KU LEUVEN

FACULTY OF ENGINEERING TECHNOLOGY

CAMPUS DE NAYER SINT-KATELIJNE-WAVER

Analysis of yeast diversity in floral microhabitats from *Metrosideros polymorpha* Gaud.

Joon KLAPS

Supervisor:
Prof. Dr. Ir. Bart Lievens

Co-supervisor:
Dr. Sergio Álvarez-Pérez

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ABSTRACT

Flowers offer a wide variety of substrates suitable for microbial growth. However, the microbiological study of flowers has only recently begun to be systematically addressed, and our knowledge about floral-inhabiting microorganisms is yet very limited. The aim of this study was to assess the diversity of culturable yeasts associated with three floral microhabitats –namely, nectar, stamina and styles– of *Metrosideros polymorpha* Gaud. (Myrtaceae), a tree endemic to Hawaii. Yeast isolates recovered from floral samples were identified by sequencing different regions of the ribosomal RNA operon and classified into operational taxonomic units (OTUs). Furthermore, rarefaction-based estimators were used to assess the diversity and composition of the flower-dwelling yeast communities. Our results showed that *M. polymorpha* flowers are inhabited by species-poor yeast communities (<42 predicted OTUs, of which we recovered *c.*50%) that are dominated by ascomycetous taxa (75% of observed OTUs). Furthermore, the yeast communities associated to specific floral structures showed certain differentiation in their OTU richness and phylogenetic diversity, both of which were higher for styles and stamina than for nectar. Finally, our mycological survey led to the discovery of two novel yeast lineages that diverged phylogenetically and phenotypically from previously described taxa, and might represent new species within the anamorphic genus *Candida*. Future work should clarify the taxonomic status and ecology of these new yeast lineages.

SAMENVATTING

Bloemen vormen een geschikte habitat voor tal van micro-organismen. Desalniettemin is nog maar weinig geweten over de microbiële gemeenschappen die huisvesten in bloemen. Het doel van deze studie bestond erin om de diversiteit aan kweekbare gisten in drie microhabitats (nectar, meeldraad en stijl) in een bloem te analyseren. Als studieobject werd gekozen voor bloemen van de Hawaiiaanse endemische boom *Metrosideros polymorpha* Gaud (Myrtaceae). Bekomen gistisolaten werden geïdentificeerd door het sequeneren van ribosomale RNA genen, waarna de gisten op basis van sequentiehomologie werden opgedeeld in *operational taxonomic units* (OTUs). Vervolgens werden *rarefaction* gebaseerde schatters gebruikt om de diversiteit en compositie van de gistgemeenschap vast te stellen. Onze resultaten geven aan dat gistgemeenschappen van de bloemen van *M. polymorpha* voornamelijk bestaan uit ascomyceten (75% van de geobserveerde OTUs), met een relatief beperkt aantal soorten (ongeveer 50% van de voorspelde 42 OTUs werden gedetecteerd). Verder geven de data ook aan dat de aanwezige gistgemeenschappen van nectar, meeldraad en stijl significant verschillen. Hierbij vertonen meeldraad en stijl een grotere fylogenetische diversiteit. Tot slot leidde de studie tot de ontdekking van twee nieuwe potentiële gistsoorten die zowel fenotypisch als fylogenetisch verschillen van reeds beschreven gisttaxa. Hoogstwaarschijnlijk behoren deze gisten tot het geslacht *Candida*. Verder onderzoek zal hun taxonomische indeling en ecologie moeten verduidelijken.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AN	Artificial Nectar
approx.	Approximately
BLAST	Basic Local Alignment Search Tool
BSC	Biological Species Concept
c.	*circa* “approximately”
CAS	Chemical Abstracts Service
CFU	Colony forming unit
DNA	DeoxyriboNucleic Acid
e. g.	*exempli gratia* "for example"
etc.	*et cetera* “and other similar things" or "and so forth"
g	Standard gravity; unit of measurement of the force resulting from acceleration
GCPSC	Genealogical Concordance Phylogenetic Species Concept
GSC	Genotypic Species Concept
GTR	General Time Reversible
i. e.	*id est* "that is"
ICE	Incidence-based Coverage Estimator
ITS	Internal Transcribed Spacer
LB	Lysogeny Broth
LBG	Latitudinal Biodiversity Gradient
LSU	Large SubUnit
Ma	*Megaannum* (10^6 years)
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MP	Maximum Parsimony
MSC	Morphological Species Concept
NGS	Next-Generation Sequencing
N.B.	*Nota Bene* “note well“
NJ	Neighbour-Joining
NNI	Nearest Neighbor Interchanges
OD	Optical Density
OTU	Operational Taxonomic Units
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD	Phylogenetic Diversity
PDA	Potato Dextrose Agar
PR	Pathogenesis-Related protein
PSC	Phylogenetic Species Concept
RAPD	Random Amplification of Polymorphic DNA

rpm	Revolutions per minute
rRNA	Ribosomal RiboNucleic Acid
SEM	Scanning Electron Microscope
SPR	Subtree-Pruning-Regrafting
SSU	Small SubUnit
var.	Variety
YM	Yeast Malt

AIMS OF THIS STUDY

The aim of this study was to assess the yeast diversity associated with three floral microhabitats –namely, nectar, stamina and styles– of *Metrosideros polymorpha* Gaud. (Myrtaceae), a tree endemic to Hawaii. Yeast isolates recovered from floral samples were identified by molecular methods and classified into operational taxonomic units (OTUs). Furthermore, rarefaction-based estimators were applied to presence-absence data to assess the diversity and composition of the flower-dwelling yeast communities. Finally, isolates that could potentially represent novel yeast taxa were genetically and phenotypically characterized following the current standards in fungal taxonomy.

1 LITERATURE

1.1 Introduction

Nearly 90% of all plant species, including 75% of domesticated crops, benefit from animal-mediated pollination (Roy *et al.,* 2017). As the main reward for their services, flowers offer nectar to the visiting pollinators (Canto *et al.,* 2008; Herrera *et al.,* 2010, 2013). Given the nutrient-rich nature of nectar, bacteria, yeasts, and other fungi are commonly found in the nectar of a wide diversity of plant species worldwide (Stockwell, 2005; Álvarez-Pérez and Herrera, 2013; Herrera, 2017; de Vega *et al.*, 2017; Vannette and Fukami, 2018). Furthermore, as a result of their metabolic activity, nectar microbes can alter the composition of their habitat (Herrera *et al.,* 2008; Wiens *et al.,* 2009; Peay *et al.*, 2012; Sobhy *et al.,* 2018; Vannette and Fukami, 2018), which, in turn, may affect pollinators' foraging behavior and eventually have an indirect impact on the reproductive success of the plant (de Vega and Herrera, 2012, 2013).

The downstream effect of microbes in nectar properties and floral biology might have important ecological consequences on plant-animal interactions (Herrera *et al.,* 2009, 2013; Peay *et al.,* 2011), mediate agriculture gains (Bailes *et al.*, 2015) and augment pollinator's health (Davis, 2001; Richardson *et al.*, 2015; Conroy *et al.,* 2016). The studies on this subject could not come at a more pressing time, as both world population and food demand are constantly increasing (Godfray, *et al.*, 2010).

1.2 The Flower

Flowers are essential structures in the reproductive cycle of angiosperms (*i.e.* flowering plants) (Schooley, 1997). Accordingly, plants spend plenty of resources to make their flowers attractive to pollinators by, for example, displaying catchy colours, secreting specific odours and forming characteristic shapes (*e.g.*, that mimic insect females). However, other flowers are wind- or water-pollinated and, thus, do not require to attract pollinators (De Craene, 2010).

Flowers are formed by modified leaves arranged in several whorls (De Craene, 2010). A typical bisexual flower contains four main organ types, namely sepals, petals, stamens, and carpels (Figure 1.1A; Becker *et al.,* 2011). However, the diversity of shapes and displays of floral structures is remarkable. Such diversity includes, for example, variation in the sexual identity of flowers (bisexual, male monosexual, female monosexual, *etc.*), abortion of organs, variation in colours, and variation in number of whorls of the same organ (Becker *et al.,* 2011).

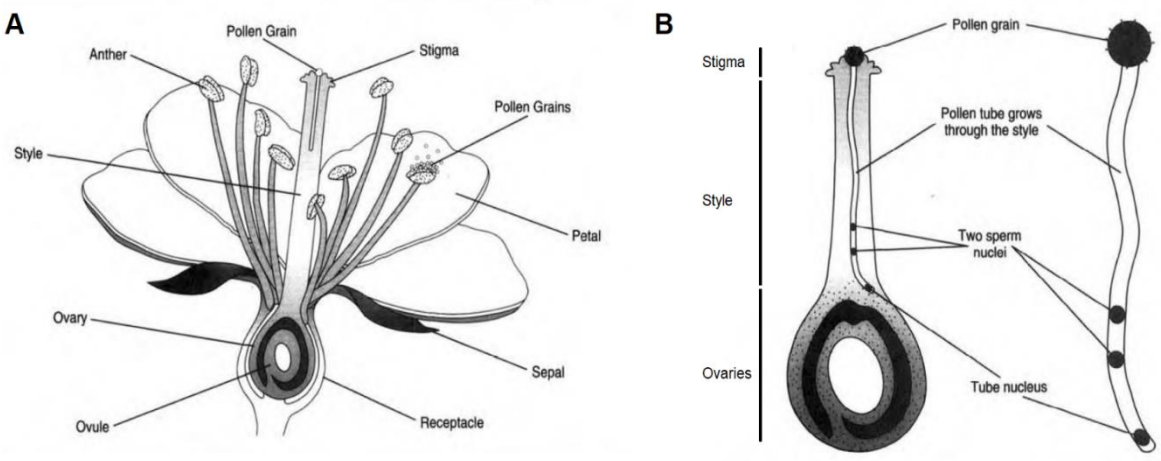

Figure 1.1: A) General overview of the morphology of a bisexual flower. B) Germinated pollen grain (*right*) and its development inside a pistil after pollination (*left*). Source: modified from Schooley (1997)

The innermost whorl of a bisexual flower (Figure 1.1A), generally constitutes the female part and receives the name of pistil. It possesses a distal stigma generally continued by a long, slender style and an ovary containing ovules at the base. The ovules, once successfully fertilized by pollen, will become seeds (Schooley, 1997; De Craene, 2010). The masculine part of the bisexual flower typically surrounds the pistil and contains the *stamens* or *stamina* (Schooley, 1997). Each individual stamen is composed of a filament and an anther that produces and store pollen grains until released for

dispersion. The next whorl contains the petals, whose ensemble forms the corolla and, finally, the outermost whorl contains the sepals that build the calyx (Schooley, 1997).

1.3 Pollinators and nectar robbers as vectors of floral microbes

Flowers are a valuable resource to a variety of animals for several reasons. Firstly, flowers provide nutrients to pollinating and non-pollinating visitors, mainly through the secretion of sugar-rich nectar but also by producing pollen, oils, and resins (Simpson and Neff, 1983). In addition, flowers also offer diverse non-nutritional rewards to floral visitors. For example, floral trichomes can be used in nest construction by some bees, and the use of flowers as mating and/or sleeping places is also quite common (Simpson and Neff, 1983). Finally, the flowers of some plant species can collect heat, and/or even produce it actively, and therefore act as "heaters" for floral visitors (Simpson and Neff, 1983, Rands and Whitney, 2008). Floral warming is particularly common in the Arctic and at high elevations, and some features thought to increase floral temperature include flower shape, size and angle to the sun. Moreover, floral warming can also increase nectar's temperature, thus making it easier to drink by nectarivorous animals due to its reduced viscosity (Nicolson *et al.,* 2013).

When animals are feeding on nectar, pollen can get attached to their body surfaces and subsequently be spread to new flowers. Pollinators and non-pollinating florivores are not sterile but carry microorganisms, including yeasts, that may inoculate into nectar and other floral parts (de Vega and Herrera, 2012) (Figure 1.2). Remarkably, as different insects carry a different microbiota, flowers pollinated by specific groups of pollinators are bound to exhibit non-random or even unique microbial communities (Brysch-Herzberg, 2004; Lachance *et al.*, 2006; Canto *et al.*, 2008; Herrera *et al.,* 2009, 2010; de Vega *et al.,* 2012). For example, some studies have demonstrated that plants pollinated by beetles tend to harbour a yeast microbial community mainly composed by the genera *Wicherhamiella, Kodamaea* and large-spored members of the *Metschnikowia* clade (Lachance *et al.*, 2006, 2008, 2011; de Vega *et al.*, 2012, 2017). In contrast, plants pollinated by bees or butterflies harbour mainly small-spored members of *Metschnikowia* and *Starmerella* (Brysch-Herzberg, 2004; Pozo *et al.,* 2011).

Yeast species that are present in great density in the flower have increased likelihood of being dispersed to a new flower when pollinators go in search of nectar. The dispersal of microbes from flower to flower is a dynamic process that keeps ongoing during the flower lifetime (Vannette and Fukami, 2017). Moreover, several studies have demonstrated that the same yeast species can be found in the same plant community over consecutive years (de Vega *et al.*, 2012a; Herrera *et al.*, 2014; Mittelbach *et al.,* 2015; Toju *et al.,* 2018).

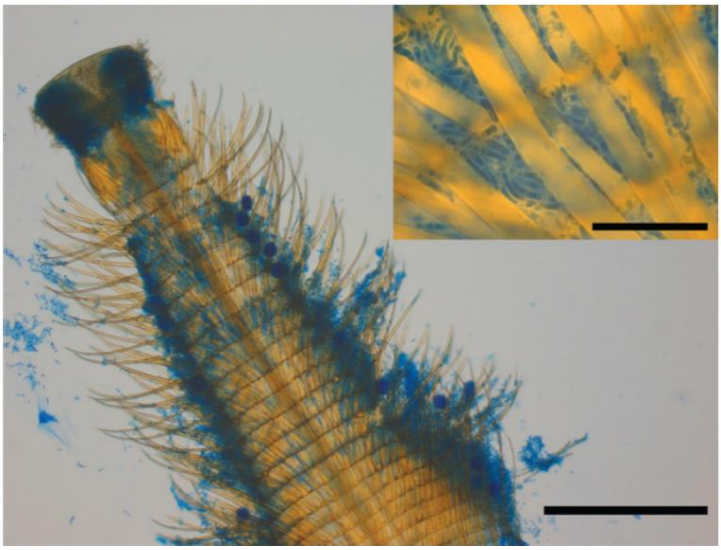

Figure 1.2: Micrographs of the terminal portion of the glossa of *Bombus pratorum* stained with lacto-cotton blue without phenol. Aside from a few pollen grains, the rest of the blue areas in the main image are dense groups of yeast cells (bar= 200 µm). The high density and close association of yeast cells can be better observed in the inset (bar= 20 µm). Source: Canto *et al.* (2008).

Comparison of the microbiota associated with floral structures and animal visitors can help to identify which taxa are dispersed by these latter and not from other sources such as wind, raindrops or contamination by endophytes. For example, by using next-generation sequencing (NGS) technologies, Aizenberg-Gershtein *et al.* (2013) compared the bacterial communities of honeybee surfaces and the nectar of *Citrus paradisi* (grapefruit) and *Amygdalus communis* (almond) plants. The authors demonstrated that the bacterial communities of the nectar from covered and uncovered flowers (*i.e.* non-exposed and exposed to pollinators, respectively) differed significantly. At the same time, the bacterial communities of honeybees differed from those of the nectar from covered flowers but were similar to those present in the nectar of the uncovered flowers (Aizenberg-Gershtein *et al.*, 2013). This suggests that there is an overlap between the surface community of the pollinator and exposed floral parts, but

if pollination is hindered, the microbial communities of the flower and the pollinators tend to diverge, which eventually leads to more distinct communities (Aizenberg-Gershtein *et al.,* 2013).

Finally, some floral visitors commonly known as 'nectar robbers' can circumvent the floral opening and remove nectar (*e.g.* through holes, slits, tears or even bitten punctures) without contacting the anthers and/or stigma and, therefore, do not provide any pollination service to the plant (Figure 1.3) (Inouye, 1980). Among insects, bees, wasps, and ants are the most common nectar robbers (Irwin *et al.*, 2010). A recent study of Zemenick *et al.* (2018) investigated the influence of legitimate nectar foragers and nectar robbers on nectar microbial communities. They demonstrated that different types of flower visitors can differentially influence nectar microbial community composition both within and among flowers, and hypothesized that by puncturing a hole in the corolla, the habitability of nectar is modified (Zemenick *et al.,* 2018). In particular, the plant can react by producing defensive substances (*e.g.* secondary metabolites), and nectar evaporation can be increased and result in a higher osmotic pressure, both of which can hinder microbial growth in nectar (Lachance, 2006; Irwin *et al.,* 2010; Vannette and Fukami, 2016; Section 1.5).

Figure 1.3: A *Tecoma stans* ('yellow elder', Bignoniaceae) flower displaying slits made by *Xylocopa cubaecola* (Apidae) (black arrow) and holes made by the hummingbird *Chlorostilbon ricordii* (blue arrow) (Source: modified from Rojas-Nossa *et al.*, 2016)

1.4 Flowers as microbial habitats

Flowers, and in particular their reproductive parts, are nutrient-rich environments that provide beneficial conditions for microbial growth. The exudates produced by floral parts contain a great variety of nutrients (*e.g.* carbohydrates, lipids, amino acids) on which microbes can thrive (Stockwell, 2005). The microbial communities that develop in floral parts may have important impacts on the reproductive success of plants (de Vega and Herrera, 2012, 2013). For instance, nectar microbial communities can influence nectar chemical composition and nutritional value, which, in turn, may affect pollinators' foraging behaviour and, eventually, have an indirect impact on plant reproduction (de Vega and Herrera, 2012; 2013). Additionally, Herrera and Pozo (2010) demonstrated that the metabolic activity of yeasts results in a temperature increase of the flower, which enhances pollinator visitation (Herrera and Pozo, 2010). However, not all influences of yeasts on plant reproduction are indirect (*i.e.* by affecting pollinator behaviour) as, for example, Eisikowitch *et al.* (1990) found that some yeasts are able to inhibit pollen germination.

Although the corolla and the calyx are also important microbial habitats, the focus of this section is on pistils, stamens, and nectar, which are the three floral structures that were studied in the experiments described in section 2.

1.4.1 Pistil

The pistil is the female reproductive part of the flower. The stigma serves as a pollen receptor surface, and the style is the structure where pollen tubes develop, as shown in Figure 1.1B. Once pollen tubes reach the ovaries, the flower is fertilized.

Morphologically, styles can be open, closed or semisolid (Sanchez *et al.,* 2004). Closed styles have transmitting tissue where pollen can grow. The cells of the transmitting tissue secrete a mixture of polysaccharides, free amino acids, proteins and other compounds which aid in pollen tube nutrition, recognition and guidance (Cheung, 1996). These nutrients are also valuable resources for bacterial and fungal communities, including commensal, symbiotic and pathogenic representatives (Aleklett *et al.*, 2014). Pathogenic fungi can hijack that natural wound to get access to floral tissues (Ngugi and Scherm, 2006). However, it is currently unknown if non-pathogenic

microbes use this entrance gate and benefit from the resources that are secreted by the transmitting tissue (Aleklett *et al.,* 2014).

The structure of the microbial community which develops on stigmas depends on several factors. For example, the amount and composition of stigma exudates can affect microbial colonization and, in particular, wet stigmas have the potential to harbour more microorganisms than dry stigmas (Ngugi and Scherm, 2006). Furthermore, external conditions such as air temperature and humidity may have an impact on stigmatic microbial communities, as demonstrated for apple flowers by Pusey *et al.* (2009).

Ovaries carry and protect the ovules and, generally, the ovary walls develop into the flesh of fruits (De Craene, 2010). This makes ovaries a suitable environment for microbial colonization because of the resources and shelter that they can provide.

1.4.2 Stamen

Only a few studies have analyzed directly the microbial communities of stamens, even when pollen sacs secrete nutrient-rich exudates and provide protection from external stresses (Aleklett *et al.*, 2014). Pollen grains exude sugars and lipids, creating a wet and sticky surface that aids in pollen adherence (Heslop-Harrison and Heslop-Harrison, 1985). Therefore, not only the pollen sacs but also the pollen grains themselves are suitable habitats for microbial colonizers. Furthermore, it is known that certain filamentous fungi are able to hijack anthers for dispersion of their own spores (Jennersten, 1988; Alexander, 1990). In contrast, the lack of exudates and epidermal cavities for colonization are the main reasons why the filaments of stamens generally do not contain high cell densities (Aleklett *et al.,* 2014).

1.4.3 Nectar

Nectar is a sugar-rich liquid substance produced, and sometimes secreted, by parenchyma cells called nectaries (Bernardello, 2007). In general, the secretion of nectar begins as soon as the flower opens (*i.e.* anthesis onset) and keeps ongoing while the flower remains receptive to pollination (Carter and Thornburg, 2004). There are different floral parts that produce nectar, including the receptacles, hypanthia, tepals, stamens, and pistils (Schmid, 1988). According to some authors, the conventional view

that nectar originates from phloem sap but may be modified by the nectary parenchyma is perhaps oversimplified (Pacini and Nicolson, 2007). The sugar component of nectar originates from the photosynthesis carried out by the nectary itself or, more commonly, from the translocation of photosynthesis products from other parts of the plant (Pacini *et al.,* 2003).

Floral nectar is usually a viscous combination of different solutes, with a predominant presence of sucrose, glucose, and fructose (Roshchina, 1993; Nicolson and Thornburg, 2007). Apart from these sugars, nectar can contain other carbohydrates (mainly lipids and organic acids), amino acids (although normally in low concentration; Herrera, 2017), and secondary metabolites, such as phenolics, alkaloids, and terpenoids (Baker and Baker, 1973, 1982; Nicolson and Thornburg, 2007).

Depending on its origin, nectar can be floral or extrafloral (Pacini and Nicolson, 2007; Nepi *et al.,* 2009; Roy *et al.,* 2017). Although these types contain some similarities in their chemical composition, they differ considerably in their properties, source of origin and mode of presentation (Table 1.1) (Davis *et al.,* 1988; Pacini *et al., 2003*; Pacini and Nicolson, 2007). Moreover, despite both functioning as a reward for animals that provide services which plants lack, floral nectar is generally a reward for pollinators (mostly insects, but also some birds, mammals, and lizards), whereas extrafloral nectar serves as retribution for plant defenders (mainly ants).

Table 1.1: Systematic distribution and chemical, functional and ecological characteristics of floral and extrafloral nectar (adapted from Nepi *et al.,* 2009).

Character	**Extrafloral nectar**	**Floral nectar**
Distribution		
Pteridophytes	Some species	Not applicable*
Gymnosperms	Gnetophytes	Not applicable*
Angiosperms	Widespread	Widespread
Main functions	Reward animals (ants) that protect from predators Defence against microbes	Rewards animals involved in pollination Defence against microbes
Reabsorption	Not demonstrated	Facultative and present especially when nectar volume is high
Main dissolved substances	Carbohydrates (glucose, fructose, and saccharose), amino acids, proteins	Carbohydrates (glucose, fructose, and saccharose), amino acids, proteins
Presentation	From a few days to some months. Herbivory increases nectar production	From a few hours to several days Natural or artificial removal may increase or reduce the secretion Pollination may decrease or stop the secretion
Predation	Predated by adults and larvae of insects like aphids, and also mites	Primary and secondary predation by insects and rarely vertebrates in many species

**N.B.*: Pteridophytes and gymnosperms do not produce flowers.

Floral nectaries can be extremely diverse with respect to their localization, their structure, and even their secretion mechanisms (Elias, 1983; Fahn, 1988; Pate *et al.*, 1985). Besides, the amount of nectar secreted by an individual flower depends on three factors: (I) ambient humidity and temperature (Corbet *et al.* 1979), (II) selective

reabsorption of solutes and/or water (Búrquez and Corbet, 1991; Nicolson, 1995), and possibly, (III) changes in the concentration at which nectar is secreted (Corbet, 2003).

