), butane-2,3-diol (–), D-glucarate (–), D-galactonate (–), nitrate (–), nitrite (–), cadaverine (+), creatine (–), creatinine (–), imidazole (–); growth on media without *myo*-inositol (+), pantothenate (+), biotin (–), thiamin (+), biotin and thiamin (–), pyridoxine (+), without pyridoxine and thiamin (+), niacin (+), *p*-aminobenzoic acid (+); growth on 0.01 % cycloheximide (–), 0.1 % cycloheximide (–), 1 % acetic acid (–); growth at 30 °C (+); starch formation (–); urea hydrolysis (–). Abbreviations: +, positive reaction; –, negative reaction; d, delayed positive reaction; w, weak positive reaction; v, variable reaction.

Characteristic	<i>M. pulcherrima</i> *	<i>M. chrysoperlae</i>	<i>Candida picachoensis</i>	<i>Candida pimensis</i>
Fermentation of:				
D-Glucose	v	+	+	+
D-Galactose	w/–	d/–	–	–
Assimilation of:				
D-Galactose	+ /d	+ /d	+ /d	d
L-Sorbose	v	+ /d	–	–
D-Glucosamine	d/–	–	d/w	w
D-Ribose	v	w/–	d/w	d
D-Xylose	v	–	–	–
Maltose	v	+	+	+
Trehalose	+	+	+	+ /d
Methyl α -D-glucoside	v	+ /d	+ /d	+ /d
Cellobiose	v	+	–	–
Salicin	v	+ /d	w	w
Arbutin	+ /d	+	w	w
Melezitose	+	+	+ /d	+
Glycerol	+	+	w	+ /d
Ribitol	+ /d	+ /w	w	d/w
Xylitol	+	+	w	d/w
D-Glucitol	+	+	+ /d	+ /d
D-Mannitol	+	+	d	+
D-Glucono-1,5-lactone	+	+	w	+ /d
2-Keto-D-gluconate	v	+ /d	–	–
D-Gluconate	+ /d	+	w	+ /w
Succinate	+ /w	d	w	w
Citrate	v	d/w	w/–	w
Ethanol	v	+	–	–
Propane-1,2-diol	d/–	d	–	–
Quinic acid	v	–	–	–
Ethylamine	v	+	d/w	+ /w
L-Lysine	v	+	–	–
D-Glucosamine	–	w	w/–	–
D-Tryptophan	v	w	–	–
Growth:				
At 35 °C	v	w	–	–
On 50 % D-glucose	+	+	+	–
On 60 % D-glucose	v	+	–	–
On 10 % NaCl	+	+	–	–
On 16 % NaCl	d/–	–	–	–

*Data from Barnett *et al.* (2000).

Fig. 1. (a–c) *Metschnikowia chrysoperlae*. Budding yeast cells and chlamydospores (arrows; a) of NRRL Y-27613 after 7 days at 25 °C on YM agar; ascospores of NRRL Y-27615^T (b) and NRRL Y-27616 (c), after 2 weeks at 15 °C on diluted V8 juice agar (1:19). (d–e) *Candida picachoensis*. Budding yeast cells of NRRL Y-27607^T (d) and Y-27611 (e), after 7 days at 25 °C on YM agar. (f–g) *Candida pimensis*. Budding yeast cells of NRRL Y-27619^T (f) and Y-27620 (g), after 7 days at 25 °C on YM agar. The magnification for (d)–(g) is the same. Bars, 5 µm.

typicum. Isolata a ile *Chrysoperla* sp., Picacho Peak, AZ, USA, depositata in Collectione Culturarum ARS (NRRL), Peoria, IL, USA.

Description of *Candida picachoensis* Suh, Gibson & Blackwell sp. nov.

Candida picachoensis (pi.cach.o.en'sis. L. gen. plur. fem. adj. *picachoensis* referring to Picacho Peak, AZ, USA, the collection locality of the type strain).

Growth in YM broth: cells globose with thick cell wall after 7 days at 25 °C, 5–10 × 5–10 µm; occurring mostly singly or in pairs (Fig. 1d, e); pseudohyphae not present. Growth on YM agar: colonies white, viscous and smooth on surface after 7 days at 25 °C. Dalmau plate culture on corn meal agar: pseudohyphae or true hyphae not present after 10 days at 25 °C; aerobic growth white to cream in colour, and somewhat transparent. No ascospores present on cornmeal agar or diluted V8 juice agar media after 2 months. See Table 2 for a summary of physiological and other characteristics.

The type strain is NRRL Y-27607^T (= CBS 9804^T).

Latin diagnosis of *Candida pimensis* Suh, Gibson & Blackwell sp. nov.

