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Fungal diversity of cocoa (*Theobroma cacao*) in Cuba: from field to fermentation

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To my son Cristhian Alejandro

You are the reason of my life!

Abstract

In Cuba, a national program intends to rehabilitate the cocoa sector. The objective is to reinforce the production chain of cocoa and to support a protected designation of origin. One of the actions for the sustainable development of cocoa in the Cuban environmental and economic context requires a rational and integrated control of pathogens affecting productivity and bean quality.

Black pod disease caused by several *Phytophthora* species is the most damaging disease in cocoa production areas in Cuba. In the present work, we studied a set of *Phytophthora* strains isolated from diseased pods originating from Cuba, combining morphological, biological and molecular approaches to identify the species involved. Our results showed that two species are damaging cocoa plantations in Cuba, viz. *P. palmivora* and *P. tropicalis*.

Different forms of crop protection are employed to reduce the impact of this disease, cultural practices such as pod stripping and the use of pesticides. However, biological control is an important alternative. Nowadays, fungal endophytes seem to be an interesting option for black pod disease control. In this study fungal endophytes present in cocoa were identified to potentially use them in the context of integrated management systems for disease control. Our findings highlight the possibility to use the endophytic fungus *Colletotrichum gloesporioides* as biological control agent against black pod disease. Proper control of the disease would strengthen cocoa plantations and reduce crop losses, increasing the quantity of healthy cocoa pods.

Healthy cocoa pods are the starting material for cocoa bean fermentation, the first step in the production of chocolate and crucial for the final chocolate flavor. Without fermentation there will not be any chocolate flavor. The fermentation of cocoa beans is a well-defined microbial succession that is dominated by yeast during the first hours. In the current work, the yeast diversity of spontaneous cocoa bean fermentation heaps in east Cuba was investigated. Yeasts were isolated from seven fermentations and their environments such as surfaces, tools, insects and plants. The most frequently detected species were *P. manshurica*, *Hanseniaspora opuntiae*, and *P. kudriavzevii*. Seventeen potentially new species were discovered, of which two, referred to as *Yamadazyma* sp. and *Saccharomycopsis cf. crataegensis*, were isolated repeatedly from fermentations and equipment.

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List of abbreviations

Act	Actin gene
CI	Consistency index
DNA	Deoxyribonucleic acid
DYPA	Dextrose Yeast Extract Peptone Agar
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
FAO	Food and Agriculture Organization of the United Nations
HCl	Hydrochloric acid
MEA	Malt extract agar
mtDNA	Mitochondrial DNA
NaCl	Sodium Chloride
NaClO	Sodium hypochlorite
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
rDNA	Ribosomal DNA
RI	Retention index
RNase	Ribonuclease
Rpm	Revolutions per minute

Introduction

Introduction

In Cuba, cocoa trees (*Theobroma cacao* L.) are cultivated in the easternmost Province of Guantánamo, more specifically in the area of Baracoa, where around 75% of the national cocoa production takes place. The local climatic conditions are very favorable to the culture of cocoa. The mountainous barrier Nipe-Sagua-Baracoa creates an effect of ascending air currents towards the northern slope, causing abundant regular rainfall, while the southern slope remains dry. However, Cuba is not a significant producer on a world scale in comparison with the top producing countries like Ivory Coast (1 448 992 t) and Ghana (835 466 t) in 2013 (International Cocoa Organization 2014, FAO 2015). With a production of 1425 t in 2013, Cuba is placed at position 34 in the list of cocoa-producing countries (FAO 2015). The agricultural production in Cuba is centred in tuber-or-root-crops (1 580 500 t), cereals (1 098 800 t) and citrics (166 900) (FAO 2015).