Previous studies have shown that microbes are prevalent in the nectar of diverse plant species around the world and can reach high densities (typically $>10^5$ cells/mm^3) (Brysch-Herzberg, 2004; Herrera *et al.,* 2008, 2009; de Vega *et al.*, 2009; Pozo *et al.,* 2011). As a consequence of their metabolic activity, microbes can have a profound impact on nectar chemistry (*e.g.* by reducing its total sugar and amino acid content and/or releasing ethanol as fermentation by-product) (Herrera *et al.,* 2008; Wiens *et al.,* 2009; Peay *et al.*, 2012; Vannette and Fukami, 2018). Therefore, nectar characteristics depend not only on external conditions but are also influenced by biotic factors.

Even when the composition, origin and secretion patterns of floral nectar have been thoroughly studied over hundreds of years, there are still some relevant questions that remain unanswered (Roy *et al.,* 2017). Some general aspects of nectar biology that clearly deserve further research are nectary development, regulation, and modes of nectar production, and influence on pollinator behaviour and health (Roy *et al.,* 2017).

1.5 Filters to microbial growth in floral nectar

Floral parts are exceptionally rich habitats with substances that can be used as nutrient sources not only by pollinators and other floral visitors, but also by diverse microorganisms. However, only a small array of microbial species can withstand the prevailing conditions in floral parts. In particular, there are several biotic and abiotic factors that contribute to the filtering effect of nectar on microbial colonization, and which may not always be present in other parts of the plant (Junker and Tholl, 2013). Other floral structures could also hinder microbial growth in multiple ways, but this topic has not been extensively studied yet (Aleklett *et al.,* 2014). Additionally, previous research has demonstrated that nectar can efficiently filter the yeast communities present on the glossae of bumblebees (Herrera *et al.,* 2010).

1.5.1 Abiotic conditions

Flowers are ephemeral structures with generally much shorter lifespans than that of leaves or other plant organs (Schooley, 1997). Some floral parts show increased humidity and moisture (Huang *et al.,* 2012) or low pH (Harrison *et al.,* 2013). In particular, the increased osmotic pressure found in nectar, which results in water activity levels ranging from 0.99 to below 0.90 (Figure 1.4), exerts strong stress on microbial growth (Ferro Fontán and Chirife, 1981; Nicolson, 1994, 2002; Lievens *et al.,* 2015). Moreover, the specific interaction of temperature and humidity on floral surfaces can act as additional selective pressure on microbial inhabitants (Lievens *et al.*, 2015).

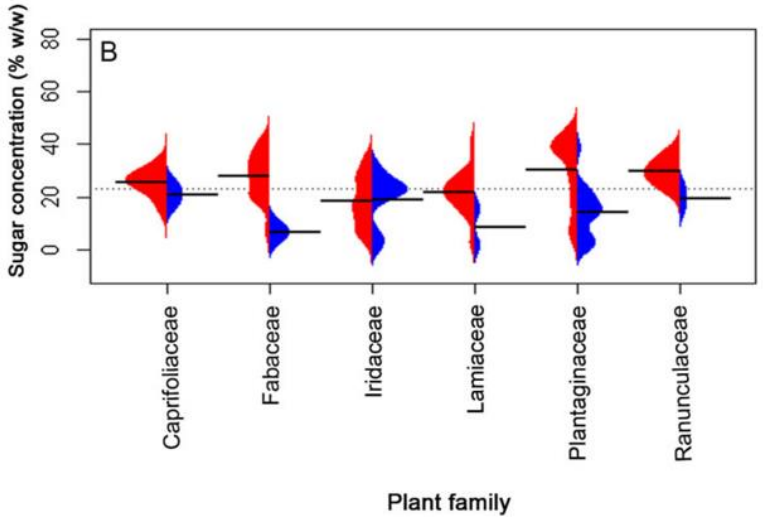

Figure 1.4: Beanplots showing differences between virgin (sterile) floral nectar (left, red) and nectar isolated from plants that could be accessed by insects (potentially contaminated with microorganisms; right, blue) for total sugar concentration (sucrose, glucose, and fructose, in percentage of weight/weight). Virgin nectar was obtained from plants protected from insect visitation. Data were obtained from 16 plant species belonging to six plant families. The overall average for each plot is drawn as a horizontal black line (Source: modified from Lievens *et al.,* 2015).

Individual plants of the same species can face different abiotic conditions, which could contribute to variation in the microbial community within a specific floral structure (Aleklett *et al.,* 2014). A previous study measured microclimatic conditions (temperature, relative humidity, and irradiance) of individual flowers and demonstrated that high abiotic variability did not always influence pollinator composition (Herrera, 1995). However, pollinator composition was significantly influenced by the sunlight mosaic, with highly irradiated flowers being preferably chosen by some particular pollinators (Herrera, 1995).

1.5.2 Biotic conditions

Flowers release a variety of volatiles, some of which are directly produced by floral organs (Knudsen *et al.,* 2006) and others that result from microbial metabolism (Wiens *et al.,* 2009). The volatiles emitted by flowers (*e.g.* aromatics, terpenoids and aliphatics) are derived from diverse biochemical pathways (Knudsen *et al.,* 2006). Additionally, the flower accumulates non-volatile substances such as alkaloids, phenolics, and proteins (Adler, 2000). Many of these compounds can exert antimicrobial activity (Cowan, 1999). For example, hydrogen peroxide and other reactive oxygen species activate a number of defence genes in the plant genome (Alvarez *et al,* 1998; Chamnongpol *et al.,* 1998; Sasabe *et al.,* 2000; Carter and Thornburg, 2004; Huang *et al.,* 2012). Some examples of defence proteins whose expression is upregulated during nectary development are listed in Table 1.2.

Table 1.2: Defense genes upregulated during nectary development (Source: Thornburg, 2007)

Protein/cDNA	Function
Snakin 1	Antimicrobial peptide
γ -thionin	Antifungal peptides
PR1	Antifungal protein
PR5	Antifungal protein
Chalcone synthase	Flavonoid biochemistry
Wound-induced *win1*	Antifungal protein
Wound-induced *pin1*	Antiherbivore protein
Wound-induced *pin2*	Antiherbivore protein

Interaction with resident microbes may also contribute to facilitate or hinder the establishment of microbial immigrants in floral microhabitats (Álvarez-Pérez *et al.*, 2019). For example, bacterial antagonists belonging to the genera *Pseudomonas* and *Pantoea* have been shown to exert moderate control of the pathogen *Erwinia amylovora*. (Wilson and Lindow, 1993; Pusey, 2002). Furthermore, it is known that some yeasts are able to alter the pH of the flower and produce ethanol (Wiens *et al.*, 2009), which might create unsuitable conditions for the growth of other microbes. Observations of community assembly on flowers suggest that dispersed microorganisms interact by competing for niche space and that the first yeast to colonize the nectar precludes the successful establishment of subsequent immigrants

(Aleklett *et al.*, 2014; Toju *et al.,* 2018). Peay *et al.* (2012) confirmed this hypothesis by demonstrating that the outcome of species competition in floral nectar depended on the arrival order, with the early arriving species hindering the growth of latecomers. The strength of this priority effect was increased when the competing species were close phylogenetic relatives (Peay *et al.,* 2012) so that late arriving yeast could less easily colonize nectar whenever this habitat was already occupied by sibling species.

1.6 Flowers as a reservoir of yeast diversity

Biodiversity peaks near the equator and decreases closer to polar regions (Figure 1.5). The fossil record offers a unique perspective on the evolution of this latitudinal biodiversity gradient (LBG) (Willig *et al.,* 2003; Hillebrand, 2004). Understanding the causes and evolution of the LBG is critical to explaining present-day geographical variation in biodiversity and modelling biotic responses to climate change (Orme *et al.,* 2005; Jenkins *et al.,* 2013). Net diversification rates are higher in the tropics than elsewhere (Wiens and Donogue, 2004; Mittelbach *et al.,* 2007; Weir and Schluter, 2007). However, it is unclear whether such pattern reflects relatively higher speciation rates, lower extinction rates or both. The LBG has been extensively discussed for plants but, in contrast, much less is known about the biogeographical distribution of microbes.

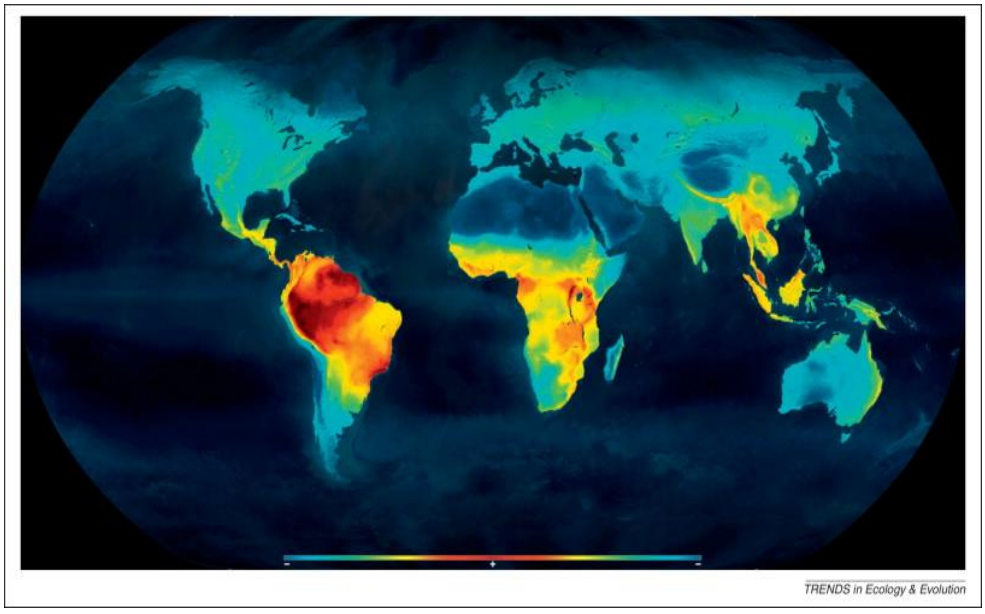

Figure 1.5: Representation of the current latitudinal plant biodiversity gradient. Plant diversity peaks in equatorial regions (red end of color spectrum) and declines polewards (blue end of the color spectrum). (Source: Mannion *et al.,* 2014)

Evaluating the biogeographic diversity of yeast communities associated to flowers remains a challenging task because of the limited number of studies performed so far in some locations and, in particular, in tropical regions where, on the other hand, most angiosperm's diversity is distributed. Therefore, a large-scale survey in different continents is needed to adequately asses the diversity of floral microbes (de Vega *et al.,* 2017). In this regard, de Vega *et al.* (2017) predicted that nectar yeast diversity should increase in habitats with a higher phylogenetic diversity of plants and a concomitant higher diversity of functional pollinator guilds (Mannion *et al.,* 2014).

The geographical distribution of floral yeasts not only depends on their plant hosts but also on their animal vectors. As discussed in Section 1.3, animal visitors influence the composition of the floral microbiota. Each pollinator or illegitimate visitor has its own preference of flowers and carries its own microbiota (Brysch-Herzberg, 2004; de Vega *et al.,* 2012). Nearly monospecific populations of *Metschnikowia koreensis, M. reukauffi* or *M. rancensis* were found in more than 40 plants species primarily pollinated by bees and butterflies (de Vega *et al.,* 2017). In contrast, the nectar of plant species pollinated by beetles was dominated by large-spored *Metschnikowia* and *Wickerhamiella* clade representatives (Lachance *et al.,* 2006, 2011; de Vega *et al.*, 2012, 2017). This might suggest that different yeast species have evolved intimate symbiotic relationships with different plants and insects. However, the fundamental nature of such interaction remains an open question (de Vega *et al.,* 2017).

Although the microbial communities of flowers contain a broad diversity of yeast species, only a few taxa appear to be consistent members of floral microbiomes (Herrera *et al.,* 2009; Pozo *et al.,* 2011; Belisle *et al.,* 2012; Alvaréz-Pérez and Herrera, 2013; Jacquemyn *et al.,* 2013a, 2013b; Canto *et al.,* 2017; Álvarez-Pérez *et al.,* 2019). Among these, the genus *Metschnikowia* (Ascomycota) is an ancient and diverse group of yeasts regularly detected in the floral nectar of phylogenetically diverse plants (Aleklett *et al.*, 2014; Pozo *et al.*, 2015). Around 70 different species of *Metschnikowia* are currently recognized (Index Fungorum, 2018), some of which have a broad geographic distribution encompassing all the continents (Buzzini *et al.,* 2017). In contrast, other species of the genus have a fairly restricted distribution, as for instance, four endemic species which are only found in the Hawaiian islands (namely *M. hawaiiensis, M. hamakuensis, M. kamakouana* and *M. mauinuiana*; Lachance *et al.,* 1990, 2005), or *M. maroccana*, a nectar inhabitant whose geographical distribution

seems to be confined to the Alhucema Bay in Morocco (Figure 1.6; de Vega *et al.*, 2018). The origin of the Metschnikowiaceae family is placed in the Late Cretaceous (71.7 Ma) with most extant species arising from the Early Eocene. Therefore, the family likely radiated long after the Mid Cretaceous radiations of angiosperms, which suggests that the diversification of the Metschniowiaceae was driven by repeated radiation on a pre-existing resource (*e.g.* plants; Guzmán *et al.,* 2013). Other yeast genera commonly found in flowers are *Candida* (Ascomycota), and *Cryptococcus*, *Rhodotorula*, and *Sporobolomyces* (Basidiomycota) (Pozo *et al.*, 2011; 2015; Belisle *et al.*, 2012; Mittelbach *et al.,* 2015; Álvarez-Pérez *et al.*, 2019). In this regard, it is widely known that the lifestyles and physiological properties of ascomycetous and basidiomycetous yeasts differ substantially, suggesting different ecological strategies. For example, while ascomycetous nectar yeasts such as *Metschnikowia reukaufii* and *M. gruessii* have been almost exclusively isolated from flowers, honey and insects, basidiomycetous taxa have been hardly associated with nectar-foraging insects (Brysch-Herzberg, 2004; Magyar *et al.*, 2005; Mittelbach *et al.,* 2015). This suggests that the ascomycetous nectar specialists spend most of their life cycle (if not all) inside the insect-flower system, whereas basidiomycetes seem to thrive in a broader variety of alternative substrates (Bandoni, 1995; Meirinho *et al.*, 2011; Mittelbach *et al.,* 2015).

Finally, flowers, and in particular floral nectar, are being increasingly recognized as a rich source of novel yeast species, and the study of these habitats has led to the discovery of more than 50 new species of *Wickerhamiella, Candida, Metschnikowia, Starmerella,* and *Kodamaea* during the last decade. (de Vega *et al.*, 2017, 2018). In any case, the role of floral microhabitats as reservoirs of unknown yeast diversity has only recently begun to be realized (de Vega *et al.*, 2017).

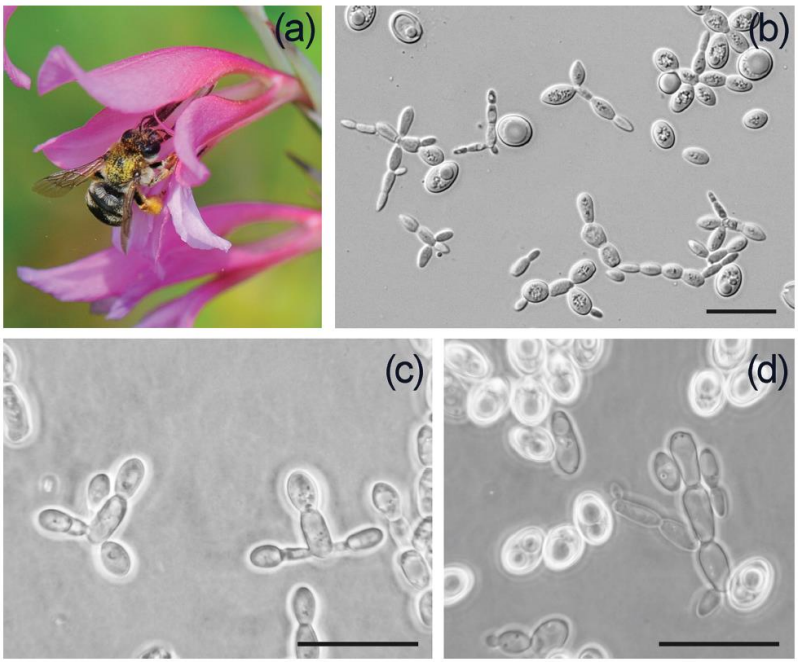

Figure 1.6: Flowers of *Gladiolus italicus* ('common sword-lily', Iridaceae) from which *Metschnikowia maroccana* f.a., sp. nov. was isolated (a). Photomicrograph of *M. maroccana* displaying the typical 'airplane' configuration of budding cells (b, c) and refringent chlamydospores (d). Bar = 25 µm (Source: de Vega *et al.,* 2018)

1.7 Taxonomic study of yeasts

1.7.1 Phenotypic tests

Before the use of deoxyribonucleic acid (DNA) sequencing in yeast taxonomy, phenotypic tests were the only method of studying the relatedness between different species or higher taxa (*i.e.* genera, families, orders, *etc.*) and assess their applied interest. In this regard, some yeasts species share unique physiological characters that can be used for reliable identification (Wickerham, 1951). Furthermore, regardless of their taxonomic value, some phenotypic traits can have a biotechnological interest. For example, diverse species of basidiomycetous oleaginous yeasts such as *Cryptococcus curvatus*, *Trichosporon cacaoliposimilis*, *Trichosporon oleaginosus*, and *Rhodotorula* spp. have been used in the production of biofuels (*e.g.* biodiesel), food, and feed additives (Hassan *et al.,* 1995; Gujjari *et al.*, 2011; Thiru *et al.,* 2011; Zhu *et al.,* 2012; Lin *et al.*, 2014; Tchakouteu *et al.,* 2015). In addition, *Saccharomyces cerevisiae* is the preferred organism for industrial production of many food and beverages, and,

according to recent studies, some strains have a great potential in bioethanol production (Mukherjee *et al.,* 2014, 2017).

Physiological and biochemical tests that are commonly used for identification of yeasts are the following ones (Kurtzman *et al.*, 2011b): fermentation of carbohydrates, growth on (assimilation of) various carbon and nitrogen sources, determination of requirement for vitamins, growth at various temperatures, determination of the osmotolerance (*i.e.* growth on media with high content of sugars or salts), hydrolysis of urea, and resistance to antifungals. Unfortunately, there is currently no standard method for phenotyping yeasts. However, most taxonomic papers follow the guidelines provided in by Kurtzman *et al.* (2011a) (see details in subsequent sections of this thesis).

1.7.2 Molecular identification and phylogenetics

Molecular methods are the current standard for identifying microbial species and analyzing their phylogenetic and taxonomic affiliation (White *et al.,* 1990; Kurtzman and Robnett, 1998). Single gene sequences, such as those from the D1 and D2 regions of the large subunit (LSU) of the gene encoding for ribosomal RNA (rRNA) or the internal transcribed spacers (ITS1 and ITS2) are commonly used as the basis for recognizing new yeast taxa (Figure 1.7; Kurtzman, 2010). However, inter-species hybrids and intra-species genetic polymorphism may not be recognized by analyzing the sequence of a single gene. In these cases, multigene approaches, generally including the study of both ribosomal and protein-encoding genes, are recommended.

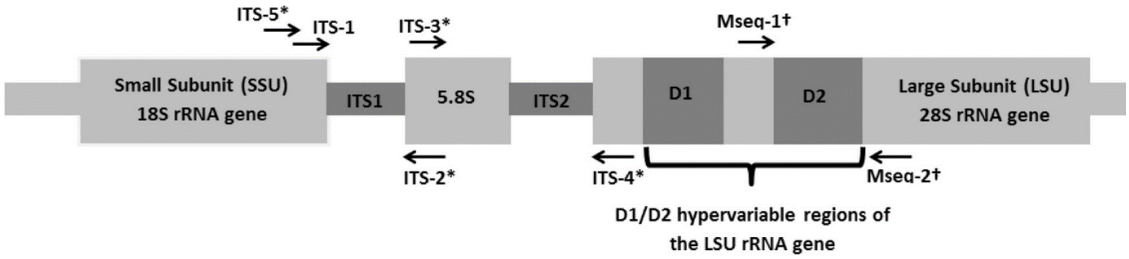

Figure 1.7: Diagram of the ribosomal gene operon (not drawn to scale). The organization of this complex includes a sequence coding for the 18S (small subunit, SSU) rRNA gene, the internal transcribed regions 1 and 2 (ITS1 and 2) separated by the highly conserved 5.8S rRNA gene coding region, and the sequence coding for the D1 and D2 regions of the 28S (large subunit, LSU) rRNA gene. Arrows indicate approximate positions of primers that can be used to amplify regions of interest. (Source: Arbefeville *et al.*, 2017)

The resolution provided by sequencing the ITS sometimes exceeds that obtained from the D1/D2 regions, but the reverse is also fairly common (Kurtzman, 2010). Therefore, the combined study of both ITS and LSU rRNA gene sequences is included in most recent descriptions of novel yeast species (Sipiczki and Tap, 2016; de Vega *et al.,* 2017, 2018; Kobayashi, 2017; Morais *et al.,* 2017; Polburee *et al.,* 2017: Varize *et al.,* 2018; Lopes *et al*., 2019).

1.7.3 The species concept in yeasts

Even when the species concept is central to biology and derived disciplines, there is a long-standing debate about what a species is. In fact, over 24 different species concepts can be found in the literature (Mayden, 1997), among which the morphological, the biological, the genotypic and the phylogenetic species concepts are the most commonly applied (Table 1.3). This current lack of consensus on defining species boundaries is even more apparent in the case of microbes, as these mostly (or exclusively, as in the case of prokaryotes) reproduce asexually and thus rarely meet the conditions of the biological species concept (Simpson, 1961; de Queiroz, 1998, 2007).

Table 1.3: Overview of some of the main species concepts available in the literature (Source: modified from Lagache *et al.,* 2013)

Species concept	Species definition according to this concept	Possible criteria for species delimitation derived from this definition
Biological species concept (BSC)	Species are "*groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups*" (Mayr, 1940).	Higher natural interfertility between individuals *within* than *among* species.
Phylogenetic species concept (PSC)	A species is "*a diagnosable cluster of individuals within which there is a parental pattern of ancestry and descent, beyond which there is not, and which exhibits a pattern of phylogenetic ancestry and descent among units of like kind*" (Eldredge and Cracraft, 1980).	Higher genetic relatedness between individuals *within* than *among* species.
Genotypic species concept (GSC)	A species is a "*genotypic cluster of individuals that can overlap without fusing with its siblings*" (Mallet, 1995; Hausdorf, 2011)	Higher genotypic similarity between individuals *within* a species.
Morphological species concept (MSC)	Species are "*the smallest detected samples of self-perpetuating organisms that have unique sets of characters*" (Nelson and Plantick, 1981; Mishler, 1985).	Higher morphological similarity between individuals *within* than *among* species.

In the particular case of yeasts, some species share morphological and/or other phenotypic characters with close and non-close relatives (Wickerham, 1951) and, therefore, the morphological species concept cannot be easily applied. The biological species concept also presents some problems when applied to yeasts, as not all yeasts are capable to reproduce sexually or their teleomorphs (*i.e.* sexual states) have not been found yet in nature or are difficult to induce in cultivation under laboratory conditions (Reynolds,1993; Kurtzman, 2011a). Furthermore, application of the genotypic species concept depends on the availability of genotyping techniques suitable to be applied to broad taxonomic categories (*e.g.* beyond the genus level) but, at the same time, have enough discriminative power to resolve sibling groups (species) which, in most cases, need to be defined (or confirmed) on the basis of other criteria (morphology, physiology, mating compatibility, pathogenicity, *etc*.). In addition, genetic clusters can be recognized not only at the species level, but also at the subpopulation level (Liti *et al*., 2006), thus hindering the recognition of genotypic species. Finally, also the phylogenetic species concept, which is based on the incongruence of gene genealogies, faces problems when applied to yeasts, as the phylogenies inferred by multiple single-gene data sets may be significantly different, and the decision about where to place the limit of species according to such single-gene data is sometimes subjective (Taylor *et al.,* 2000). For instance, if a gene is polymorphic among individuals within a species (*e.g.* having two possible alleles, A and B; Figure 1.8), individuals sharing one of the alleles could also be classified as a different species when compared with individuals carrying any of the other alleles.