Fig. 1(f, g). In medio liquido dextrosus et peptonum et extractum levidinis continente post 7 dies ad 25 °C cellulae vegetative globosae aut ellipsoidae (3–7 × 3–9 µm). Plerumque globosae et subglobosae, singulae vel binae; pseudohyphae non fiunt. Cultura in agar extramalti et faecis continente post 7 dies ad 25 °C, candida aut crenea, et teres. In agar farina Zeae maydis confecto post 10 dies ad 25 °C, pseudohyphae et hyphae verae non fiunt. Ascospores non fiunt. Glucosum fermentantur. Galactosum, maltosum, methyl α-D-glucosidum, sucrosus, trehalosum, melibiosum, lactosum, cellobiosum, melezitum, raffinum, inulinum, amyllum solubile et D-xylosum non fermentantur. Assimilantur glucosum, galactosum (lente), D-glucosaminum (infirmum), D-ribosum (lente), sucrosus, maltosum, trehalosum, methyl α-D-glucosidum, salicinum (infirmum), arbutinum (infirmum), melezitum, glycerolum, ribitolum (lente, infirmum), xylitolum (lente, infirmum), D-glucitolum, D-mannitolum, gluconolactonum, D-gluconatum (infirmum), acidum succinicum (infirmum) et acidum citricum (variabile). Non assimilantur L-sorbosum, D-xylosum, L-arabiosum, D-arabiosum, L-rhamnosum, cellobiosum, melibiosum, lactosum, raffinum, inulinum, amyllum solubile, erythritolum, L-arabinitolum, galactitolum, inositolum, 2-keto-D-gluconatum, D-glucuronatum, DL-acidum lacticum, methanolum, ethanolum, propane-1,2-diolum, butano-2,3-diolum, acidum quinicum, D-glucaratum et D-galactonatum. Assimilantur ethylaminum et cadaverinum. Non assimilantur kali nitratum, sodii nitritum, L-lysinum, creatinum, creatininum, glucosaminum, imidazolium et D-tryptophanum. Amyllum non formatur. Biotinum externum ad crescentiam necessarium est. Augmentum non fiunt in temperatura 35 °C. Non crescit in medio 10 µg ml⁻¹ cycloheximido addito. Holotypus: NRRL Y-27619^T (= CBS 9805^T), designat stirpem typicum. Isolata a ile *Chrysoperla carnea*, Tucson, AZ, USA, depositata in Collectione Culturarum ARS (NRRL), Peoria, IL, USA.

Description of *Candida pimensis* Suh, Gibson & Blackwell sp. nov.

Candida pimensis (pi.men'sis. N.L. fem. adj. *pimensis* referring to Pima County, AZ, USA, the collection locality of the type strain).

Growth in YM broth: cells globose to ellipsoidal after 7 days at 25 °C, 3–7 × 3–9 µm, mostly globose and subglobose; occurring singly or in pairs (Fig. 1f, g). Pseudohyphae not present. Growth on YM agar: colonies white to cream-coloured, smooth on surface to somewhat mucoid around edge after 7 days at 25 °C. Dalmau plate culture on corn meal agar: pseudohyphae not present after 10 days at 25 °C; aerobic growth white to cream in colour. No ascospores present on cornmeal agar or diluted V8 juice agar media after 2 months. See Table 2 for a summary of physiological and other characteristics.

The type strain is NRRL Y-27619^T (= CBS 9805^T).

Characterization of yeasts from lacewings and their phylogeny

Chrysopidae, the green lacewings, have been used in biological control because the larvae are generalist predators of many pest insects. Hagen & Tassan (1966, 1972) and Hagen *et al.* (1970) first reported that lacewing adults possess yeast symbionts and proposed that the yeasts supplied essential amino acids to their hosts. The yeasts, identified as *Torulopsis* sp., had no detailed description of taxonomic traits, and we do not know if these yeasts are related to our isolates because the cultures are not available. *Torulopsis* has had a problematic taxonomic history, and the genus is no longer valid in current yeast classification (Kurtzman & Fell, 1998). More recently, Woolfolk & Inglis (2003) studied micro-organisms associated with *Chrysoperla rufilabris* and reported large numbers of *M. pulcherrima*, as well as some basidiomycetous yeasts, in the gut of field-collected adults in MS, USA. *M. pulcherrima* is best known from flowers, fruits and nectar, but some isolates have come from insects, including *Drosophila* spp. and bees, a known habitat for other *Metschnikowia* species (Barnett *et al.*, 2000; Miller & Phaff, 1998). Interestingly, the three species described here are closely related to *M. pulcherrima*. A consensus of 14 parsimonious trees generated from D1/D2 sequences showed that the three novel species, *M. chrysoperlae*, *Candida picachoensis* and *Candida pimensis*, formed a statistically well-supported clade with *M. pulcherrima*, *Metschnikowia fructicola* and several undescribed *Metschnikowia* species (Fig. 2). The tree from the combined dataset

of SSU and LSU rRNA gene sequences supported the same result (data not shown). *Metschnikowia* sp. NRRL Y-6148 (GenBank accession no. AF017401) was the closest yeast to *M. chrysoperlae* based on its D1/D2 sequence, which was 3 bp different from that of *M. chrysoperlae*. The closest previously described species to the three novel species was *M. pulcherrima*, which had 6 bp differences from *M. chrysoperlae* and 48 and 50 bp differences from *Candida picachoensis* and *Candida pimensis*, respectively, in D1/D2 sequences. *M. fructicola*, which has biocontrol activity against *Botrytis* rot of stored grapes (Kurtzman & Droby, 2001), was also close to *M. chrysoperlae*, but the two were distinguished by more than 15 bp of substitutions in the D1/D2 sequences (Fig. 2).