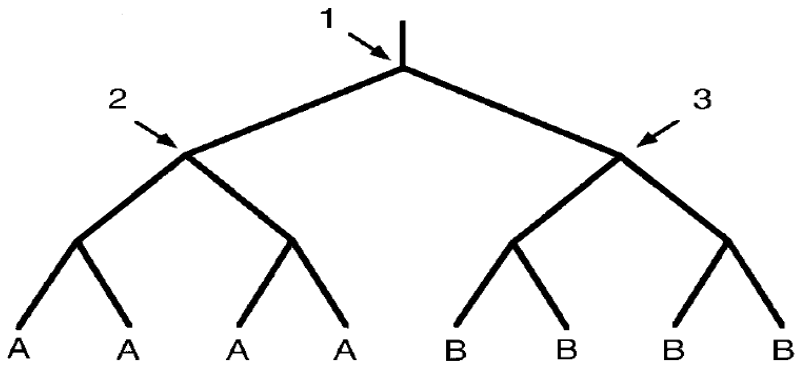

Figure 1.8: Example of the limitations of defining species boundaries on the basis of a single gene genealogy. Phylogenetic analysis of single genes may help to classify individuals in monophyletic groups, but cannot rank them into species. For example, should the species limit be at arrow 1, therefore implying that alleles A and B are intraspecific variations? Or, should the limit of species be at arrow 2 and 3, implying that alleles A and B define two genetically distinct species? (Source: Taylor *et al.,* 2000)

The subjectivity in determining species boundaries can easily be avoided by relying on more than one gene genealogy, an approach that constitutes the basis of the Genealogical Concordance Phylogenetic Species Concept (GCPSC; Avise and Ball, 1990). However, despite the strengths of the GCPSC, recombination among individuals within a given species may cause conflict between different gene phylogenies and thus blur species boundaries. Nevertheless, the transition bifurcation between concordance and incongruence of phylogenies can be used to infer species boundaries (Figure 1.9; Taylor *et al.,* 2000).

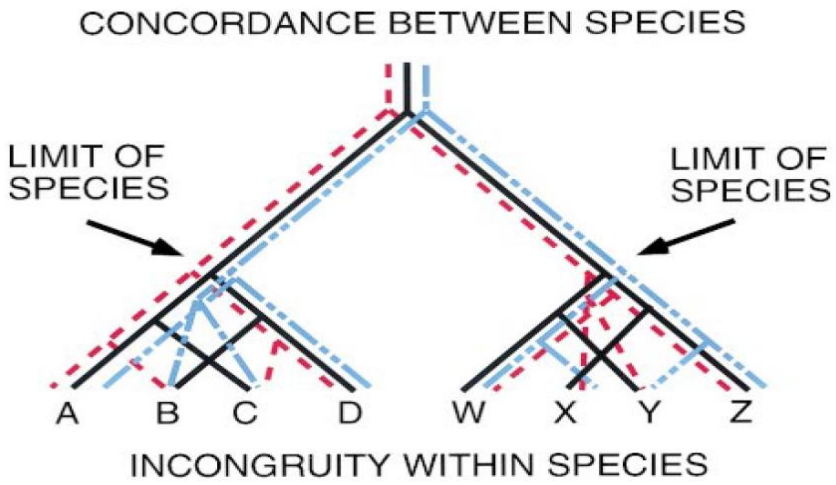

Figure 1.9: Simultaneous phylogenetic analysis of three gene genealogies (black continuous, and blue and magenta discontinuous lines) for the same individuals (A-D and W-Z), showing the transition from concordance to incongruence among branches frequently observed in multi-gene approaches, and which can be used to determine species boundaries according to the GCPSC (Source: Taylor *et al.,* 2000)

2 MATERIAL AND METHODS

2.1 Study system

The native Hawaiian *ʻōhiʻa lehua* (*Metrosideros polymorpha* Gaud., Family Myrtaceae) is the most abundant tree in the Hawaiian Islands (USA). It is found in all the major islands and distributed in a variety of habitats ranging from near the sea level to approx. 2500 m of altitude. Furthermore, *M. polymorpha* can grow on a broad range of substrates including recent lava flows (Figure 2.1) and exhibits high resistance to different environmental stressors such as freezing, volcanic vapours, excessive moisture or dryness (Mueller-Dombois and Fosberg, 1998). According to the Hawaiian mythology, the vernacular name of the plant species comes from two lovers called *ʻŌhiʻa*. and *Lehua*. ʻŌhiʻa. was a brave young warrior who had fallen in love with the beautiful Lehua. In the evenings, ʻŌhiʻa used to play his bamboo flute to call on Lehua for a walk in the forest. However, the gentle melodies of ʻŌhiʻa's flute also attracted the great volcano goddess *Pele*, who became jealous of Lehua. Pele wanted ʻŌhiʻa to leave Lehua and for them to be together, but ʻŌhiʻa refused to do so. Pele, who got filled with hatred, turned then ʻŌhiʻa into a tree. The forest's spirits tried to rescue ʻŌhiʻa, but they were not powerful enough to stop Pele. However, as those spirits had power over the growth of plants and forest creatures, they turned the heartbroken Lehua into flame-coloured flowers which, from that day on, would be held tenderly by the leafy arms of her lover (Yuen, 2016).

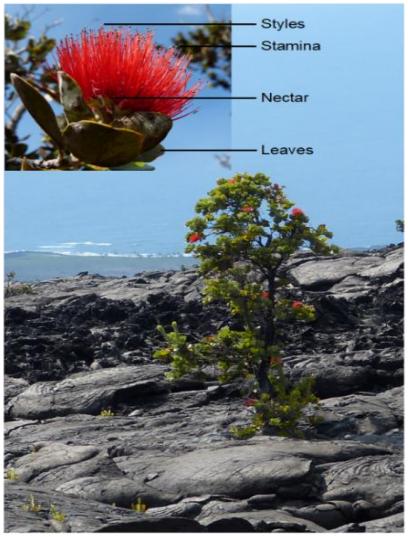

Figure 2.1: *Metrosideros polymorpha* (*ʻōhiʻa lehua*, Myrtaceae) tree growing on solidified lava. A detail of an inflorescence is shown in the upper left insert (Source: Junker and Keller, 2015).

On the island of Hawaii, there are currently recognized six varieties of *M. polymorpha* that differ in the morphology of vegetative and reproductive organs (Table 2.1). The corolla of *M. polymorpha* flowers is usually red, but can also be pink, salmon, orange, yellow, and different shades in-between. Such flowers are pollinated by both birds and insects. Interestingly, yellow flowers have shown to be self-compatible and are thus able to form seeds from their own pollen, while red flowers are partially self-incompatible and require a pollinator for good seed set (Carpenter, 1976).

Table 2.1: Morphological characteristics of *Metrosideros polymorpha* varieties present on the island of Hawaii, as described by Dawson and Stemmermann (1999) (Source: modified from James *et al.,* 2004).

Character	***Metrosideros polymorpha* var.**					
	glaberrima	*macrophylla*	*newellii*	*incana*	*polymorpha*	*nuda*
Plant size	shrubs to tall trees	small to tall trees	shrubs to tall trees	shrubs to tall trees	shrubs to tall trees	shrubs to tall trees
Leaf shape	ovate or obovate to elliptic	broadly ovate, large	elliptic	ovate to surbicular	ovate to surbicular	ovate to surbicular
Leaf pubescence (lower surface)	glabrous	glabrous	glabrous	sericeous pubescent (silky)	sericeous pubescent (silky)	glabrous
Petiole length	petiolate	petiolate	petiolate	subsessile to petiolate	subsessile	subsessile
Inflorescence	pubescent (glabrous)	pubescent	pubescent	pubescent	pubescent	glabrous

2.2 Samples

The samples analyzed in this study were kindly provided by Professor Robert R. Junker (University of Salzburg, Austria, http://www.uni-salzburg.at/orgbiol/robert.junker). These samples consisted of nectar, stamina and styles coming from the flowers of 33 individual *M. polymorpha* trees from 14 populations distributed across the island of Hawaii, USA (Figure 2.2).

Samples were collected in the field with sterile forceps (stamina and styles) or glass microcapillaries (nectar). A total of 91 samples were analyzed for microbial presence in the present study, including nectar (n = 30), stamina (n = 31) and styles (n = 30). All flower organs from one plant individual were sampled from the same inflorescence (*i.e.* cluster of flowers on a branch or a system of branches; Junker and Keller, 2015). The samples were stored in plastic vials (2 mL; BRAND GmbH +Co KG) containing lysogeny

broth (LB; 1.0% tryptone (Sigma-Aldrich), 1.0% NaCl (Merck), and 0.5% yeast extract (Oxoid)) or phosphate-buffered saline (PBS, Sigma-Aldrich) at -80°C until being analyzed.

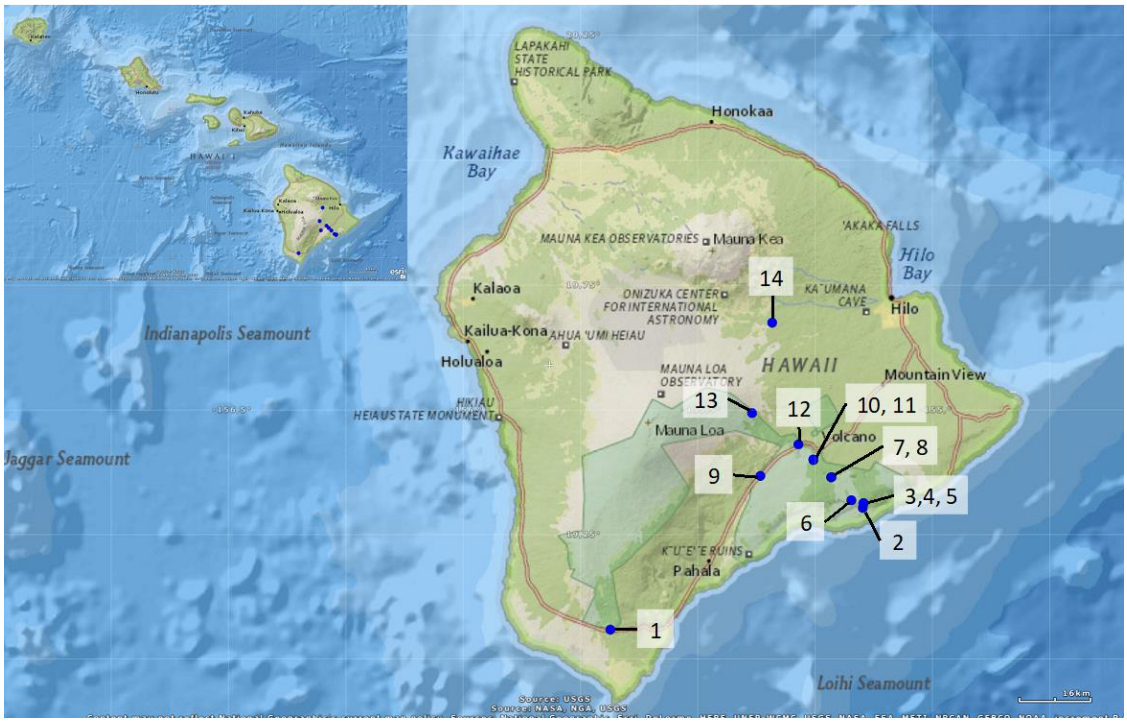

Figure 2.2: Location of the sampled *M. polymorpha* populations in Hawaii, USA. In each population, samples of, nectar, stamina and styles were taken from one to five trees. The area corresponding to Hawaii Volcanoes National Park is depicted in green (Source: Junker and Keller, 2015).

2.3 Isolation of yeasts

The vials containing the samples were defrosted, agitated by vortexing and, in the case of stamen and style samples, introduced in a sonication bath for 60 seconds so as to facilitate the detachment of epiphytic yeasts (Pusey *et al.*, 2011), and then vortexed again. Twenty-five microliters of the samples were streaked on yeast malt agar plates (YM; 2.0% agar (Oxoid), 1.0% glucose (Sigma-Aldrich), 0.5% Bacto Peptone (Becton, Dickinson and Company, BD) , 0.3% malt extract (Oxoid), 0.3% yeast extract (Oxoid), pH 6.2 ± 0.2) containing 0.01% chloramphenicol (Sigma-Aldrich) to inhibit bacterial growth. Inoculated plates were incubated at 25°C for 6 days. A representative colony of each different morphotype was purified by repeated streaking on YM agar and preserved until further use at -80°C in LB medium containing 25% of glycerol (Prolabo).

2.4 Molecular identification and subtyping

Genomic DNA was isolated from fresh (2 to 4 day-old) yeast cultures growing on YM agar. Briefly, yeast colonies were suspended into a microtube containing 500 µL of lysis buffer (400 mM Tris-HCl (Sigma-Aldrich), 60 mM ethylenediaminetetraacetic acid (Sigma-Aldrich), 150 mM NaCl (Merck) and 1% sodium dodecyl sulfate (Amresco)) and heated for 2 h at 56°C in a Multiblock heater. One hundred and fifty microliters of potassium acetate solution (60% of 5 M potassium acetate (Merck) and 11.5% of glacial acetic acid (Merck)) were added afterwards to each microtube and the cell debris was removed by two consecutive centrifugations at 8000 *g* for 5 min, followed by transferring of the supernatant to a new microtube. The same volume (approx. 600 µL) of 2-propanol (VWR) was added to each microtube and the mixture was briefly mixed by inverting the tubes and centrifuged for 15 min at 16000 *g*. The DNA pellet was washed with 300 µL of 70% cold ethanol and centrifuged again at 16000 *g* for 10 min. The remaining pellet was finally resuspended in 50 µL of nuclease-free water (Ambion) and the concentration and purity of DNA was assessed by determining the optical density at 260 nm and 280 nm using the Nanodrop system (Thermo Fisher Scientific).

The D1/D2 regions of the LSU rRNA gene were PCR amplified using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'- GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett, 1998). Additionally, for some isolates for which a better taxonomic resolution was required, the ITS regions were amplified using primers ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White, 1990). Finally, for the yeast isolates representing potential new taxa (see Results and Discussion) the SSU rRNA gene was partially amplified using the universal primers 515F (5'- GTGCCAGCMGCCGCGGTAA-3') (Turner *et al.,* 1999) and 917R (5'-CCGTCAATTCMTTTRAGTTT-3') (Lane *et al.,* 1991). In all cases, reaction mixtures contained 5 µL of Titanium Buffer (10X, Clontech), 0.25 mM $MgCl_2$ (Thermo Scientific), 0.25 mM of each primer (Sigma-Aldrich), 0.5 U Titanium Taq polymerase (50X, Clontech) and 50 ng of template DNA in a final volume of 50 µL. PCR amplifications were carried out in a BioRad T100 thermocycler and consisted of a denaturation step of 2 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C and 2 min at 72°C, and a final extension of 10 min at 72°C. PCR products were then purified using Thermo Scientific's GeneJET PCR Purification Kit and sent for Sanger sequencing to Macrogen Europe (Amsterdam, The Netherlands). In all cases, DNA sequences were assembled

and manually edited with the program Geneious Prime2019.1.1 (https://www.geneious.com) or SeqTrace (Stucky, 2012).

In order to determine the interspecies variability of the strains representing potentially new yeast taxa (see Results and Discussion), we subtyped those strains by PCR fingerprinting using primer M13 (5'-GAGGGTGGCGGTTCT-3'), which corresponds to a minisatellite core sequence derived from the wild-type phage M13 (Vassart *et al.,* 1987; Meyer and Mitchell, 1995). Reaction mixtures contained 2.5 µL of Titanium Buffer (10X, Clontech), 1.5 mM $MgCl_2$ (Thermo Scientific), 6 µM of M13 primer (Sigma-Aldrich), 1 U Titanium Taq polymerase (50X, Clontech) and 10 ng of template DNA in a final volume of 50 µL. PCR amplifications were carried out in a BioRad thermocycler and consisted of a denaturation step of 2 min at 95°C, followed by 40 cycles of 30 s at 94°C, 60 s at 50°C and 60 s at 72°C, and a final extension of 10 min at 72°C. Five microliters of each PCR product were electrophoresed in a 2% agarose gel. Gels were stained with ethidium bromide (Sigma-Aldrich) and visualized under UV light. Digitalized images of the gels were captured with a Epi Chemi II darkroom image analyzer (UVP) and visually inspected. Different band profiles were classified as different genotypes.

2.5 Delineation of Operational Taxonomic Units

The LSU rRNA gene sequence (and, when available, the ITS and SSU rRNA gene sequences) obtained from flower-inhabiting yeasts were compared with reference sequences from type strains available in the GenBank databases using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, LSU rRNA gene sequences were included multiple alignments (one for ascomycetous and another for basidiomycetous yeasts) generated by MUSCLE (Edgar, 2004), and the resulting alignments were trimmed with Gblocks (Castresana, 2000) to eliminate poorly aligned regions, using 'allowed gap positions = with half', 'minimum length of a block = 5' and default settings for all other options (Álvarez-Pérez *et al.*, 2012; Álvarez-Pérez and Herrera, 2013).

Determination of the number of OTUs occurring in the sets of LSU rRNA gene sequences obtained for ascomycetous and basidiomycetous yeasts and assignment of those sequences to individual OTUs was done with mothur v.1.41.3 (Schloss *et al.,* 2009). A sequence dissimilarity cut-off value of 3% was used in these analyses, as

recommended in previous diversity analyses of nectar yeasts (Pozo *et al.,* 2011; Álvarez-Pérez and Herrera, 2013). In order to asses the overall richness of yeast OTUs, rarefaction methods were applied to presence-absence (1/0) data sets. Sample-based average rarefaction curves for yeast communities were computed with EstimateS v.9.1.0 (Colwell, 2009), using 50 randomisations and sampling without replacement. Because the data consisted on presence-absence matrices, the ICE (Incidence-based Coverage Estimator) and Chao2 non-parametric estimators of expected OTU richness were used (Pozo *et al.,* 2011; Álvarez-Pérez *et al., 2012;* Álvarez-Pérez and Herrera, 2013; Jacquemyn *et al.,* 2013a; 2013b). Furthermore, a Venn diagram of OTU distribution across the different floral microenvironments analyzed (*i.e.* nectar, stamina, and styles) and rank-abundance plots were created using Bioinformatics & Evolutionary Genomics tools (http://bioinformatics.psb.ugent.be/webtools/Venn/) and Microsoft Excel, respectively. Finally, the diversity of the yeast OTUs recovered from different floral parts of *M. polymorpha* was compared by calculating Faith's phylogenetic diversity (PD; Faith, 1992), as implemented in the R v.3.6.0. package 'picante' (Kembel *et al.,* 2010; R Core Team, 2019), using phylogenetic trees obtained for the LSU rRNA gene (see section 2.6). Faith's PD is the most common measure of phylogenetic diversity and, for a given set of species (or OTUs), it is defined as equal to the sum of the lengths of all those branches on the tree that span the members of the set (Faith, 1992).

2.6 Phylogenetic reconstruction

Phylogenetic trees for the LSU rRNA gene alignments were built using three different methods: neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). The NJ and MP trees were obtained using MEGA version X (Kumar *et al.,* 2018). In NJ analyses, evolutionary distances were calculated based on the Jukes-Cantor model with the rate of variation among sites modelled by a gamma distribution, as estimated by jModelTest v2.1.10 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (ten replicates). ML analyses were carried out using the online version of PhyML v3.0 (http://www.atgc-montpellier.fr/phyml/, Guindon *et al.,* 2010) under the GTR+G+I model of sequence evolution (which was selected by jModelTest v2.1.10 as the simplest model among those available in PhyML that best fitted our sequence data),

with four substitution rate categories, starting trees generated by BIONJ, and Nearest Neighbor Interchanges (NNIs) tree search algorithms. For OTU-based phylogenetic trees, 1000 bootstrap replications were used to define consensus trees. Trees based on the LSU- and ITS-region of the ribosomal RNA for yeast presenting potential new taxa were inferred using 100 bootstrap replications. All reference sequences were downloaded from the GenBank.

2.7 Phenotypic characterization

Phenotypic characterization was based on the protocols described by Kurtzman *et al.* (2011b), which represent the current standard methods in yeast taxonomy and have been applied to the recent description of new species of yeasts (Freitas *et al.,* 2015; de Vega *et al.,* 2017, 2018; Morais *et al.,* 2017; Lachance *et al.,* 2018; Varize *et al.,* 2018; Fotedar *et al.*, 2019a, 2019b; Lopes *et al*., 2019;Tsuji *et al.,* 2019).

2.7.1 Morphological characterization

Description of the morphology of yeast colonies was performed using plate cultures on YM agar and potato dextrose agar (PDA, Neogen) that had been incubated for 48-72 h at 25°C. Colonies were analyzed underneath an SZX10 stereomicroscope (Olympus). The following aspects were considered for colony description (Kurtzman *et al.,* 2011b; Figure 2.3): colour, texture, surface, edge (or margin), and elevation.

Microscopic inspection of cells was performed with an optical microscope (DP74 Microscope Digital Camera, Olympus) using a phase contrast objective (100x magnification) and a scanning electron microscope (VEGA3, TESCAN). The samples for the optical microscope were prepared by suspending the cells in saline solution (0.85% NaCl; Merck). A small drop of each cell suspension was pipetted between a glass slide and a cover slide (Thermo Scientific) and was analyzed underneath the microscope. Samples for the scanning electron microscope (SEM) were prepared by air-drying the same cell suspensions on carbon layered stubs, which were subsequently coated in gold by applying high voltage in vacuum pressure. Both techniques (optical and SEM microscopy) were carried out at KU Leuven's Campus De Nayer (laboratories of Dr. Houtmeyers and Prof. Peeters, respectively; Sint-Katelijne-Waver, Belgium). Special attention was paid in all cases to measuring cell size, analyzing budding

patterns, and checking for possible formation of storage bodies (*e.g.* vacuoles) and pseudohyphae.

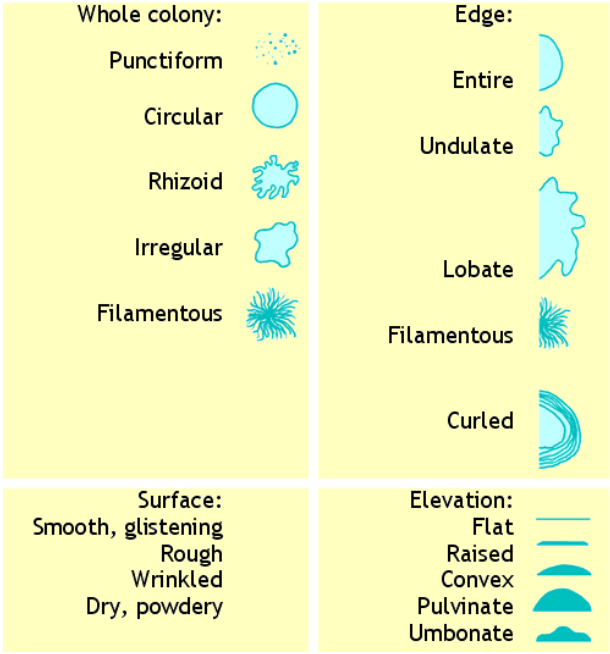

Figure 2.3: Overview of colony morphology features
(Source: modified from Seeley and Vandemark 1962).

Apart from the aforementioned vegetative structures, many yeasts reproduce sexually resulting in the formation of characteristic cells. In the particular case of ascomycetous yeasts, the site of meiosis is the ascus, where haploid ascospores are formed. The ability to sporulate sometimes declines when strains are maintained under laboratory conditions and may even be lost. However, some strains never lose this ability and form ascospores within a short time (Kurtzman, 2011a). Conditions for inducing ascospore formation vary greatly. Therefore, four different agars were used in this study for inducing production of ascospores: malt extract agar (2% of agar and 5% of malt extract), restriction growth agar (0.02% of yeast extract, 0.02% of peptone, 0.1% of glucose and 2% agar) and V8 agar (diluted V8 juice (Campbell Soup Company), 1:9 and 1:19, and 2% agar; pH = 5.5). All these media were inoculated by spotting portions of yeast colonies on the agar surface using sterile plastic loops (VWR). Strains from a same OTU were tested alone and in pairwise combinations, and inoculated plates were incubated for 7 weeks at 25 °C. Formation of ascospores was checked under the optical microscope, following the procedure detailed in the previous paragraph.

2.7.2 Physiological and biochemical tests

Unless otherwise indicated, all test described in subsequent paragraphs were performed by duplicate on different days, and incubation temperature was in most cases 25ºC. Furthermore, the sterilization of materials and media by autoclaving was always performed at 121°C for 15 minutes. Finally, the following reference strains were included in all tests for quality control: *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^T), *M. koreensis* (CBS 8854^T), *M. reukaufii* (CECT 10671^T) and *M. gruessii* (CBS 7657^T).

2.7.2.1 Fermentation of carbohydrates

Production of carbon dioxide (CO_2) as a result from fermentation of diverse carbohydrates was assessed using the fermentation basal medium described by Wickerham (1951), which contains 4.5 g of powdered yeast extract (Oxoid), 7.5 g bacto peptone (Becton Dickinson) and 2.7 mg of bromothymol blue (Alfa Aesar) per 100 mL of the medium. Bromothymol blue, which is added as a pH indicator, has a yellow colour in acidic conditions and turns to dark green or blue in alkaline conditions. The medium is sterilized by autoclaving and then distributed in different sterile flasks to which a certain volume of a filter-sterilized carbohydrate stock solution (10% w/v) is added so as to yield a final concentration of 2% (except for raffinose, for which the concentration is 4%, since some strains cleave and ferment only part of the molecule of this trisaccharide; Kurtzman *et al.,* 2011b). Tested compounds are shown in Table 2.2. Aliquots (9 mL) of the fermentation medium containing the diluted carbohydrates were added to glass tubes containing a Durham insert, which collects any gas that may be produced by the strain tested (Figure 2.4).

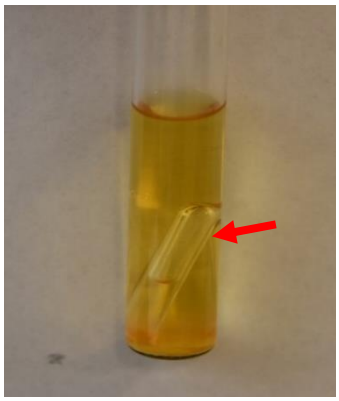

Figure 2.4: Example of a positive fermentation reaction, in which a Durham insert has collected the gas resulting from fermentation of the carbohydrates present in the medium (red arrow).

Finally, 300 µL of a cell suspension in saline solution (0.85% NaCl, Merck; 85% transmittance, as determined by a bench-top Biolog™ turbidimeter) was added to each test tube, and were incubated at 25°C for up to 33 days. Due to time and material constraints (lack of enough space in the incubators for such a long incubation period), fermentation tests were performed just once. Nevertheless, results obtained for the quality control strains generally matched the expectations according to the literature (Lachance, 2011; see section 3.4.4). Scoring of results was as follows (Kurtzman *et al.,* 2011b):

- +, strongly positive: Durham insert completely filled with gas within 7 days;
- l, delayed (latent) positive: insert rapidly filled, but only after more than 7 days;
- s, slowly positive: insert slowly filled after more than 7 days;
- w, weakly positive: the insert was not completely filled (<1/3 of its length) with gas;
- -, negative: no accumulation of gas in the insert.

2.7.2.2 Assimilation of carbon compounds

These phenotypic tests determine the ability of yeasts to grow aerobically on particular carbon compounds serving as the sole energy source for growth. The tests were carried out by a replica plating method in which a set of plates, containing each a single carbon source in basal agar medium (0.67% of Bacto Yeast Nitrogen Base (Becton Dickinson), 0.5% of carbon compound and 2% of agar), are inoculated by spotting starved yeast cells and incubated at 25°C for up to 7 days. Starvation of cells was achieved by inoculating these into a broth containing very limited nutrient sources (0.1% glucose

and 0.67% of Bacto Yeast Nitrogen Base). After 48 h of incubation at 25ºC and 150 rpm, starved cells were diluted with carbon-free broth up to an 85% transmittance (as determined by a bench-top Biolog™ turbidimeter) and used for spotting the aforementioned carbon assimilation plates. Results were read by inspecting the plates and comparing them to negative controls which contained no carbon compound. A detailed list of tested compounds is given in Table 2.2.

Scoring of results was as follows for the tests carried out in solid medium:

- +, strongly positive: significant growth in 3 days;
- l, delayed (latent) positive: growth in 7 days;
- -, no growth or scarce growth when compared to the negative control plate.

One of the carbon sources tested, namely 2-keto-D-gluconate (1 g), was difficult to dissolve in the recommended volume of 0.67% Bacto Yeast Nitrogen Base solution (*i.e.* 20 mL) and, therefore, it was dissolved in a higher volume (180 mL) to reach the final concentration of 0.5% (w/v), filter sterilized and tested in a broth medium setting instead of on agar plates. Scoring of results in this particular case was performed as recommended by *Kurtzman et al*. (2011b), where the degree of growth is determined by visual inspection. Briefly, tubes were shaken and placed in front of a card or piece of paper on which two lines (0.75 mm thick and 5 mm apart) had been drawn with black ink. Results were scored as 3+ if the lines were completely obscured, as 2+ if the lines appeared diffuse, as 1+ if the lines were distinguishable but blurred, and negative if the lines were clearly seen and had sharp edges. Finally, results were ranked as follows:

- +, positive: either 2+ or 3+ reading after 1or 2 weeks;
- l, delayed positive (latent): either 2+ or 3+ developing rapidly after 2 weeks;
- s, slow positive: a 2+ or 3+ reading developing slowly over a period exceeding 2 weeks;
- w, weakly positive (1+);
- -, negative.

Table 2.2: Detailed list of carbon compounds used in assimilation (A) and fermentation (F) tests, with the corresponding Chemical Abstracts Service (CAS) number and the used commercial brand.

Carbon source categories	Compound	CAS Number	Brand	Use
Hexose-based	Sucrose	57-50-1	Sigma-Aldrich	A, F
	D-(+)-Glucose	50-99-7	VWR	A, F
	D-(-)-Fructose	57-48-7	VWR	A, F
	D-(+)-Galactose	59-23-4	Sigma-Aldrich	A, F
	D-(+)-Maltose monohydrate	6363-53-7	Sigma-Aldrich	A; F
	D-(+)-Trehalose dihydrate	6138-23-4	Sigma-Aldrich	A, F
	Inulin	9005-80-5	Alfa Aesar	A
	Methyl-α-D-glucoside	97-30-3	Acros Organics	A
	D-(+)-Melezitose hydrate	207511-10-2	Sigma-Aldrich	A, F
	L-Sorbose	87-79-6	Acros Organics	A
	D-(+)-Raffinose pentahydrate	17629-30-0	Sigma-Aldrich	A, F
	D-(+)-Cellobiose	528-50-7	Alfa Aesar	A, F
	D-Lactose monohydrate	64044-51-5	Sigma-Aldrich	A, F
	D-(+)-Melibiose	585-99-9	Sigma-Aldrich	A, F
	D-(-)-Salicin	138-52-3	Alfa Aesar	A
Methyl-pentoses	L(+)-Rhamnose monohydrate	10030-85-0	VWR	A
Pentoses	D-(+)-Xylose	58-86-6	Tokyo Chemical Industry	A
	L-(+)-Arabinose	5328-37-0	Sigma-Aldrich	A
	D-Arabinose	10323-20-3	Alfa Aesar	A
	D-Ribose	50-69-1	Alfa Aesar	A
Alcohols	Ethanol	64-17-5	Merck	A
	Ribitol (= adonitol)	488-81-3	Alfa Aesar	A
	Xylitol	87-99-0	Alfa Aesar	A
	Dulcitol (= galacitol)	608-66-2	Acros Organics	A
	myo-Inositol	87-89-8	Merck	A
	Glycerol	56-81-5	VWR	A
	D-Sorbitol (= glucitol)	50-70-4	Sigma-Aldrich	A
	D-Mannitol	69-65-8	Sigma-Aldrich	A
	meso-Erythritol	149-32-6	Sigma-Aldrich	A
Acids	D,L-Lactic acid	72-17-3	Alfa Aesar	A
	Gluconolactone	90-80-2	Alfa Aesar	A
	2-Keto-D-gluconate	3470-37-9	Alfa Aesar	A
	5-Keto-D-gluconate	5477-60-9	Alfa Aesar	A
	Citric acid monohydrate	7643-75-6	Alfa Aesar	A
	Succinic acid	110-15-6	Merck	A
	Gluconic acid	526-95-4	ACROS	A
Amines	D-Glucosamine	66-84-2	Alfa Aesar	A
	N-Acetyl-D-glucosamine	7512-17-6	Sigma-Aldrich	A
Alkanes	Hexadecane	544-76-3	Alfa Aesar	A

2.7.2.3 Assimilation of nitrogen compounds

The method for testing growth on nitrogen compounds is similar to that used in the carbon assimilation tests, *i.e.* replica plating. However, the basal growth agar for nitrogen assimilation contains 1.17% of Bacto Yeast Carbon Base (Sigma-Aldrich) and 2% of agar. After autoclaving the basal agar, the nitrogen source to be tested is added from a filter-sterilized stock so as to yield a final concentration of 0.0108% (w/v) of nitrogen. Plates were inoculated by spotting starved cells and incubated for 7 days at 25°C. Starvation of yeast cells was achieved by inoculating these into a starvation broth containing 1.17% of Bacto Yeast Carbon Base and incubating for 72 h at 25°C, after which these were diluted to an 85% transmittance using the same nitrogen-free broth. The list of nitrogen sources used in this study is shown in Table 2.3. Evaluation of results was done as described for carbon assimilation test on solid medium (see previous section).

Table 2.3: Nitrogen sources used in assimilation tests. For each compound, the Chemical Abstracts Service (CAS) number, the commercial brand, and the amount of compound required to yield 0.0108% (w/v) of total nitrogen are shown.

Product	Weight (in 100mL)	CAS Number	Brand
Potassium nitrate	78 mg	7757-79-1	Merck
Sodium nitrite	26 mg	7632-00-0	Sigma-Aldrich
Ethylamine hydrochloride	68 mg	557-66-4	VWR
1,5-Diaminopentane (= cadaverine)	42 mg	462-94-2	Alfa Aesar
Ammonium chloride	41 mg	12125-02-9	Merck
L-Ornithine monohydrochloride	76 mg	3184-13-2	Sigma-Aldrich
D-tryptophan	92 mg	153-94-6	Fluka
Urea	27 mg	57-13-6	Merck

2.7.2.4 *Growth in vitamin-free medium*

The ability of strains to grow in a medium lacking vitamins was determined by inoculating these into a vitamin-free medium (Yeast Carbon Base without vitamins and 60% (w/w) of D-glucose). In order to get rid of all vitamins stored by yeast cells in their vacuoles, these were starved in a saline solution for 4 hours (25°C) and then further starved in the vitamin-free medium (5 mL) for 72 h at 25ºC and 150 rpm. Next, 200 µL of the starved cells were added to 9.8 mL of vitamin-free medium and incubated for 3 weeks at 25°C. Scoring of results was done as for the carbon assimilation tests performed in liquid medium (see 2.7.2.2).

2.7.2.5 Growth in media of high osmotic pressure

Most yeast species are able to grow at glucose concentrations up to 40%. Nevertheless, yeasts isolated from habitats with high sugar and salt concentrations are usually resistant to high osmotic pressures ranging from 50% to 70% of glucose (Lievens *et al.,* 2015). Three different types of broth media were used in this study to test for osmotolerance:

- 50% (w/w) glucose dissolved in a 1% (w/v) solution of yeast extract;
- 60% (w/w) glucose dissolved in a 1% (w/v) solution of yeast extract;
- and 10% sodium chloride, 5% glucose and 0.67% of Bacto Yeast Nitrogen Base in demineralized water (w/v, in all cases).

All these media were filter sterilized to prevent caramelization of sugars (Figure 2.5) and aliquoted in sterile glass tubes (4.9 mL each). Tubes were inoculated with 100 µL of a cell suspension (prepared as explained in previous tests) and incubated at 25°C for up to 12 days. The ability to grow in the different test media was visually assessed and scored as a positive result.

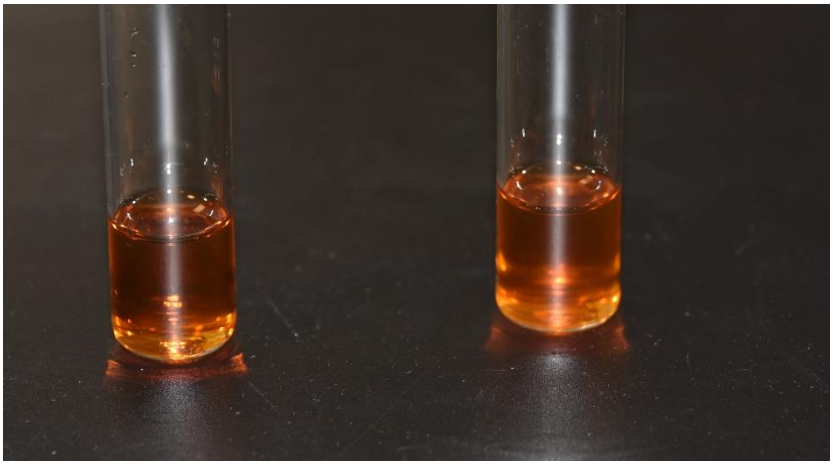

Figure 2.5: Typical caramelization of sugars observed after autoclaving a broth containing a high concentration (≥50%, w/w) of glucose.

2.7.2.6 Growth at different temperatures

The optimal growth temperature of most yeasts ranges between 20°C and 28°C. Nonetheless, optimal growth temperatures vary greatly, particularly if the strains were isolated from specific habitats (Kurtzman *et al.*, 2011a).

In this study, the growth ability of isolates at different temperatures was determined in both liquid (0.67% Bacto Yeast Nitrogen Base, 1% glucose) and solid (glucose-peptone-yeast extract agar: 8% glucose, 1% Bacto Peptone and 4% agar) media. The liquid medium was filter sterilized, aliquoted into sterile glass tubes (4.9 mL each) and inoculated with 0.1 mL of an 85% transmittance cell suspension prepared as previously explained. Inoculated tubes were incubated at 4°C, 12°C, 25°C, 30°C, 37°C and 40°C for up to 3 weeks. Development of growth in the test tubes was checked visually. On the other hand, plates of the solid medium were streaked with the strains, wrapped in aluminium foil to prevent desiccation and incubated at 4°C, 12°C, 25°C, 30°C, 37°C and 40°C for up to 3 weeks. Plates were inspected for growth frequently and those containing colonies were scored as positive results.

2.7.2.7 Splitting of arbutin

The test for the splitting of arbutin is an alternative to the arbutin assimilitation asay (performed as detailed in section 2.7.2.2 for other carbon sources). The basal test medium for this test contained 0.5% arbutin (w/v; Molekula), 1% yeast extract and 2% agar, and it was sterilized by autoclaving. Once the basal medium had been cooled down to 50ºC, 2 mL of a filter-sterilized 1% solution of ferric ammonium citrate (w/v; Sigma-Aldrich) were added for every 100 mL of agar base. The medium was poured into sterile Petri dishes and left to settle. This arbutin agar was then streaked with young cell cultures and incubated for 2-7 days at 25°C. Development of a dark brown colour around yeast colonies, due to the reaction of the hydroxyquinone released from the splitting of arbutin with the ferric salts present in the medium (Figure 2.6), was interpreted as a positive result.

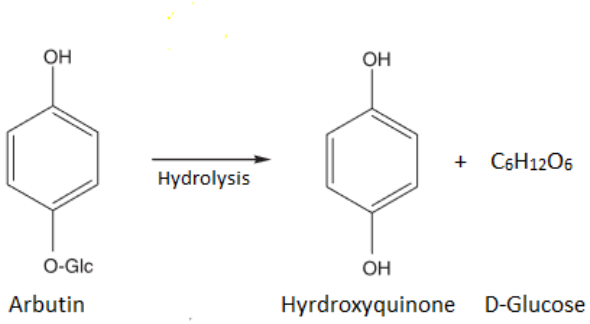

Figure 2.6: Metabolic hydrolysis of arbutin to hydroxyquinone and D-glucose in yeasts (Source: modified from Bang *et al.,* 2008)

2.7.2.8 Acid production from glucose

This phenotypic test was carried out using Custer's chalk agar, which contains 5% of glucose, 0.5% of calcium carbonate (Prolabo), 0.5% of yeast extract and 2% of agar (w/v, in all cases). If the yeast strains produce significant amounts of acids, the calcium carbonate slightly dissolves creating transparent halos in the area surrounding yeast colonies. In this study, test plates were inoculated in a straight line with yeast colonies grown on YM agar or PDA and incubated at 25°C for up to 2 weeks. Strains yielding transparent halos were considered positive for this test.

2.7.2.9 Hydrolysis of urea

Most ascomycetous yeasts are unable to hydrolyze urea, whereas this phenotype is common among basidiomycetous genera such as *Cryptococcus* and *Rhodotorula* (Hagler and Ahearn, 1981). Determining the presence of urease activity in yeasts can be done by culturing these on Christensen's urea agar (Christensen, 1946). This medium was prepared by dissolving 0.1 g of Bacto Peptone, 0.5 g of sodium chloride (Merck), 0.2 g of dihydrogen phosphate (Sigma-Aldrich), and 1.2 µg of phenol red per 100 mL of demineralized water and adjusting the pH to 6.8. Next, 2 g of agar were dissolved and the mixture was autoclaved. Finally, once the agar was cooled down to 50°C, 4.5 mL of the mixture and 0.5 mL of a filter-sterilized solution containing 20% (w/v) of urea were added to glass tubes. After mixing, tubes were slanted and the agar was allowed to solidify. Tubes were inoculated by streaking the strains on the slant surface and incubated at 25°C for up to 2 weeks. Development of a red colour in the agar was interpreted as a positive result.

2.7.2.10 Lipase activity

Lipase activity of the strains was assessed by the Tween opacity test developed by Slifkin (2000). Briefly, this medium was prepared by dissolving 1 g of Bacto Peptone, 0.5 g of sodium chloride, 10 mg of calcium chloride and 1.5 g of agar per 100 mL of demineralized water and sterilized by autoclaving. After cooling down to 50ºC, 0.5 mL of sterile Tween 80 (Sigma-Aldrich; autoclaved in a different bottle than the basal medium) were added per each 100 mL of the agar base. Inoculation of the test plates was done by spotting the strains in 10-mm circular areas. If the yeasts can secrete lipolytic enzymes, such as esterases and phospholipases, liberated fatty acids bind with

the calcium present in the medium and forms visible soluble crystals around the inoculation site. Plates were incubated at 25°C for 6 days.

2.7.2.11 Resistance to cycloheximide

Resistance to cycloheximide was tested in filter-sterilized broth media containing 0.67% Bacto Yeast Nitrogen Base, 0.5% of D-glucose and 0.1% or 0.01% of cycloheximide (Sigma-Aldrich) (w/v, in all cases). Tubes were inoculated with an 85% transmittance cell suspension and incubated at 25°C up to 12 days. Development of growth in the media containing cycloheximide was visually evaluated.

2.7.2.12 Tolerance to 1% Acetic acid

Tolerance to acetic acid was tested in a solid medium containing this organic acid. The basal agar medium contained 1% of D-glucose, 0.1% of tryptone (Sigma), 0.1% of yeast extract, and 2% of agar (w/v, in all cases), and was sterilized by autoclaving. Once the media was cooled down to 50°C, 1% (v/v) of glacial acetic acid (Merck) was added, and the resulting mixture was poured into sterile plastic Petri dishes. Test plates were inoculated by streaking the strains on the agar surface and incubated at 25°C for up to 6 days. Plates were visually inspected at regular intervals and those containing yeast colonies were considered as positive results.

2.7.2.13 Gelatin liquefaction

The ability to liquefy gelatin (gelatinase activity) is of reduced diagnostic value since the majority of yeasts are non-proteolytic (Kurtzman, 2011b). Nevertheless, this is a test generally performed in taxonomic descriptions and, therefore, it was also included in the present study. The test medium was prepared by dissolving 10 g of gelatin (Sigma-Aldrich), 0.5 g of D-glucose and 0.67 g of Bacto Yeast Nitrogen Base per 100 mL of demineralized water. The medium was then autoclaved and aliquoted into sterile glass tubes (9 mL each). Yeasts were inoculated by stabbing the tubes with young colonies grown on YM agar or PDA, and test tubes were incubated at 25°C for up to 3 weeks. Yeast-mediated liquefaction of the gelatin contained in the tube was scored as a positive result. As gelatin normally liquefies at ≥25ºC, test tubes were introduced into the refrigerator for *c.*30 minutes in order to confirm that liquefaction was due to the gelatinase activity of tested strains. A gelatinase-producing bacterial strain (*Staphylococcus aureus* ATCC 29213^{T}) was used as a positive control in these tests (Figure 2.7).

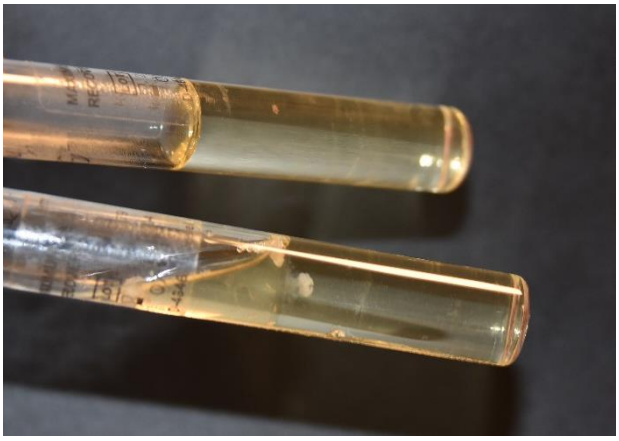

Figure 2.7: Example of a positive result for gelatinase production observed for a bacterial strain (bottom) used as positive control, and comparison with a negative result (top).

2.7.2.14 Microaerobiosis and anaerobiosis

Growth under microaerobiosis was determined by culturing isolates on PDA and incubating the plates at 25°C for 5 days into a candle jar (Figure 2.8A). Additionally, growth under anaerobic conditions was tested by streaking isolates on PDA and incubating the plates into a jar containing an anaerobiosis generator sachet and anaerobiosis indicator (Microbiology Anaerocult, Merck; Figure 2.8B) for 4 days at

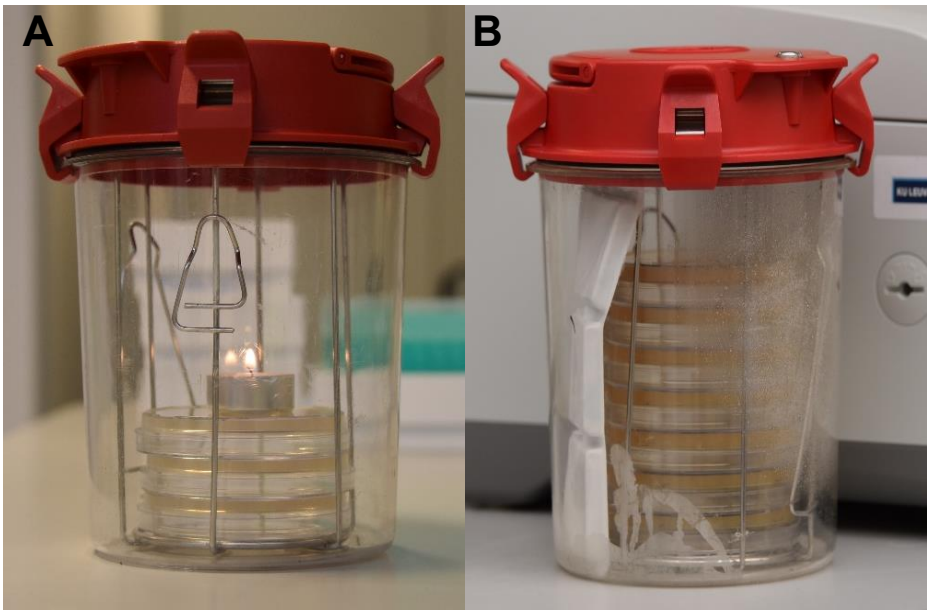

Figure 2.8: Experimental setup of a candle jar (A) and a jar with an anaerobiosis generator sachet (B) for testing growth under microaerobic and anaerobic conditions, respectively.