M. chrysoperlae may be conspecific with some yeasts from *Chrysoperla rufilabris* isolated by Woolfolk & Inglis (2003), e.g. S1740 that they identified as *M. pulcherrima*. There was only 1 bp difference in the ITS and 5-8S rRNA gene sequences between S1740 (GenBank accession no. AY301026) and *M. chrysoperlae* (GenBank accession nos AY494782 and AY494783, from this study), and they share the ability to produce needle-shaped ascospores (Fig. 1b, c). Although the data provided for S1740 are too limited to compare fully, S1740 has similar physiological characters except for variation in the ability to assimilate several carbon sources such as cellobiose (Table 2; Woolfolk & Inglis, 2003).

The variable D1/D2 sequence of the LSU rRNA gene (about 600 bp) was determined for all currently recognized

Fig. 2. Strict consensus of 14 most-parsimonious trees obtained from D1/D2 loop sequences of the LSU rRNA gene sequence data. Reference sequences of *Metschnikowia* species were selected to show the phylogeny of the three novel species based on comparison of SSU and LSU rRNA gene sequences. *Saccharomyces cerevisiae* was used as an outgroup taxon. Tree length = 928; consistency index = 0.5075; homoplasy index = 0.4925; retention index = 0.6543; rescaled consistency index = 0.3321. Upper or single numbers indicate support above 50% in 1000 bootstrap replicates with parsimony analysis. Numbers below branches represent probability of nodes in Bayesian analysis.

ascomycete yeasts (e.g. Kurtzman & Robnett, 1995, 1997, 1998), and comparisons of the D1/D2 sequences are a useful tool for rapid identification of yeasts and detection of novel species (e.g. Kurtzman, 2000). Many yeast species have been defined by a phenetic species concept that recognizes strains with greater than 1% sequence divergence as separate species; strains with less divergence are usually considered to be conspecific. Our species concept differs from this because it is based on a phylogenetic concept in which unique monophyletic lineages identified through phylogenetic studies distinguish the novel species described here (Hibbett & Donoghue, 1996). The outcome, however, is similar because species recognition (Taylor *et al.*, 2000; Dettman *et al.*, 2003) ultimately rests on differences in the D1/D2 loop. For example, the phylogenetically defined species *M. chrysoperlae* has a 6 bp substitution difference from its sister taxon, *M. pulcherrima*, in the D1/D2 region, sufficient molecular variation to distinguish the two taxa at species level by the phenetic concept as well. Physiological characters of the two taxa are similar to each other except for a few carbon assimilation tests (Table 2).

The two novel *Candida* species, *Candida picachoensis* and *Candida pimensis*, are closely related sister taxa of *M. pulcherrima* and *M. chrysoperlae*; the two species, however, are clearly distinguished from *M. chrysoperlae*, *M. pulcherrima* and related taxa based on sequence comparison and other taxonomic characters (Fig. 2 and Table 2). No ascospores were observed in any cultures of these isolates during the incubation at 15 °C for up to 2 months on diluted V8 juice agar and cornmeal agar after crosses were made. Morphological and physiological characters are similar for both *Candida* species, but their D1/D2 and ITS sequences clearly separate them (Table 2).

Host relations

It is not possible to determine a clear pattern of host relations, because our study has been applied to only a limited number of *Chrysoperla* species, and some of the hosts were not identified to the species level (Table 1). Based on our data, however, *Chrysoperla comanche* and *Chrysoperla carnea* appear to have different yeast gut mycota (Table 1). When the lacewing species were known, female adults of *Chrysoperla comanche* were hosts for *M. chrysoperlae*, while the males possessed *Candida picachoensis*. The *Candida pimensis* isolates came from known *Chrysoperla carnea* only. NRRL Y-27619^T and NRRL Y-27620 were isolated from the female and male of the species, respectively. The two strains have identical D1/D2 sequences but were distinctive with 11 bp different out of 339 bp of the ITS and 5-8S rRNA gene sequences compared. Interestingly, *M. chrysoperlae* was also isolated from eggs of females. This finding might suggest that the yeasts have been transmitted vertically to subsequent generations through eggs, as observed in some obligate insect–endosymbiont associations (Jurzitza, 1979; Fukatsu & Hosokawa, 2002). Unlike Woolfolk & Inglis (2003), however, we were unable to isolate yeasts from larvae. Without such information we cannot draw conclusions as

to the nature of the interactions between insects and fungi. The source of the lacewing yeasts is not clear; although the yeasts could be obtained repeatedly from plants in the environment, their repeated isolation and apparent specificity to lacewings suggests the possibility of a host–symbiont relationship in nature. More yeast strains will need to be isolated from known lacewing species before we can understand the interactions between the organisms.

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