25°C. In both tests, the appearance of colonies on the plates was recorded as a positive result.

2.7.2.15 Growth on blood agar

Certain yeast species, including some members of the ascomycetous genus *Candida*, can lyse red blood cells, which is considered to be an important virulence factor that helps pathogenic microorganisms to survive inside animal hosts (Wan *et al.,* 2015). The hemolytic activity of the strains tested in this study was determined by streaking these onto Columbia blood agar plates (VWR) and incubating these for 3 days at 25°C. Hemolytic strains typically form an halo of blood digestion around the colonies, whereas non-hemolytic strains do not yield such an halo. The Gram-positive bacterium *Staphylococcus aureus* ATCC 29213^{T} was used as a positive control in these tests (Figure 2.9).

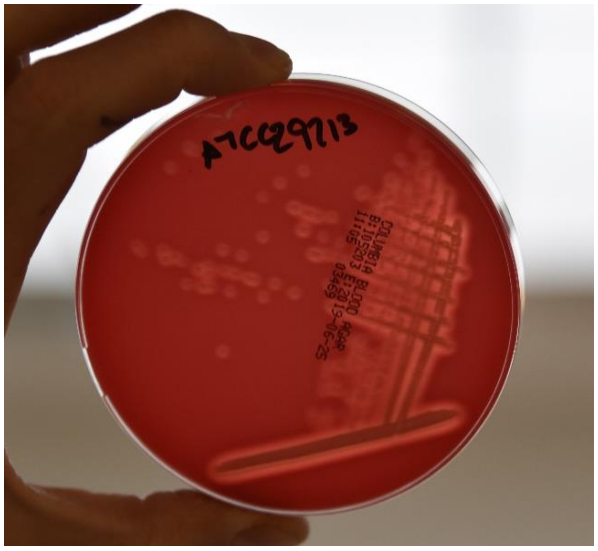

Figure 2.9: Hemolytic phenotype of *Staphylococcus aureus* ATCC 29213^{T}, used in this study as positive control for hemolysin production.

2.7.3 Growth ability in different types of nectar

Flowers are highly variable in nectar chemical composition, which can affect the ability of microorganisms to survive and thrive in this habitat (Pozo *et al.*, 2015). Therefore, we tested the ability of the potentially new yeast species characterized in this study (see Results) to grow in different types on nectar. Additionally, some reference strains from genus *Metschnikowia*, namely *M. bicuspidata* var. *bicuspidata*

(CBS 5575^T), *M. gruessii* (CBS 7657^T), *M. reukaufii* (CECT 10671^T), and *M. koreensis* (CBS 8854^T), were included in the tests for comparative analysis. Twelve different artificial nectars varying in total sugar concentration and composition, and nitrogen level were used in these experiments (Table 2.4), and the experimental setting involved a 96-well plate assay. The whole experiment was replicated three times on different days.

Table 2.4: Characteristics of the artificial nectars included in the 96-well plate assay used to test the growth ability of yeast strains in different types of nectar.

Type of artificial nectar	Total sugar concentration (% w/v)	Sugar composition†	Nitrogen level (%peptone, w/v)
AN01	15%	S	Low (0.003%)
AN02	15%	S	High (0.3%)
AN03	50%	S	Low (0.003%)
AN04	50%	S	High (0.3%)
AN05	15%	⅓S, ⅓G, ⅓F	Low (0.003%)
AN06	15%	⅓S, ⅓G, ⅓F	High (0.3%)
AN07	50%	⅓S, ⅓G, ⅓F	Low (0.003%)
AN08	50%	⅓S, ⅓G, ⅓F	High (0.3%)
AN09	15%	½G, ½F	Low (0.003%)
AN10	15%	½G, ½F	High (0.3%)
AN11	50%	½G, ½F	Low (0.003%)
AN12	50%	½G, ½F	High (0.3%)

† S: sucrose; G: glucose; F: fructose.

Briefly, artificial nectars were prepared by dissolving the corresponding amounts of each sugar (*i.e.* sucrose, glucose and/or fructose) and peptone in 50 mL of distilled water. Heating of these mixtures at 50ºC for approx. 30 min was required to completely dissolve the sugars. Subsequently, the artificial nectars were filter sterilized to prevent caramelization (Figure 2.5) and added (180 µL per well) to different rows of sterile 96-well plates (BRAND GmbH +Co KG). A positive control (1/10× tryptic soy broth; Sigma-Aldrich) and a negative control (sterile distilled water) treatment were included in all plates, and the order of the artificial nectars and controls in the rows of the plates (6 artificial nectar + 2 controls) was randomized in each replicate of the assay. Then, 20 µL of yeast cell suspensions (prepared as explained in section 2.7.2) were inoculated per well in columns 1–11, and 20 µL of sterile saline solution (0.85% w/v NaCl) per well in column 12. Inoculated plates were covered with a breathable membrane (Breathe-Easy®, Diversified Biotech) to prevent evaporation of the media and incubated for 7

days at 25ºC. Assay plates were read in a bench spectrophotometer (Multiskan GO, Thermo Fisher) at a wavelength of 600 nm after 72 h and 7 days of incubation. Results obtained for each strain were normalized (*i.e.* substraction of the optical density (OD) values obtained in the negative controls, followed by division by the OD of the corresponding positive control) and further analyzed by Principal Component Analysis (PCA), as implemented in R. PCA biplots were created using the R package 'ggbiplot' (Wickham, 2006; Vu, 2011).

3 RESULTS

3.1 Occurrence of yeasts in floral microhabitats

The overall frequency of yeast occurrence in the 91 samples analyzed was 55%. Mycelial fungi and chloramphenicol resistant bacteria were detected in a small number of samples (12% and 11%, respectively), and were excluded from further analyses. Distribution of yeast occurrence across the samples originating from different floral microhabitat was as follows: 15/30 (50%) for nectar, 12/31 (38.7%) for stamina, and 23/30 (76.7%) for styles. Therefore, there was a significant difference in yeast occurrence between the three floral microhabitats analyzed (chi-square statistic = 9.315, P = 0.0095).

3.2 Observed and estimated OTU richness

A total of 20 yeast OTUs were identified by using a 3% DNA dissimilarity cut-off value ($OTU_{0.03}$) in the mothur analyses when all samples from the three floral microhabitats considered were combined into a single analysis (Table 3.1). A Venn diagram was constructed then to compare the observed OTU richness found per floral part (Figure 3.1). Stamina and styles showed a higher OTU richness than nectar (14 and 11 OTUs, respectively, *vs.* 7), but five OTUs (*i.e.* 25% of the total) were shared by the three habitats.

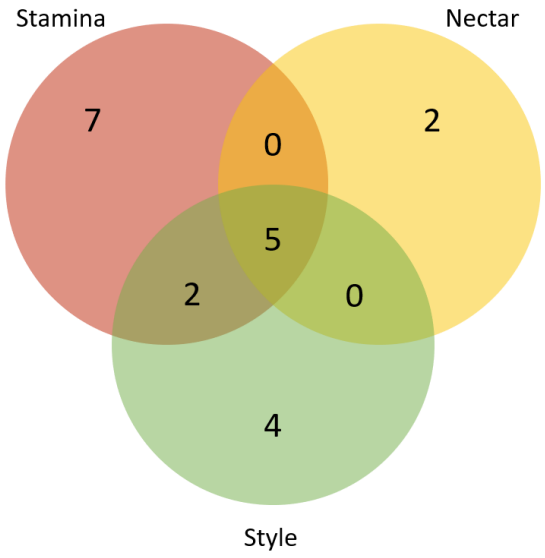

Figure 3.1: Venn diagram displaying the OTU richness found in the floral microhabitats analyzed in this study.

Recovered OTUs varied widely in their relative abundance (1 to 21 samples, mean ± S.D. = 3.85 ± 5.36). Nevertheless, most OTUs occurred just in a small proportion of samples, which led to a rank-abundance plot displaying a long right-hand tail (Figure 3.2).

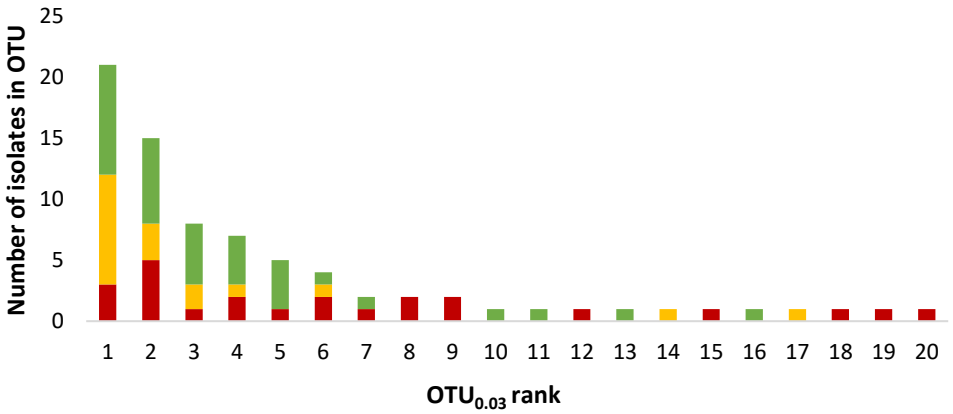

Figure 3.2: Bar chart displaying the rank abundance of observed yeast OTUs. The relative frequency of occurrence in the studied floral microhabitats is indicated by different colors: stamina (**red**), nectar (**orange**), and styles (**green**).

The $OTU_{0.03}$ accumulation curve obtained for the whole data set was close to reaching a plateau for the number of samples analyzed (*n* = 91, Figure 3.3). However, rarefaction-based methods estimated that the richness for the studied yeast community ranged from 40 to 42 OTUs (according to the Chao2 and ICE estimators, respectively; Figure 3.3, Table 3.1). Therefore, our sampling and culturing protocols recovered only 47%-50% of the estimated number of the yeast OTUs occurring in floral parts of *M. polymorpha* (Table 3.1).

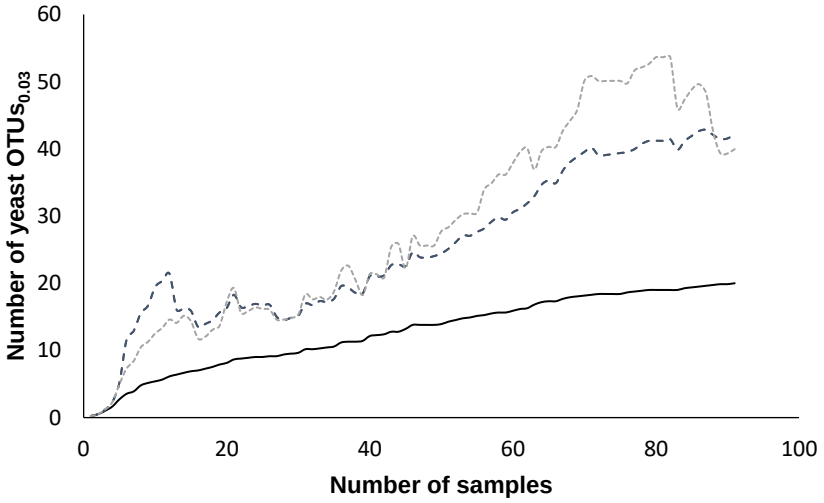

Figure 3.3: Graphical representation of sample based rarefaction curves and nonparametric estimators (ICE, long dashes; and Chao2, dotted line) of $OTU_{0.03}$ richness of floral microorganisms obtained for our data set.

Separate analyses of OTU richness for nectar, stamen and style samples yielded different results depending on the floral microhabitat considered. Yeast $OTU_{0.03}$ accumulation curves reached in all cases a plateau for the number of samples analyzed (*n* = 30, 31, and 30, for nectar, stamina, and styles, respectively), but the number of observed OTUs was uneven (7, 14 and 11, respectively; Figure 3.4). Non-parametric estimators yielded in all cases values of expected OTU richness that exceeded those actually observed, but the proportion of OTUs recovered by our methods varied widely depending on the estimator and floral part, being higher for stamina than for styles and nectar (Table 3.1).

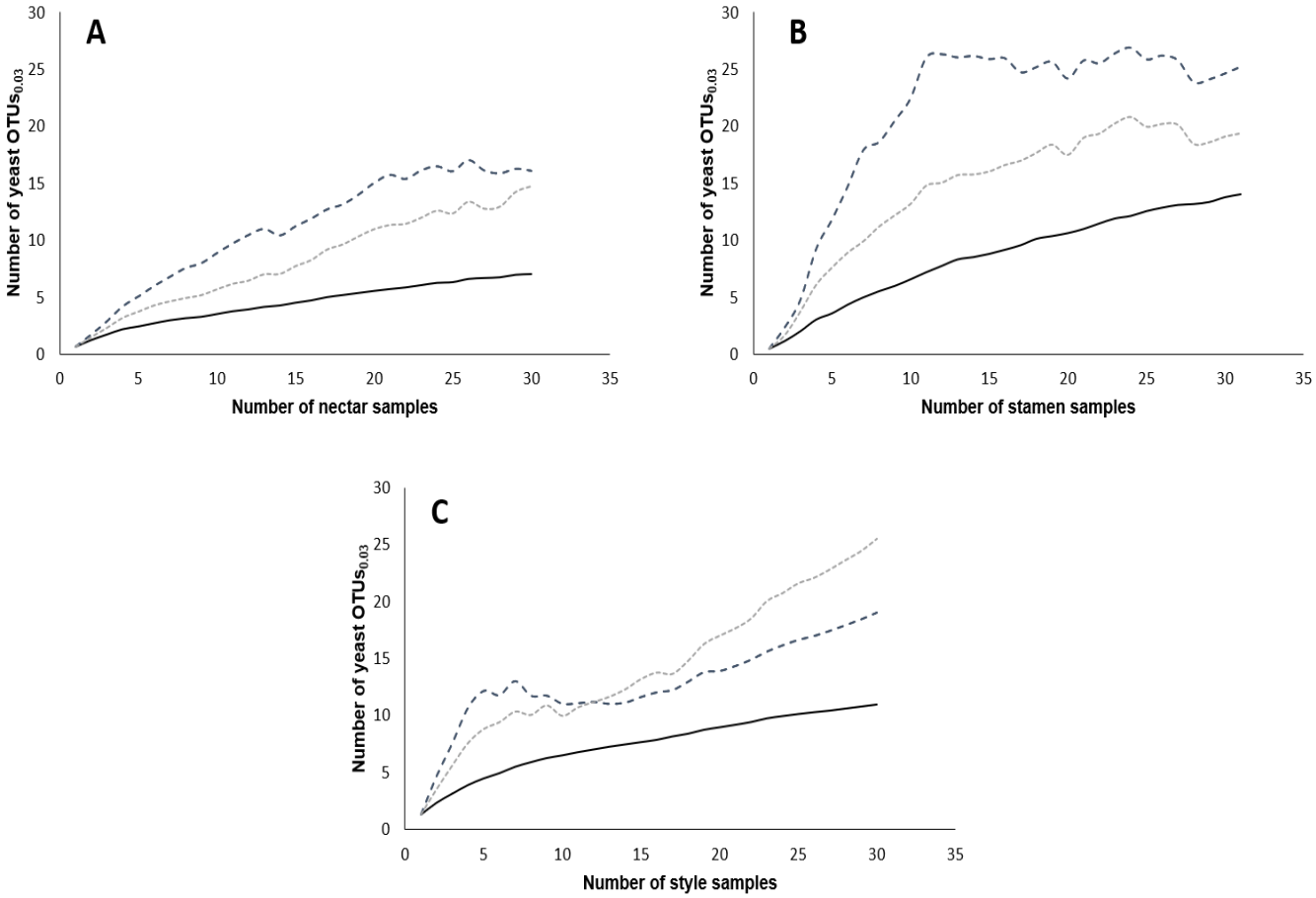

Figure 3.4: Graphical representation of the sample-based rarefaction curves and nonparametric estimators (ICE, long dashes; and Chao2, dotted line) of $OTU_{0.03}$ richness of floral microorganisms obtained for nectar (a), stamina (b), and styles (c).

Table 3.1: Observed and expected $OTU_{0.03}$ richness of floral yeast communities of *Metrosideros polymorpha.*

Floral microhabitat	Observed number of OTUs	ICE estimator	Chao2 estimator	OTU recovery*
Nectar	7	16	15	43.8% / 46.7%
Stamina	14	25	22	56% / 63.6%
Styles	11	19	26	57.9% / 42.3%
TOTAL	20	42	40	47.6% / 50%

*Percentages of $OTUs_{0.03}$ recovery as calculated in relation to the ICE and Chao2 non-parametric estimators of expected richness, respectively.

3.3 Phylogenetic diversity and taxonomic affiliation

Recovered yeast OTUs mostly belonged to the Ascomycota phylum (75%, *vs.* the 25% representing members of the Basidiomycota). The taxonomic status of such OTUs is indicated in Table 3.2. *Metschnikowia* and *Aureobasidium* were the two yeast genera most commonly recovered (23% and 16% of samples yielding yeast isolates, respectively). Other genera identified were *Starmerella* (9%)*, Debaryomyces* (7.7%)*, Rhizosphaera* (5.5%)*, Wickerhamiella* (4.4%)*, Candida* (6.5%)*, Rhodotorula* (3.3%)*, Papiliotrema* (2.2%)*, Kodamaea* (1.1%), *Priceomyces* (2.2%*), Filobasidium* (1.1%), and *Dimennazyma* (1.1%). Notably, two of the OTUs found in the microbiological survey (OTU_11 and OTU_12, each occurring in a single sample coming from styles and stamina, respectively) had low LSU rRNA gene sequence identity with other type strains (91% and 95%, respectively, as determined in BLAST searches; Table 3.2) and, therefore, were considered as potentially new yeast taxa worth of further characterization. A detailed analysis of such isolates is included in sections 3.4 and 3.5.

Figures 3.5 and 3.6 show the LSU rRNA gene ML phylogenetic trees built for the ascomycetous and basidiomycetous OTUs, respectively, that were isolated from *M. polymorpha* flowers, together with their best matches in the Genbank. NJ and MP trees were also constructed and, in general, these confirmed the robustness of the phylogenetic ML inference.

Table 3.2: Yeast operational taxonomic units (OTUs) that were identified in this study based on a 3% DNA dissimilarity cut-off value.†

OTU	Representative isolate	Phylum	Family	Closest match in GenBank	Sequence identity	No. of isolates
OTU_01	JK 43	Ascomycota	Metschnikowiaceae	*Metschnikowia rancensis*	98%	21
OTU_02	JK 96	Ascomycota	Saccotheciaceae	*Aureobasidium melanogenum*	100%	15
OTU_03	JK 74	Ascomycota	*Incertae sedis*	*Starmerella bombicola*	98%	8
OTU_04	JK 55	Ascomycota	Saccharomycetaceae	*Debaryomyces vindobonensis*	100%	7
OTU_05	JK 93	Ascomycota	Venturiaceae	*Rhizosphaera macrospora*	98%	5
OTU_06	JK 85	Ascomycota	Trichomonascaceae	*Wickerhamiella azyma*	98%	4
OTU_07	JK 47	Ascomycota	*Incertae sedis*	*Candida hawaiiana*	100%	2
OTU_08	JK 24	Basidiomycota	*Incertae sedis*	*Rhodotorula nothofagi*	100%	2
OTU_09	JK 29	Basidiomycota	Tremellaceae	*Papiliotrema terrestris*	99%	2
OTU_10	JK 88	Ascomycota	*Incertae sedis*	*Candida sorbosivorans*	100%	1
OTU_11*	JK 58	Ascomycota	*Incertae sedis*	*Candida heveicola*	91%	1
OTU_12*	JK 22	Ascomycota	*Incertae sedis*	*Candida duobushaemulonis*	95%	1
OTU_13	JK 86	Ascomycota	*Incertae sedis*	*Kodamaea ohmeri*	100%	1
OTU_14	JK 12	Ascomycota	Debaryomycetaceae	*Priceomyces melissophilus*	99%	1
OTU_15	JK 02	Ascomycota	Debaryomycetaceae	*Priceomyces melissophilus*	99%	1
OTU_16	JK 65	Ascomycota	*Incertae sedis*	*Candida apicola*	100%	1
OTU_17	JK 09	Ascomycota	*Incertae sedis*	*Candida bombi*	100%	1
OTU_18	JK 77	Basidiomycota	*Incertae sedis*	*Rhodotorula mucilaginosa*	100%	1
OTU_19	JK 27	Basidiomycota	Filobasidiaceae	*Filobasidium globisporum*	97%	1
OTU_20	JK 94	Basidiomycota	Naemateliaceae	*Dimennazyma cisti-albidi*	99%	1

† Matched species were based on BLAST search results against type strains. The taxonomic affiliation (phylum and family) of the different species identified was confirmed by searching the Index Fungorum database (Index Fungorum Partnership, 2018). OTUs representing potentially new taxa are marked by an asterisk.

Interestingly, the different clades observed in the tree built for ascomycetous OTUs grouped together strains originating from the three different floral habitats under study (Figure 3.5). Nevertheless, the highest phylogenetic diversity of the ascomycetous OTUs was found for styles (Faith's PD = 2.37; 11 OTUs), followed by stamina (PD = 1.92; 9 OTUs), and then nectar (PD = 1.47; 7 OTUs). In contrast, all strains belonging to phylum Basidiomycota were isolated from stamina (Figure 3.6).

OTU_11 and OTU_12 clustered in the ML, NJ and MP trees together with *Candida heveicola* and *Candida duobushaemulonis* (100% bootstrap support in all cases). Furthermore, those OTUs formed a sister clade with *Metschnikowia rancensis* and *Candida hawaiiana*, which were the best BLAST hits of OTU_1 and OTU_7, respectively.

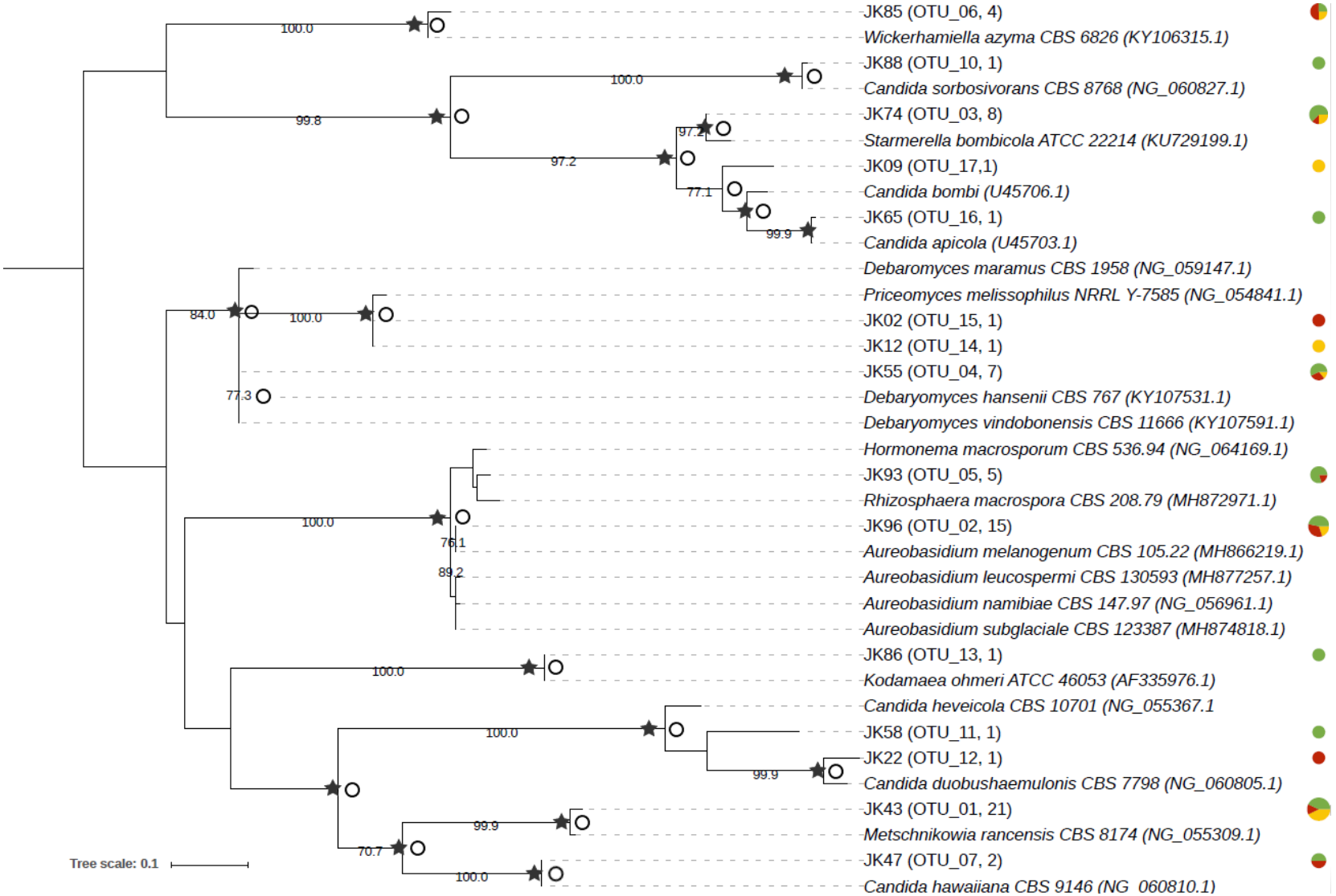

Figure 3.5: Maximum likelihood (ML) phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene from floral inhabiting ascomycetous yeasts (one representative strain per OTU) and representative type strains (best hits found in BLAST searches). For the OTUs retrieved from *Metrosideros polymorpha* flowers, the total number of isolates obtained in this study is shown between parenthesis. The pie charts on the tips of branches represent sample origin (in percentage): stamina (**red**), nectar (**orange**), and style (**green**). The size of the pie charts represents the number of isolates. Bootstrap percentages (based on 1000 replicates) above 70% are shown on branches. Stars and circles designate clades displaying >70% bootstrap support in the neighbor-joining and maximum parsimony trees, respectively. A time scale is included in the left bottom corner of the tree.

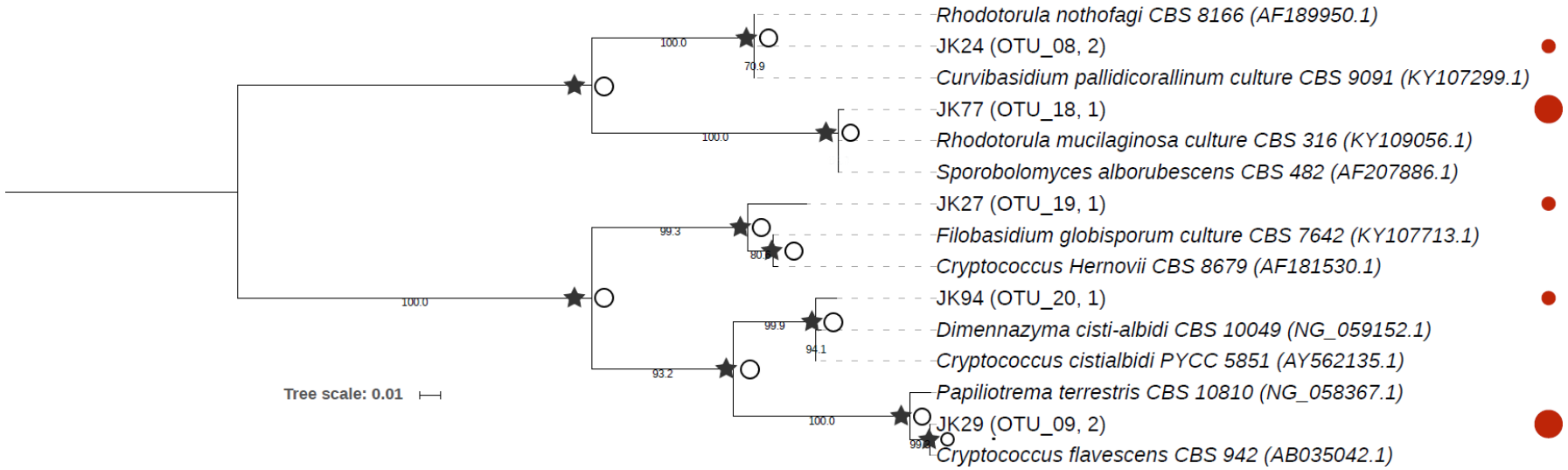

Figure 3.6: Maximum likelihood (ML) phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene from floral inhabiting basidiomycetous yeasts (one representative strain per OTU) and representative type strains (best hits found in BLAST searches). For the OTUs retrieved from *Metrosideros polymorpha* flowers, the total number of isolates obtained in this study is shown between parenthesis. The pie charts on the tips of branches represent sample origin (in percentage): stamina (**red**), nectar (**orange**), and style (**green**). The size of the pie charts represents the number of isolates. Bootstrap percentages (based on 1000 replicates) above 70% are shown on branches. Stars and circles designate clades displaying >70% bootstrap support in the neighbor-joining and maximum parsimony trees, respectively. A time scale is included in the left bottom corner of the tree.

3.4 Phylogenetic and phenotypic characterization of potentially new yeast taxa

As described above (section 3.3), community analysis of flower-dwelling yeasts of *M. polymorpha* identified two OTUs (OTU_11 and OTU_12; Table 3.2) that potentially represent new ascomycetous species. In order to clarify the taxonomic status of such OTUs, their phenotypic and (phylo)genetic characterization was performed according to the current standards in yeast taxonomy.

Reanalysis of the samples of *Metrosideros polymorpha* yielding OTU_11 and OTU_12 in search of additional yeast colonies of similar morphology yielded three additional strains from OTU_11, but none from OTU_12. Therefore, subsequent (phylo)genetic and phenotypic analyses (see 3.4.1 to 3.4.4 and 3.5) were performed on a total of five flower strains (four belonging to OTU_11 [designated as JK58_2, JK58_4, JK58_5, and JK58_6] and one to OTU_12 [JK22]) and the reference strains from genus *Metschnikowia* listed in section 2.7.2.

A transmittance of approx. 85% resulted in a colony forming unit (CFU) of 10^6 per mL for OTU_11 and OTU_12.

3.4.1 Phylogenetic relatedness

The sequences of the D1/D2, ITS and the SSU regions of the rRNA operon from all strains representing undescribed taxa were analyzed along with those retrieved from the GenBank for closely related species (Figures 3.7 and 3.8). However, due to the scarcity of SSU sequences in the GenBank for type strains closely related to OTU_11 and OTU_12, no phylogenetic tree was obtained for this region. Instead, Table 3.3 provides an overview of the SSU rRNA gene sequence identity of different type strains with OTU_11 and OTU_12. Unfortunately, due to inconvenient circumstances, only three ITS sequences of high quality were obtained for our strains (JK22 from OTU_12, and JK58_2 and JK58_5, both from OTU_11).

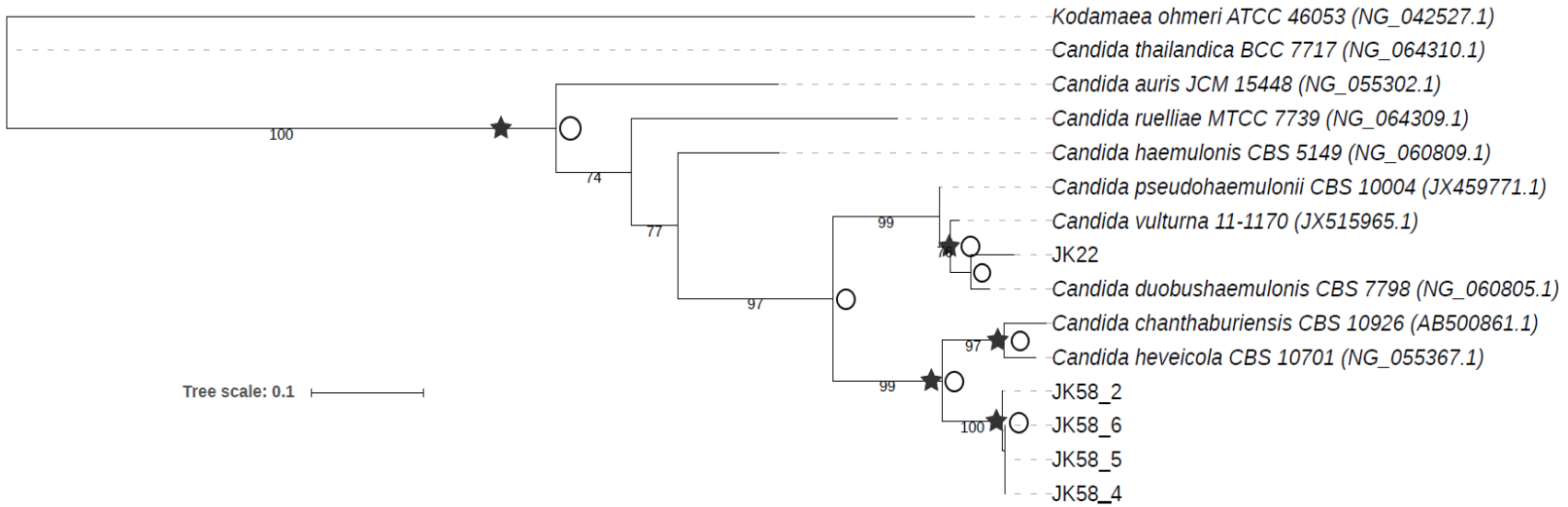

Figure 3.7: Maximum likelihood (ML) phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene from OTU_11 (strains JK58_2, JK58_4, JK58_5, and JK58_6) and OTU_12 (JK22), and representative type strains (best hits found in BLAST searches). Bootstrap percentages (based on 100 replicates) above 70% are shown on branches. Stars and circles designate clades displaying >70% bootstrap support in the neighbor-joining and maximum parsimony trees, respectively. A time scale is included in the left bottom corner of the tree.

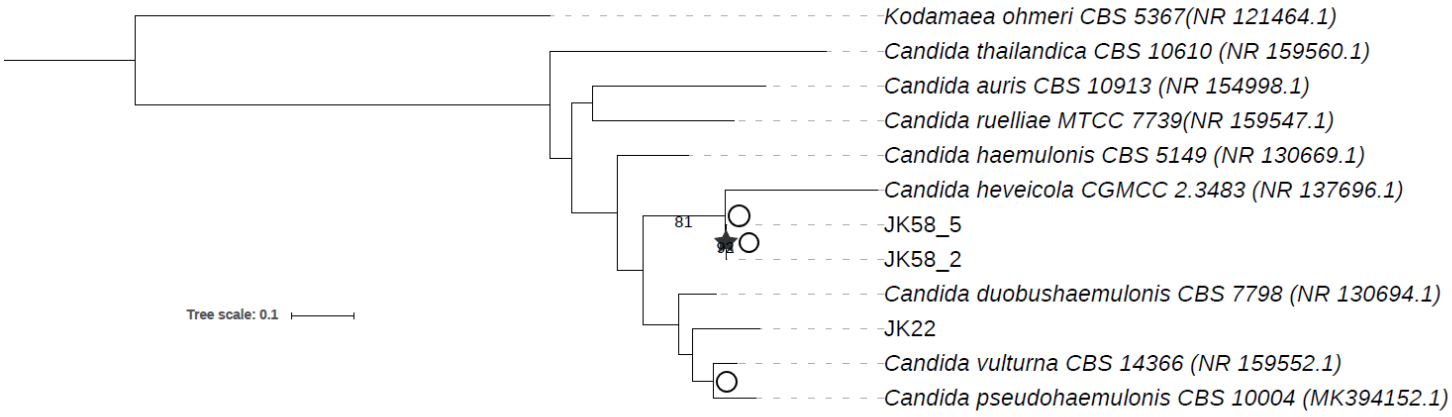

Figure 3.8: Maximum likelihood (ML) phylogenetic tree based on the ITS region of the rRNA operon from OTU_11 (strains JK58_2 and JK58_5) and OTU_12 (JK22), and representative type strains (best hits found in BLAST searches). Bootstrap percentages (based on 100 replicates) above 70% are shown on branches. Stars and circles designate clades displaying >70% bootstrap support in the neighbor-joining and maximum parsimony trees, respectively. A time scale is included in the left bottom corner of the tree.

Table 3.3: Overview of the closest relative type strains of OTU_11 and OTU_12 based on the sequence of the SSU region of ribosomal RNA operon, as determined in BLAST searches of the GenBank database.

Type strains	GenBank accession no.	Percentage of identity	
		OTU_12	OTU_11†
Candida duobushaemulonis CBS 7798^T	MK394153.1	99.27%	98.00%
Candida pseudohaemulonis CBS 10004^T	MK394152.1	99.27%	98.18%
Candida haemulonii JCM 3762^T	NG_063413.1	98.54%	97.82%
Clavispora optuntiae CBS 7068^T	MK394148.1	95.99%	96.01%
Clavispora reshetovae CBS 11556^T	NG_062775.1	95.26%	95.46%
Candida fructus NRRL Y-17072^T	MG050883.1	95.43%	95.11%

† The four different strains of OTU_11 (JK58_2, JK58_4, JK58_5, and JK58_6) had identical sequences and are therefore excluded from the table.

OTU_11 clustered in the phylogenetic tree built from LSU rRNA sequences as a sister clade of *Candida heveicola* and *Candida chanthaburiensis* (8.7% and 9.5% of variation in the nucleotide sequences, respectively), and such placement was well supported by all methods used for phylogenetic inference (Figure 3.7). Notably, OTU_11 also clustered with *C. heveicola* in the tree obtained for ITS sequences, thus suggesting the phylogenetic relatedness of these two yeast lineages (Figure 3.8). However, no ITS sequence of the type strain of *C. chantaburiensis* was found in the GenBank, nor any SSU rRNA gene sequence for the type strain *C. heveicola*. Therefore, the relatedness between the *C. heveicola/C. chantaburiensis* group and our OTU_11 could not be further assessed.

On the other hand, JK22 (OTU_12) clustered in the phylogenetic trees based on LSU rRNA and ITS sequences in a clade containing *Candida pseuodohaemulonii*, *Candida vulturna,* and *Candida duobushaemulonis* (7.1%, 6.5% and 4.9% of sequence variation, respectively; Figures 3.7 and 3.8). However, while the position of JK22 in this clade was well supported in the tree built from LSU rRNA sequences (Figure 3.7), a weaker support of such clustering was found in the phylogenetic analysis of ITS sequences (Figure 3.8). On the other hand, the SSU rRNA sequence obtained for JK22 showed >97% of similarity with those available in the GenBank for *C. pseuodohaemulonii*, *C. vulturna,* and *C. duobushaemulonis*.

3.4.2 M13 subtyping

M13-based fingerprinting resulted in 8-12 different bands per strain, as shown in Figure 3.9. Two distinct band patterns were noticed: one for JK22 and a second one shared by JK58_2, JK5858_4, JK58_5, and JK58_6, therefore confirming that OTU_11 and OTU_12 are genetically different. In contrast, no intra-OTU variability was detected by using this subtyping technique for OTU_11 strains. Some few faint bands were detected in the negative control (which contained no yeast DNA, only nuclease-free water), but these were in most cases different to those obtained for the studied yeast strains. The presence of unspecific bands in negative control has been previously reported for M13/RAPD subtyping of microorganisms, and might be due to contamination of the reaction mixture with non-target DNA and/or primer artefacts (Herrero and Klemsdal, 1998).

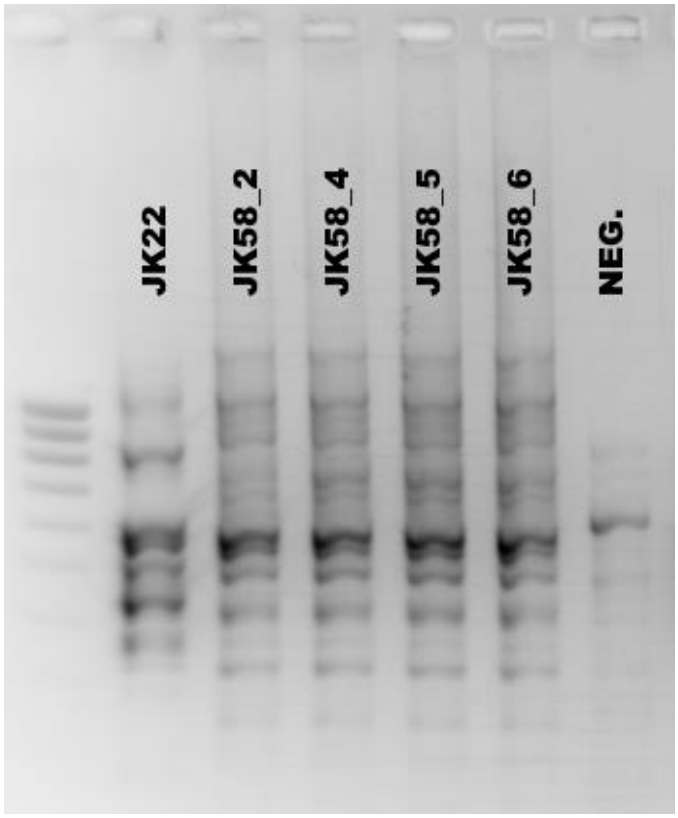

Figure 3.9: M13-based fingerprinting band profiles obtained for OTU_11 (JK58_2, JK58_4, JK58_5, JK58_6) and OTU_12 (JK22) strains. The first column corresponds to a 100-bp (range: 100-1000 bp) DNA ladder.

3.4.3 Morphological analysis

After 48 h of incubation at 25ºC on YM agar and PDA, OTU_12 (strain JK22) formed white to cream-colour, soft and butyrous, and flat to slightly domed colonies, with an entire margin and a size ranging from 0.5 mm to 1 mm (Figure 3.10A). Colonies of OTU_11 (strains JK58_2, JK58_4, JK58_5, and JK58_6) on the same culture media and incubation conditions were white, soft and butyrous, flat or slightly domed, with a smooth or slightly wrinkled surface and entire margin. The size of OTU_11 colonies ranged from 1 to 2 mm (Figure 3.10B).

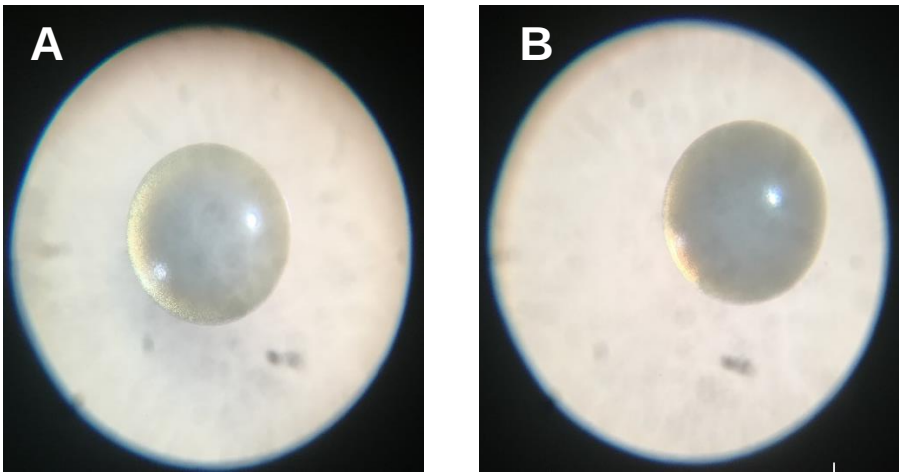

Figure 3.10: General aspect of the colonies from strains JK22 (OTU_12; A) and JK58_5 (OTU_11; B) after 48 h of incubation at 25ºC on YM agar. The size of the colonies shown in this figures was *c.*1 mm.

Cells from strain JK22 grown on PDA for 6 days at 25ºC were rounded to slightly ovoid (5.5-8.0 x 6.7-8.3 µm in size) and occurred singly or in parent-bud pairs (Figure 3.11). The presence of vacuoles of different sizes was fairly common, and cell budding was monopolar on a narrow base. No pseudohyphae were observed for this strain.

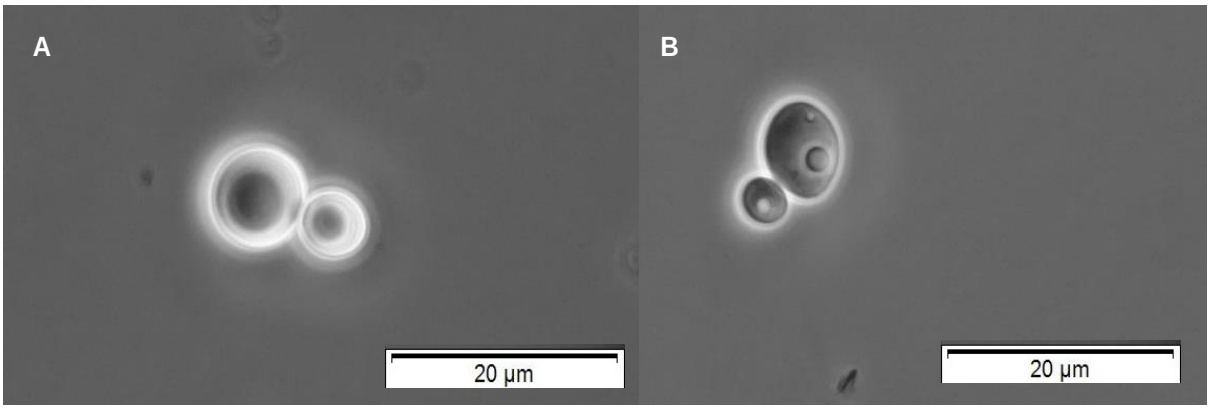

Figure 3.11: Photomicrographs of strain JK22 (OTU_12) after 6 days of growth at 25ºC on PDA.

The cells from OTU_11 strains were smaller than those of OTU_12 (3.2-4.9 x 3.3-5.0 µm; Figure 3.12). However, the cell morphology was also rounded to slightly ovoid, cells occurred singly or in small groups, and the presence of vacuoles was common. Budding of OTU_11 strains was generally multipolar on a narrow base, and no pseudohyphae were observed.

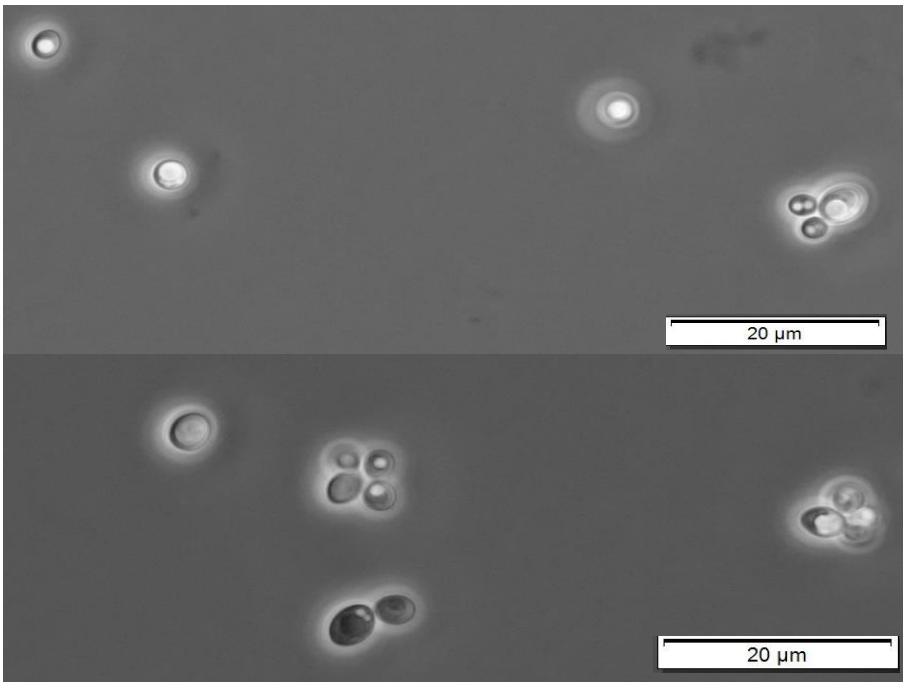

Figure 3.12: Photomicrographs of strain JK58_5 after 6 days of growth at 25ºC on PDA.

Visualization of the cells of OTU_11 and OTU_12 using a scanning electron microscope confirmed their rounded to oval shape and the size measurements obtained with the confocal optical microscope (Figure 3.13).

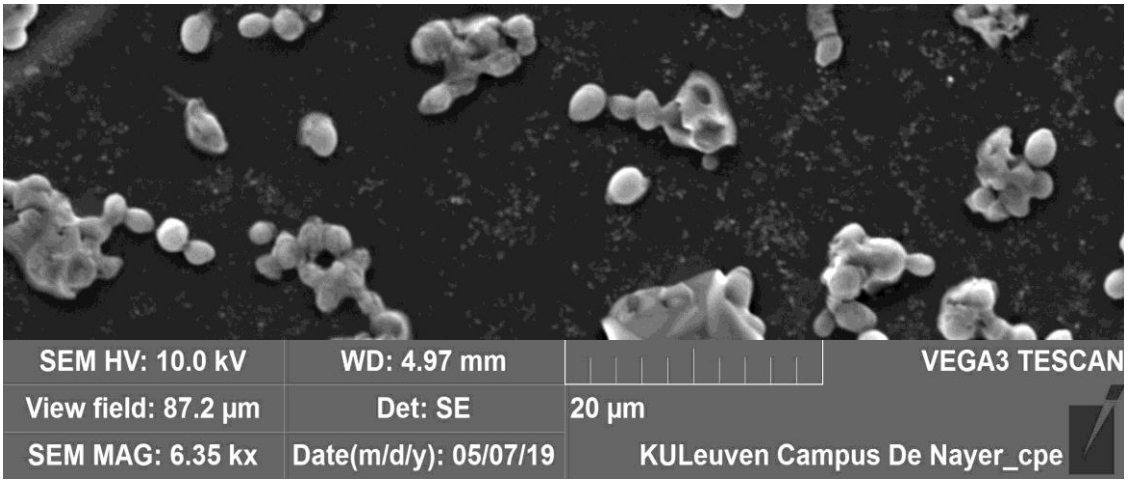

Figure 3.13: Scanning electron photomicrograph of cells from strain JK58_6 (OTU_11) after incubation on PDA for 3 days at 25ºC.

None of the mating experiments carried out in malt extract agar, restriction growth agar, and diluted (1:9 and 1:19) V8 agar gave any indication of the presence of a sexual cycle (*e.g.* ascospore production) in OTU_11 and OTU_12 strains.

3.4.4 Fermentation and assimilation of nutrient sources

The results of the fermentation assays obtained for OTU_11 and OTU_12 and the reference strains are shown in Table 3.4. Tests performed with the reference strains used for quality control yielded in most cases results that agreed with those reported in the literature (Wang *et al.,* 2008; Lachance, 2011; Cendejas-Bueno *et al.,* 2012). In general, OTU_11 and OTU_12 strains showed similar fermentation profiles. Both OTUs were able to produce gas from glucose (in less than 7 days) and trehalose (after 10 days), but not from galactose, maltose, lactose, melezitose, cellobiose and melibiose, even after prolonged incubation (33 days). However, whereas OTU_11 weakly fermented sucrose and raffinose, OTU_12 yielded a negative result for these carbohydrates. Notably, *Candida heveicola* and *C. duobushaemulonii*, which were identified as the closest relatives to OTU_11 and OTU_12, respectively, for the LSU rRNA gene (section 3.4.1), can also ferment both glucose and sucrose (Table 3.4). Three of the four OTU_11 strains tested showed a delayed positive fermentation of fructose, whereas the remaining strain (JK58_2) gave a strongly positive reaction (*i.e.* positive in less than 7 days). In contrast, OTU_12 was unable to ferment fructose. No data was found in the literature about the ability of *C. heveicola* and *C. duobushaemulonii* to ferment fructose.

Table 3.4: Fermentation characteristics of OTU_11 and OTU_12 in comparison to reference strains.†

Compound	Reference strains‡						OTU_12§	OTU_11§
	1	2	3	4	5	6		
Glucose	+	+*[s]	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	+	+	-	w/s
Maltose	-	-	-	-	-	l	-	-
Lactose	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	+	-	-	w
Trehalose	-	-	-	-	ND	ND	w/s	s
Melezitose	- [ND]	- [ND]	- [ND]	- [ND]	+	ND	-	-
Fructose	w [ND]	l [ND]	l [ND]	l [ND]	ND	ND	-	+(1)/ l(3)
Cellobiose	- [ND]	- [ND]	- [ND]	- [ND]	-	ND	-	-
Melibiose	- [ND]	- [ND]	- [ND]	- [ND]	-	ND	-	-

† Abbreviations: +, positive; -, negative; l, delayed positive; w, weak; s, slow (see section 2.7.2.1 for further details on the scoring system). ND: no data available.

‡ Reference strains used in this study for quality control: 1, *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^T); 2, *M. gruessii* (CBS 7657^T); 3, *M. reukaufii* (CECT 10671^T); and 4, *M. koreensis* (CBS 8854^T). Other reference strains not tested in this study: 5, *Candida duobushaemulonii* (CBS 7798^T); and 6, *C. heveicola* (CBS 10702^T). Results showing variations with those reported in the literature are indicated by an asterisk (*), and the previously reported observation is indicated (when available) between squared brackets.

§ Four different strains of OTU_11 (JK58_2, JK58_4, JK58_5, and JK58_6), and one of OTU_12 (JK22) were analyzed. In some cases, intra-OTU variability for OTU_11 is indicated by displaying between brackets the number of strains yielding a specific result.

The results obtained for carbon assimilation are shown in Table 3.5. Again, the results for the reference strains used as quality controls mostly agreed with those reported in previous studies (Wang *et al.,* 2008; Lachance, 2011; Cendejas-Bueno *et al.,* 2012). A remarkable exception was the inulin assimilation test, for which none of the results obtained for the reference strains matched those reported in the literature. Both OTU_11 and OTU_12 and all the reference strains were able to assimilate sucrose and glucose which, together with fructose, are the three main sugars present in floral nectar (Nicolson and Thornburg, 2007). Tested strains were also able to assimilate fructose, but no data on the ability of *C. heveicola* and *C. duobushaemulonii* to assimilate this latter monosaccharide was found in the literature. Regarding the other sugars present in trace amounts in nectar (Nicolson and Thornburg, 2007), all OTU_11 and OTU_12 strains yielded positive results for xylose, melezitose, and maltose, negative for arabinose (both L- and D- isomers) and melibiose, and variable for raffinose (slow positive and negative, respectively). Similarly, *C. heveicola* and *C. duobushaemulonii*

were able to assimilate some of these trace sugars. For OTU_11, intra-OTU variability was only observed in the melezitose assimilation assay, in which two strains (JK58_4 and JK58_5) showed profuse growth in 3 days and the remaining two (JK58_2 and JK58_6) only displayed slow growth.

Table 3.6. displays the results of the nitrogen assimilation tests. Except in a few cases (nitrate and cadaverine assimilation), the results obtained for the reference strains used as quality controls were in agreement with those reported in the literature (Wang *et al.,* 2008; Lachance, 2011; Cendejas-Bueno *et al.,* 2012). OTU_11 and OTU_12 showed almost the same nitrogen assimilation profile and yielded a positive result for all nitrogen sources tested. The only difference was observed for nitrite assimilation, as OTU_11 gave a strong positive growth reaction while OTU_12 displayed slow growth. No intra-OTU variability was detected for OTU_11. Both OTU_11 and OTU_12 but not *C. heveicola* and *C. duobushaemulonii* were able to assimilate nitrate and nitrite in two independent assays carried out on different days (Table 3.6) but, given the disagreement between our results and those reported in Lachance (2011) for reference strains of *Metschnikowia* spp., the positive reaction obtained in this study for nitrate should be further validated.

Table 3.5: Carbon assimilation profile of OTU_11 and OTU_12 in comparison to reference strains.†

Compound	Reference strains‡						OTU_12§	OTU_11 §
	1	2	3	4	5	6		
Glucose	+	+	+	+	+	+	+	+
Fructose	+ [ND]	+ [ND]	+[ND]	+[ND]	ND	ND	+	+
Inulin	+* [-]	+* [-]	+* [-]	+* [-]	+/-	-	+	+
Sucrose	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	+	+	-	s
Melibiose	-	-	-	-	-/v	-	-	-
Galactose	+	s	-	s	+	+	-	s
Lactose	-	-	-	-	-	-	-	-
Trehalose	+	-	s	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Melezitose	-	+	+	+	+	+	+	s(2)/ +(2)
Methyl-α-D-glucoside	-	-	-	+	-/w/l	w	s	-
Cellobiose	+	+	+	+	-	-	+	-
Salicin	+	-	+	-	-	+	+	-
L-Sorbose	+	s	s	+	+	+	s	+
L-Rhamnose	-	-	-	-	+	-	-	-
D-Xylose	+	-	-	+	l	l	s	s
L-Arabinose	s* [-]	-	-	-	-/w	-	-	-
D-Arabinose	-	-	-	-	l	-	-	-
D-Ribose	+* [-]	-	s	-	-/w	-	+	-
Ethanol	s	-	s	s	w/l	+	+	-
Glycerol	s	+	+	+	+	+	+	+
meso-Erythritol	-	-	-	-	-/l	-	-	-
Ribitol	+* [-]	s	s	s	+	+	s	s
Xylitol	+	+	s	+	+	ND	s	-
Galactitol	-	-	-	-	ND	+	-	s
D-Mannitol	+	s	+	+	+	+	+	+
D-Glucitol	+	s	+	+	+	+	+	+
myo-Inositol	-	-	-	-	-/w	-	-	-
Gluconolactone	-* [+]	-	+	s	+		+	+
2-Keto-D-gluconate	s	-	+	+	ND	ND	+	+
5-Keto-D-gluconate	s [ND]	- [ND]	- [ND]	- [ND]	ND	ND	-	-
DL-Lactate	-	-	-	-	-/w/l	-	-	-
Succinate	-	-	s	+	+	+	+	-
Citrate	-	-	-	-	+	+	s	-
D-Gluconate	s* [+]	s	+	+	+	ND	+	+
D-Glucosamine	-	-	s	+	+	+	+	+
N-Acetyl-D-glucosamine	+	-* [+/s]	+	+	ND	ND	+	+
Hexadecane	-	-	-* [w/s]	-	ND	-	-	-

† Abbreviations: +, positive; -, negative; l, delayed positive; w, weak; s, slow (see section 2.7.2.2 for further details on the scoring system). ND: no data available.
‡ Reference strains used in this study for quality control: 1, *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^T); 2, *M. gruessii* (CBS 7657^T); 3, *M. reukaufii* (CECT 10671^T); and 4, *M. koreensis* (CBS 8854^T). Other reference strains not tested in this study: 5, *Candida duobushaemulonii* (CBS 7798^T); and 6, *C. heveicola* (CBS 10702^T). Results showing variations with those reported in the literature are indicated by an asterisk (*), and the previously reported observation is indicated (when available) between squared brackets.
§ Four different strains of OTU_11 (JK58_2, JK58_4, JK58_5, and JK58_6) and one of OTU_12 (JK22) were analyzed. In some cases, intra-OTU variability for OTU_11 is indicated by displaying between brackets the number of strains yielding a specific result.

Table 3.6: Nitrogen assimilation profile of OTU_11 and OTU_12 in comparison to reference strains.†

Compound	Reference strains‡						OTU12§	OTU_11§
	1	2	3	4	5	6		
Nitrate	+* [-]	+* [-]	+* [-]	+* [-]	-	-	+	+
Nitrite	-	-	-	-	-	-	s	+
Ethylamine	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+
Cadaverine	-* [+]	+	+	+	+	+	+	+
Ammonium	+	+	+	+	ND	+	+	+
L-Ornithine	+	+	+	+	ND	ND	+	+
D-Tryptophan	+	+	+	+	ND	ND	+	+
Urea	+	+	+	+	ND	ND	+	+

† Abbreviations: +, positive; -, negative; l, delayed positive; w, weak; s, slow (see section 2.7.2.3 for further details on the scoring system). ND: no data available.
‡ Reference strains used in this study for quality control: 1, *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^T); 2, *M. gruessii* (CBS 7657^T); 3, *M. reukaufii* (CECT 10671^T); and 4, *M. koreensis* (CBS 8854^T). Other reference strains not tested in this study: 5, *Candida duobushaemulonii* (CBS 7798^T); and 6, *C. heveicola* (CBS 10702^T). Results showing variations with those reported in the literature are indicated by an asterisk (*), and the previously reported observation is indicated (when available) between squared brackets.
§ Four different strains of OTU_11 (JK58_2, JK58_4, JK58_5, and JK58_6) and one of OTU_12 (JK22) were analyzed. In some cases, intra-OTU variability for OTU_11 is indicated by displaying between brackets the number of strains yielding a specific result.

3.4.5 Other phenotypic traits

The results obtained in the assays testing for other relevant phenotypic traits are shown in Table 3.7. Once again, the phenotypes observed for the reference strains used as quality controls agreed with those previously reported (Wang *et al.,* 2008; Lachance, 2011; Cendejas-Bueno *et al.,* 2012). Incongruent results between our results and those of Lachance (2011) (*e.g.* for acid production and growth on vitamin-free medium) might

be due to small variations in the testing protocols (*e.g*. length of the cell starvation period before inoculating the test tubes) and should be further assessed. Unfortunately, some of the phenotypic tests carried out in the present study are not mandatory for describing new yeast taxa and, therefore, these are rarely performed, thus precluding comparison between our flower isolates and reference strains. OTU_11 and OTU_12 showed a similar profile for these additional tests and only differed in their osmotolerance (weak/slow growth *vs.* negative result, respectively, in the medium containing 60% of glucose), hydrolysis of Tween 80 (negative *vs.* positive), acid production from glucose (positive *vs.* weak), and ability to grow in anaerobiosis (positive *vs.* weak). No intra-OTU variability was detected for OTU_11.

Table 3.7: Other phenotypic traits of OTU_11 and OTU_12 in comparison to reference strains.†

Trait	Reference strains‡						OTU_12§	OTU_11§
	1	2	3	4	5	6		
Tolerance to NaCl (10% w/v)	+	+	+	+* [s]	ND	ND	+	+
Osmotolerance (50% glucose, w/w)	-	+	+/s	+* [s]	+	ND	+	+
Osmotolerance (60% glucose, w/w)	- [ND]	- [ND]	- [ND]	- [ND]	+	ND	-	w/s
Gelatin hydrolysis	-	-	-	-	ND	ND	-	-
Tween 80 hydrolysis	- [ND]	-	s	+	ND	ND	+	-
Acid production from glucose	w* [-]	+* [-]	w* [-]	+* [-]	-	ND	w	+
Tolerance to cycloheximide 0.01% (w/v)	-	-	-	-	+	ND	+	+
Tolerance to cycloheximide 0.1% (w/v)	-	-	-	-	ND	ND	+/s	+/s
Tolerance to acetic acid (1% v/v)	-	-	-	-	-	ND	-	-
Growth at 4°C	s [ND]	s [ND]	s [ND]	s [ND]	ND	ND	-	-
Growth at 10°C	s [ND]	s [ND]	s [ND]	s [ND]	ND	ND	s	s
Growth at 25°C	+	+	+	+	+	+	+	+
Growth at 30°C	+	+	+	+	+	ND	+	+
Growth at 37°C	-	-	-	+	+	+	-	-
Growth at 40°C	- [ND]	- [ND]	- [ND]	- [ND]	-	ND	-	-
Hemolysis	- [ND]	- [ND]	- [ND]	- [ND]	ND	ND	-	-
Growth in microaerobiosis	+ [ND]	+ [ND]	+ [ND]	+ [ND]	ND	ND	+	+
Growth in anaearobiosis	s [ND]	s [ND]	w [ND]	+ [ND]	ND	ND	w	+
Splitting of arbutin	+ [ND]	+ [ND]	+ [ND]	+ [ND]	ND	ND	+	+
Hydrolysis of urea	- [ND]	- [ND]	- [ND]	- [ND]	ND	-	-	-
Growth in vitamin-free medium	-	-	w* [-]	w* [-]	-	+	s	w(1)/ s(3)

† Abbreviations: +, positive; -, negative; l, delayed positive; w, weak; s, slow (see section 2.7.2 for further details on the scoring system). ND: no data available.
‡ Reference strains used in this study for quality control: 1, *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^T); 2, *M. gruessii* (CBS 7657^T); 3, *M. reukaufii* (CECT 10671^T); and 4, *M. koreensis* (CBS 8854^T). Other reference strains not tested in this study: 5, *Candida duobushaemulonii* (CBS 7798^T); and 6, *C. heveicola* (CBS 10702^T). Results showing variations with those reported in the literature are indicated by an asterisk (*), and the previously reported observation is indicated (when available) between squared brackets.
§ Four different strains of OTU_11 (JK58_2, JK58_4, JK58_5, and JK58_6) and one of OTU_12 (JK22) were analyzed. In some cases, intra-OTU variability for OTU_11 is indicated by displaying between brackets the number of strains yielding a specific result.

3.5 Growth ability in different types of nectar

The PCA plots obtained for the results of the 96-well assay testing for growth in 12 different types of artificial nectar after 72 h and 7 days of incubation are shown in Figures 3.14A and 3.14B, respectively. At both incubation times, OTU_11 strains clustered apart from all other tested strains, including JK22 (*i.e.* single representative from OTU_12) due to their better performance in a low sugar/high nitrogen nectar containing only sucrose (AN02). OTU_11 strains also showed profuse growth in similar artificial nectars that contained sucrose, glucose, and fructose, or only glucose and fructose (AN06 and AN10, respectively), but not at high osmotic pressures (AN03, AN04, AN07, AN08, AN11, or AN12; Table 3.8). Strain JK22 also showed limited or no growth at high osmotic pressures, and performed better when some glucose and fructose were available in the medium (*e.g.* AN06 and AN10). The scattering of OTU_11 strains in the PCA plot reveals some degree of intra-OTU variability in their ability to grow in the different artificial nectars.

Regarding the reference strains from genus *Metschnikowia*, it was remarkable the ability of *M. gruessii* to thrive in nectars with high total sugar concentration (*e.g.* AN08, initially containing a 1:1:1 mixture of sucrose, fructose and glucose). Finally, due to its scarce or absent growth in most artificial nectars tested, *M. bicuspidata* var. *bicuspidata* was positioned (both after 3 and 7 days) far apart from all the other strains.

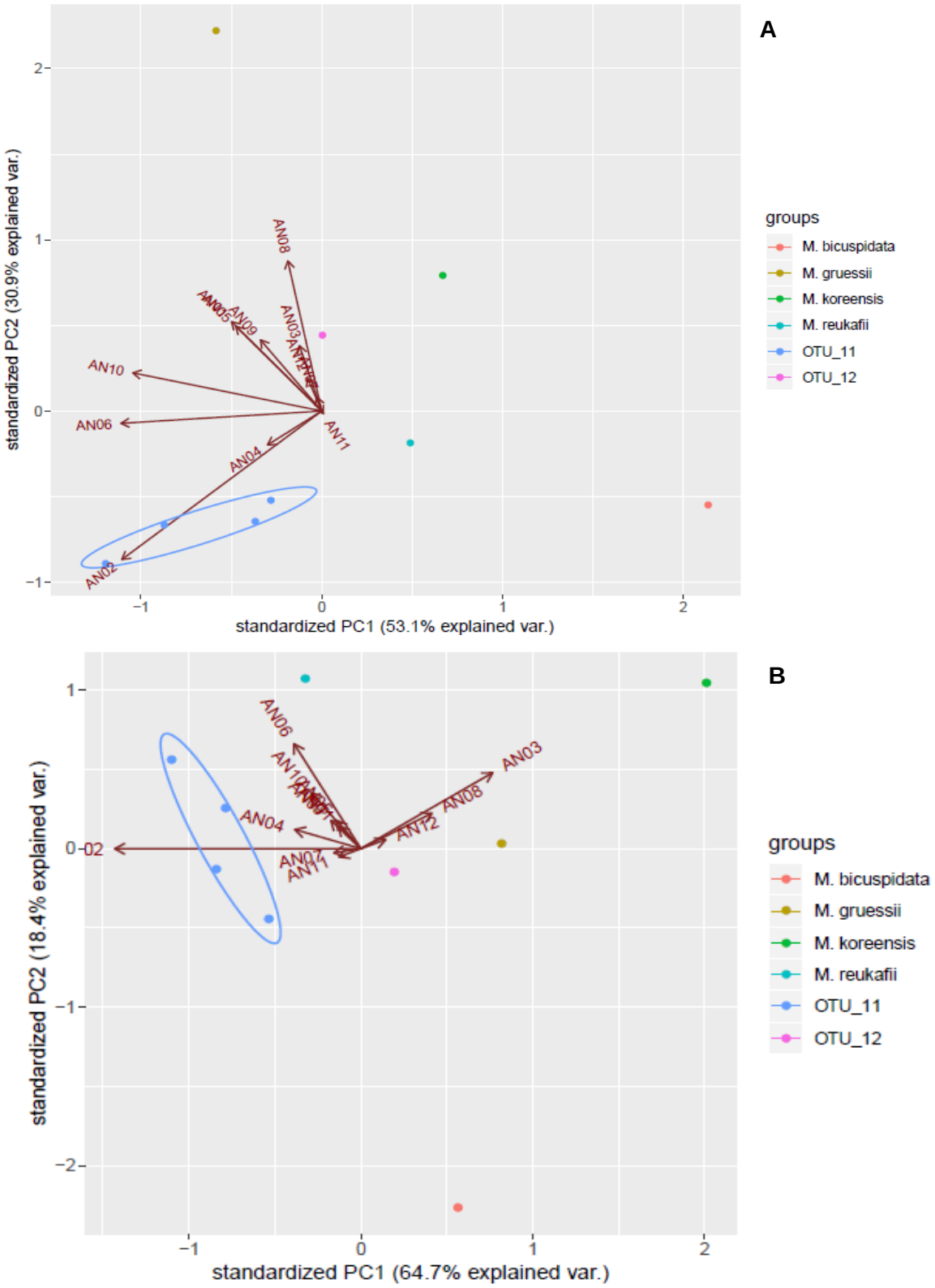

Figure 3.14: Principal component analysis (PCA) biplots obtained for growth of OTU_11, OTU_12, and reference strains in 12 types of artificial nectar (AN1 to AN12; see Table 2.4). Panels A and B show the results obtained after 72 hours and 7 days of incubation at 25ºC, respectively. The blue oval encloses the data points obtained for the four strains belonging to OTU_11. Reference strains: *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^{T}), *M. gruessii* (CBS 7657^{T}), *M. reukaufii* (CECT 10671^{T}), and *M. koreensis* (CBS 8854^{T}).

Table 3.8: Relative growth of OTU_11, OTU_12 and some reference strains from genus *Metschnikowia* in different types of artificial nectar.†

Artificial nectar	Incubation time	*M. bicuspidata*	*M. gruessii*	*M. reukaufii*	*M. koreensis*	JK22	JK58_2	JK58_4	JK58_5	JK58_6
AN01	72 h.	0.10 ± 0.03	0.55 ± 0.06	0.21 ± 0.04	0.14 ± 0.09	0.16 ± 0.08	0.22 ± 0.01	0.26 ± 0.08	0.27 ± 0.05	0.24 ± 0.01
	7 d.	0.09 ± 0.01	0.33 ± 0.07	0.24 ± 0.07	0.11 ± 0.17	0.18 ± 0.13	0.22 ± 0.03	0.21 ± 0.05	0.22 ± 0.05	0.21 ± 0.04
AN02	72 h.	0.32 ± 0.01	0.43 ± 0.20	0.52 ± 0.13	0.25 ± 0.06	0.44 ± 0.14	0.67 ± 0.06	0.72 ± 0.06	0.86 ± 0.06	0.78 ± 0.02
	7 d.	0.44 ± 0.05	0.34 ± 0.12	0.63 ± 0.24	0.08 ± 0.11	0.55 ± 0.10	0.87 ± 0.18	0.79 ± 0.03	0.95 ± 0.13	0.83 ± 0.09
AN03	72 h.	-0.01 ± 0.01	0.20 ± 0.11	0.16 ± 0.05	0.17 ± 0.05	0.18 ± 0.02	0.00 ± 0.02	0.05 ± 0.03	0.08 ± 0.01	0.10 ± 0.01
	7 d.	-0.05 ± 0.01	0.07 ± 0.09	0.07 ± 0.05	0.54 ± 0.09	0.07 ± 0.02	-0.05 ± 0.03	-0.04 ± 0.08	-0.02 ± 0.02	-0.03 ± 0.03
AN04	72 h.	0.11 ± 0.01	0.14 ± 0.09	0.21 ± 0.08	0.10 ± 0.06	0.19 ± 0.08	0.18 ± 0.06	0.16 ± 0.04	0.28 ± 0.06	0.26 ± 0.05
	7 d.	0.14 ± 0.00	0.10 ± 0.08	0.28 ± 0.08	0.07 ± 0.18	0.17 ± 0.05	0.23 ± 0.05	0.16 ± 0.06	0.33 ± 0.00	0.27 ± 0.03
AN05	72 h.	0.11 ± 0.02	0.52 ± 0.03	0.28 ± 0.07	0.17 ± 008	0.37 ± 0.08	0.23 ± 0.03	0.27 ± 0.06	0.29 ± 0.07	0.28 ± 0.01
	7 d.	0.09 ± 0.03	0.37 ± 0.07	0.27 ± 0.09	0.14 ± 0.14	0.33 ± 0.01	0.23 ± 0.03	0.25 ± 0.01	0.27 ± 0.08	0.26 ± 0.02
AN06	72 h.	0.39 ± 0.03	0.79 ± 0.13	0.71 ± 0.14	0.84 ± 0.17	0.78 ± 0.51	0.76 ± 0.04	0.84 ± 0.22	1.05 ± 0.26	0.95 ± 0.26
	7 d.	0.61 ± 0.08	0.74 ± 0.06	1.12 ± 0.40	0.91 ± 0.14	0.79 ± 0.48	0.88 ± 0.03	0.90 ± 0.32	1.07 ± 0.29	1.01 ± 0.35
AN07	72 h.	-0.02 ± 0.01	0.03 ± 0.05	-0.03 ± 0.03	-0.02 ± 0.08	-0.03 ± 0.02	-0.03 ± 0.02	-0.02 ± 0.01	-0.01 ± 0.02	-0.02 ± 0.01
	7 d.	-0.07 ± 0.01	-0.07 ± 0.07	-0.06 ± 0.04	-0.15 ± 0.10	-0.07 ± 0.02	-0.06 ± 0.02	-0.06 ± 0.01	-0.03 ± 0.03	-0.05 ± 0.01
AN08	72 h.	-0.02 ± 0.01	0.41 ± 0.08	0.02 ± 0.05	0.22 ± 0.05	0.10 ± 0.21	0.03 ± 0.04	0.02 ± 0.03	0.04 ± 0.04	0.05 ± 0.04
	7 d.	-0.08 ± 0.01	0.27 ± 0.27	-0.01 ± 0.07	0.17 ± 0.08	0.07 ± 0.20	-0.04 ± 0.02	-0.06 ± 0.04	-0.02 ± 0.02	-0.03 ± 0.00
AN09	72 h.	0.10 ± 0.05	0.43 ± 0.03	0.24 ± 0.05	0.16 ± 0.06	0.27 ± 0.05	0.27 ± 0.08	0.20 ± 0.05	0.21 ± 0.05	0.22 ± 0.02
	7 d.	0.11 ± 0.06	0.32 ± 0.01	0.36 ± 0.09	0.11 ± 0.11	0.27 ± 0.04	0.26 ± 0.08	0.20 ± 0.03	0.23 ± 0.01	0.22 ± 0.04
AN10	72 h.	0.37 ± 0.11	0.86 ± 0.13	0.58 ± 0.09	0.72 ± 0.07	0.85 ± 0.60	0.88 ± 0.10	0.78 ± 0.14	0.85 ± 0.13	0.86 ± 0.20
	7 d.	0.55 ± 0.17	0.79 ± 0.22	0.83 ± 0.09	0.74 ± 0.09	0.84 ± 0.68	0.94 ± 0.12	0.77 ± 0.15	0.84 ± 0.13	0.85 ± 0.21
AN11	72 h.	-0.01 ± 0.01	-0.03 ± 0.04	-0.02 ± 0.02	-0.01 ± 0.02	-0.03 ± 0.02	-0.02 ± 0.01	-0.05 ± 0.04	0.00 ± 0.02	-0.01 ± 0.01
	7 d.	-0.05 ± 0.01	-0.06 ± 0.08	-0.05 ± 0.04	-0.17 ± 0.08	-0.07 ± 0.02	-0.07 ± 0.03	-0.11 ± 0.08	-0.04 ± 0.01	-0.05 ± 0.04
AN12	72 h.	-0.02 ± 0.01	0.08 ± 0.02	-0.01 ± 0.03	0.14 ± 0.01	0.02 ± 0.10	0.00 ± 0.03	0.01 ± 0.06	0.05 ± 0.04	0.05 ± 0.03
	7 d.	-0.05 ± 0.01	0.06 ± 0.12	-0.05 ± 0.05	0.01 ± 0.09	-0.03 ± 0.08	-0.07 ± 0.03	-0.08 ± 0.03	-0.04 ± 0.02	-0.06 ± 0.03

† Values refer to average ± S.D. of normalized OD measurements obtained in three different replicates of the experiment. For further details see Table 2.4 and section 2.7.3. Reference type strains used in this study: *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^T), *M. gruessii* (CBS 7657^T), *M. reukaufii* (CECT 10671^T),and *M. koreensis* (CBS 8854^T).

4 DISCUSSION

Flowers offer a wide variety of substrates suitable for microbial growth. However, the development of such microbial communities is normally restricted by several biotic and abiotic conditions whose nature and strength depends on the plant host, the array of floral visitors (*e.g.* pollinators and nectar robbers), the geographical location, and other parameters such as altitude, humidity, solar irradiation, *etc.* (Pusey *et al.,* 2009; Zimmerman and Vitousek, 2012; Aleklett *et al.,* 2014; Parachnowitsch *et al.,* 2019). In any case, the microbiological study of flowers has only recently begun to be systematically addressed, and our knowledge about floral-inhabiting microorganisms is yet very limited, especially when compared with the vast amount of information available for rhizospheric, phyllospheric and endophytic microbial communities (Aleklett *et al.*, 2014).

Two particular aspects of flower microbiology that are receiving increasing attention in recent years are: (i) the composition of the microbial communities inhabiting different floral microhabitats (Herrera *et al.,* 2009; Aleklett *et al.*, 2014; Junker and Keller, 2015); and (ii) the role of flowers as reservoirs of undescribed microbial taxa (Lachance, 1990; Lachance *et al.,* 2003; de Vega *et al.,* 2012, 2017, 2018). In the present thesis, we addressed these two aspects for the culturable yeast communities inhabiting the flowers of the Hawaiian endemic tree *Metrosideros polymorpha*.

4.1 Community analysis

Analysis of the frequency of yeast occurrence in floral parts of *M. polymorpha* indicated that there was a significant difference between the three floral habitats considered in this study. Such a significant difference was a consequence of the higher yeast occurrence found in styles when compared to the other flower microhabitats. Styles secrete a variety of substrates to aid in pollen tube nutrition (section 1.4.1), which can also be used by diverse microorganisms to thrive (Ngugi and Scherm, 2006; Pusey *et al.,* 2009; Aleklett *et al.,* 2014). In contrast, the filaments of stamens generally do not produce exudates and lack of epidermal cavities, making them less suitable places for microbial colonization (Aleklett *et al.,* 2014).

All the yeast OTUs isolated in this study from *M. polymorpha* floral parts have been previously detected in plant materials (Lachance *et al.,* 2001; Buzzini *et al.,* 2017; de Vega *et al.,* 2017). For example, *Metschnikowia rancensis*, which was the best BLAST hit for the most abundant OTU in our samples, is a fairly common floral inhabitant (de Vega *et al.,* 2017). However, we did not find *M. gruessii* and *M. reukaufii* in our survey, a result which is remarkable due to the frequent occurrence of both species in flowers (Brysch-Herzberg, 2004; Pozo *et al.*, 2011; Alvarez-Perez and Herrera, 2013; de Vega *et al.*, 2017). Nevertheless, a classic assumption in biodiversity studies is that island species richness depends on the migration of species coming from large, mainland communities. Therefore, the farther the island from the mainland, the lower the chance of encountering there species with low migration rates, as it may be the case for flower-inhabiting yeasts that depend on animal vectors to get dispersed (Lachance *et al.,* 2003; Vannette *et al.*, 2017). As the Hawaiian islands are the most isolated archipelago in the world (Carlquist, 1980), dispersal limitation of flower- and other plant-associated microbes is highly plausible. Moreover, our results agree with the observations of Lachance *et al.* (2001), who analyzed the yeast communities vectored by beetles, drosophilids, and bees that visited flowers in different locations and did not obtain any *M. gruessii* or *M. reukaufii* isolate in Hawaii.

Sample-based rarefaction curves, were asymptotic or nearly asymptotic, implying that sampling depth was adequate to capture the diversity of OTUs present in *M. polymorpha* flowers. Nonetheless, ICE and Chao2 richness estimators indicated that, on average, only 50% of the estimated yeast diversity was captured by the sampling and our culturing methods, thus suggesting that further (rare) OTUs would appear if more floral samples were analyzed. Several factors may contribute to this low observed species richness, including a suboptimal sampling scheme and/or the fact that our mycological survey was exclusively based on culture-dependent methods that might not have adequately accounted for differences in the ability of floral yeasts to grow in laboratory conditions. A possible improvement would probably be to analyze the yeast microbiota of *M. polymorpha* flowers using next-generation sequencing technologies, as done in the study of Junker and Keller (2015) for the bacterial communities associated to flowers and other parts of the same plant species. However, despite the aforementioned limitations, we can conclude with some confidence that the floral yeast communities of *M. polymorpha*, at least in the geographic locations covered by our samples, are species-poor. Similar results have been obtained in previous surveys on

nectar yeasts carried out in diverse biogeographic regions (Herrera *et al.,* 2008, 2009; de Vega *et al.,* 2009, 2017; Belisle *et al.,* 2012; Jacquemyn *et al.,* 2013a, 2013b; Canto *et al.,* 2017) but, unfortunately, there is very limited data in the literature about the yeast communities associated with other floral parts (Aleklett *et al.*, 2014).

Given the high osmotic pressure and other stress factors present in floral nectar (section 1.5), it has been claimed that the yeast communities that inhabit this habitat are just a subset of those associated to floral visitors and/or other floral structures (Herrera *et al.*, 2010). Furthermore, such 'filtering' effect of nectar is not random, and only a few phylogenetically related yeast lineages (mostly members of the Metschnikowiaceae family) seem to be well adapted to nectar dwelling (Herrera *et al.*, 2010). The results of this study agree with that hypothesis, as nectar was the floral microhabitat from which a lower number of OTUs were recovered and showing lower values of expected OTU richness. Furthermore, no basidiomycetous yeasts were found in nectar, which contrasts with the five OTUs from this phylum isolated from stamina samples, and the phylogenetic diversity of the ascomycetous OTUs was lower for nectar than for stamina and styles. In any case, in addition to the filtering effect of the physical and chemical characteristics of nectar (which may be variable even within the same plant individual), dispersal limitation and microbe-microbe interactions can also determine the species composition of the nectar microbiome (Álvarez-Pérez *et al.*, 2019).

4.2 Characterization of undescribed yeast taxa

The second part of the present study consisted of the detailed characterization of two yeast OTUs retrieved from *Metrosideros polymorpha*, namely OTU_11 and OTU_12, that potentially represent new ascomycetous species. Interestingly, these OTUs clustered together with several pathogenic *Candida* species (*C. auris, C. haemulonis, C. duobushaemulonis, C. pseudohaemulonis*) that show decreased susceptibility to different antifungal compounds (Cendejas-Bueno *et al*.,2012; Sipiczki and Tap, 2016). In fact, *C. auris* and *C. duobushaemulonii* are currently regarded as emerging fungal pathogens causing life-threatening infections in specific patient populations (*e.g.* immunosuppressed individuals and people undergoing long broad-spectrum antibiotic treatment), frequently displaying multidrug resistance and capable of spreading globally (Ruben *et al.,* 2018; Forsberg *et al.,* 2019). On the other hand, phylogenetic analyses

carried out for the ITS and LSU regions of the ribosomal RNA operon revealed that OTU_11 and OTU_12 also clustered with *Candida* species that have not yet been considered as (potential) pathogens, including *C. chanthaburiensis, C. heveicola*, *C. vulturna,* and *C. ruelliae*. The OTU_11 and OTU_12 strains characterized in this study seem to belong to this second group as, for example, they were unable to grow at 37ºC (which is the average normal body temperature of humans) and therefore, might not be able to produce invasive and/or systemic infections. Furthermore, these two OTUs tested negative in the hemolysis and gelatinase assays, and OTU_11 also tested negative for lipase production (hydrolysis of Tween 80). All in all, these findings suggest that, despite their phylogenetics placement, OTU_11 and OTU_12 should not be considered as pathogenic to warm-blooded animals (mammals and birds). However, we cannot discard the possibility that these OTUs might act as opportunistic pathogens of other animal species such as invertebrates, fishes, amphibians and reptiles.

As both OTU_11 and OTU_12 were isolated from floral parts and most of their closest phylogenetic relatives were originally found in plants (Saluja and Prasad, 2008; Wang *et al.,* 2008; Limtong and Yonngmanitchai, 2010; Sipiczki and Tap, 2016), it can be presumed that the undescribed yeast taxa found in this study are naturally adapted to plant-dwelling. In this regard, our phenotypic assays confirmed that both OTU_11 and OTU_12 can assimilate the three main sugars present in nectar and other plant secretions (*i.e.* glucose, sucrose and fructose), and also other carbohydrates which are less abundant but fairly common in different plant microhabitats (*e.g.* xylose and maltose). Furthermore, OTU_11 and OTU_12 were able to grow in high osmotic- (up to 60% for OTU_11), vitamin-free- and microaerobic conditions, proving that these yeasts can endure some of the strong selective factors of nectar (Herrera *et al.*, 2010; Álvarez-Pérez *et al.*, 2012, 2019; Lievens *et al.*, 2015). They also showed some growth in artificial nectars containing a high concentration of sugars, even when they performed much better at lower osmotic pressures (Figure 3.14 and Table 3.8). Other phenotypic traits not determined for OTU_11 and OTU_12 but which have also been involved in the adaptation of yeasts to plant-dwelling include catalase activity, resistance to irradiation and desiccation, tolerance to plant toxins, and biofilm formation (Fonseca and Inácio, 2006; Álvarez-Pérez *et al.*, 2012; Pozo *et al.*, 2012, 2015, 2016). Therefore, the ability of OTU_11 and OTU_12 to colonize and thrive in flowers and other plant-associated habitats should be further assessed in future studies.

It has been postulated that the cell morphology of some flower-inhabiting yeasts, and in particular of the nectarivorous species *M. gruessii*, which typically displays aeroplane cell configurations (Figure 4.1B), might be an adaptation to efficiently attach to the mouthparts of some pollinators such as bumblebees that disperse them from flower to flower (Brysch-Herzberg, 2004). Also *M. reukauffi*, which is one of the main yeast inhabitants of floral nectar (Pozo *et al.*, 2011; Belisle *et al.*, 2012; Álvarez-Pérez and Herrera, 2013; Álvarez-Pérez *et al.*, 2019), typically produces elongated cells (Figure 4.1C). In contrast, the cells of other members from genus *Metschnikowia* do not seem to have any morphological adaptation to dispersal by animal vectors as observed, for example, for *M. koreensis* or the aquatic species *M. bicuspidata* (Figure 4.1A, D). The cells of OTU_12 show certain resemblance to those of *M. bicuspidata*, as these are rounded to slightly oval and of similar size for both species (Figure 3.11 and Figure 4.1A). The same can be said for OTU_11 and *M. koreensis* (Figure 3.12 and Figure 4.1D). As the basic biology of OTU_11, OTU_12 and some *Metschnikowia* species remains to be studied in detail, it is still difficult to infer if the morphology of their cells fits any specific ecological role and/or it may be the result of selective forces to minimize surface-to-volume ratio in yeasts that frequently inhabit highly osmotic habitats.

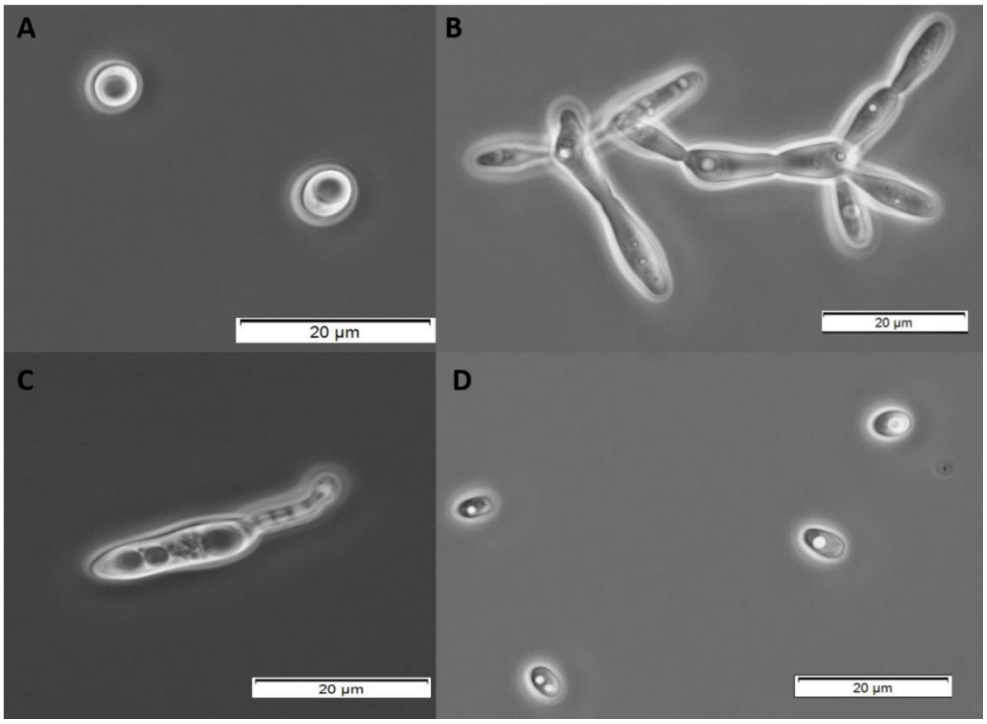

Figure 4.1: Comparison of cell morphologies between (A) *Metschnikowia bicuspidata* var. *bicuspidata* (CBS 5575^{T}); (B) *M. gruessii* (CBS 7657^{T}); (C) *M. reukaufii* (CECT 10671^{T}); and (D) *M. koreensis* (CBS 8854^{T}) after 6 days of growth at 25ºC on PDA. Note the typical 'airplane' configuration of *M. gruessii* cells.

As a general rule, and whenever it is possible, taxonomic descriptions of new yeast species should be based in more than a single strain, so that intra-species variability in genetic and phenotypic characters can be assessed (Kurtzman, 2010). The main limitation of our study was the availability of just a single isolate for OTU_12 and four genetically similar (as determined by M13 fingerprinting; Figure 3.9), but somewhat phenotypically different (*e.g.* Tables 3.4, 3.5 and 3.7) strains of OTU_11 isolated from the same sample. Nonetheless, nearly one-third of the species included in the so far more comprehensive guide on yeast taxonomy (namely *The Yeasts, a Taxonomic study*; Kurtzman *et al., 2011a*) was described from a single strain. Therefore, if such species based on a single strain would not have been formally described, our current knowledge about the diversity of yeasts would be much scarcer (Kurtzman, 2010). Additionally, the history of fungal taxonomy has taught us that once a species is described, whether it originates from a single strain or multiple ones, new similar or closely related strains are recognized from other substrates and/or geographical areas. Thus, from a perspective of understanding the yeast diversity in our planet, taxonomic

descriptions based on a single strain are still valuable, even when those based on multiple strains are always preferred (Kurtzman, 2010).

Finally, previous studies have shown that strains belonging to the same yeast species usually differ by less than 1% mismatches in the D1/D2 domain of the LSU rRNA gene (Kurtzman and Robnett, 1998; Fell *et al.,* 2000). The significant D1/D2 and ITS sequence divergence of OTU_11 and OTU_12 strains from their closest relatives (>5% in all cases) confirms that these strains represent two distinct, novel yeast species. According to the current taxonomical conventions for ascomycetous yeasts (Kurtzman *et al.,* 2011; Turland *et al.,* 2018), the studied strains should be assigned to the anamorphic genus, *Candida*. Moreover, as remarkable sequence divergence was found in more than one gene marker, any eventual taxonomic proposal involving OTU_11 and OTU_12 would be supported by the concordance phylogenetic species concept (GCPSC, section 1.7.3; Avise and Ball, 1990). However, the inability of OTU_11 and OTU_12 strains to produce asci or signs of conjugation, even after prolonged incubation of every possible strain pair in different cultivation media, precludes the application of the biological species concept to the strains characterized in this study, and these should be then considered as *forma asexualis* (*f.a.*; Lachance, 2012; de Vega *et al.*, 2017). Finally, it should also be noted here that species concepts are merely evolving hypotheses that can constantly be questioned in the light of new data, analyses or techniques, and which will probably be perceived differently by different taxonomists (Lipscomb *et al.*, 2003; Seberg *et al.*, 2003).

5 CONCLUSION

In conclusion, the flowers of *Metrosideros polymorpha* Gaud (Myrtaceae), a tree endemic to Hawaii, are inhabited by species-poor yeast communities which are dominated by ascomycetous taxa. Furthermore, the yeast communities associated to specific floral structures, such as nectar, styles and stamina show certain differentiation, for example in their OTU richness and phylogenetic diversity. Finally, our mycological survey led to the discovery of two novel yeast lineages that diverged phylogenetically and phenotypically from previously described taxa and might represent new species within the anamorphic genus *Candida*. Future work should clarify the taxonomic status of these new yeast lineages, as well as their ecological role within the plant-yeast-pollinator system.

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FACULTEIT INDUSTRIËLE INGENIEURSWETENSCHAPPEN
CAMPUS DE NAYER SINT-KATELIJNE-WAVER
J. De Nayerlaan 5
2860 SINT-KATELIJNE-WAVER, België
tel. + 32 15 31 69 44
iiw.denayer@kuleuven.be
www.iiw.kuleuven.be