A national program intends to rehabilitate the cocoa sector which reached its highest production level in 1992 with a production of 3000 t (FAO 2015). This program started in 2003 and is regulated by Cuban Law 228/2002 (Hartmann & Larramendi 2011). The objective is to reinforce the production chain of cocoa and to support a protected designation of origin. Cocoa bean quantity, quality and taste of the finished products depend on different key factors in its production chain: the choice of varieties, the control of pathogens, the cocoa bean fermentation, roasting, and further processing. Therefore, actions at different production steps have to be undertaken, such as the genetic improvement of plant material to attain varieties with the potential to produce fine cocoa, the disease control and productivity increase by cultivation methods that include sanitary protection, the adoption of good fermentation practices, followed by the roasting and transformation of raw cocoa into high-value diversified products. On the basis of the redeployment of ancient local plant varieties and the improvement of local processing to result in high-value diversified products, the objective is to obtain a local brand or a Cuban cocoa for the international market, local consumption and tourist activities.

One of the actions for the sustainable development of cocoa in the Cuban environmental and economic context requires a rational and integrated control of pathogens affecting productivity and bean quality. Black pod disease caused by several *Phytophthora* species is globally the most damaging disease, in cocoa production areas (Aime & Phillips-Mora 2005, Crozier et al. 2006, Evans 2007). In Cuba black pod disease was reported in 1958 (Venning & Gertsch 1958).

Chemical control of black pod disease can be expensive, ineffective, and has a negative impact on environmental and human health. Biological control as part of integrated pest management has been suggested as the most sustainable long-term solution (Backman & Sikora 2008). Specifically, promising results for the control of cocoa plant diseases have been obtained

using epiphytic mycoparasitic fungi (Krauss & Soberanis 2002, Ten Hoopen et al. 2003). In some cases endophytic fungi restricted cocoa pathogen growth or damage in vitro and in vivo (Arnold et al. 2003, Evans et al. 2003, Mejía et al. 2008, Holmes et al. 2004, Rubini et al. 2005, Tondje et al. 2007) highlighting their status as a new source of biological control agents for combating cocoa pathogens. Proper control of the disease would strengthen cocoa plantations and reduce crop losses.

Healthy cocoa pods are the starting material for cocoa bean fermentation, the first step in the production of chocolate and crucial for the final chocolate flavor. Without fermentation there will not be any chocolate flavor. A high quality of fermented dry cocoa beans is correlated with good agricultural and post-harvest practices on the farms, encompassing plant breeding and cocoa pod/bean selection (Papalexandratou 2011).

Cocoa fermentation in Cuba is not yet described in scientific terms. This process is centralized by government-cooperatives and the farmers deliver the harvest to these cooperatives which have fermentation centers in two different locations in Baracoa: El Jamal and Paso de Cuba. However, fermentations are poorly controlled in Baracoa, mostly because of a lack of timely transport of the harvest to the fermentation centers and consequently the uncontrolled spontaneous fermentations starting in the different collection points of cocoa pulp-bean mass.

Outline of the thesis

Outline of the thesis

Within this thesis, three major objectives are followed:

1. The identification and characterization of *Phytophthora* species causing black pod disease in Cuba. A set of *Phytophthora* strains originating from cocoa plantation in French Guiana were identified and compared with the strains from Cuba.

2. Identification of fungal endophytes isolated from leaves of *Theobroma cacao* in Cuba.

3. Detection of yeast diversity in Cuban cocoa bean fermentation heaps and associated environmental niches.

In the introduction, the subject of the thesis is presented.

In Chapter I, Context of the Study, the state of the art relative to the study is reviewed. This chapter contains an overview on current classification of *Phytophthora,* their impact on cocoa (*Theobroma cacao*) around the world, specifically in Cuba and the phylogenetic relation between different species of *Phytophthora.* The importance of fungal endophytes as biocontrol agents against cocoa pathogens is addressed, with emphasis on their effect against species of *Phytophthora*. We described the different ways in which cocoa fermentation can be carried out around the world and in Cuba, the yeast diversity during cocoa fermentation and the principal methods used to identify them.

In Chapter II, the results of the characterization of *Phytophthora* strains isolated from Cuba and French Guiana are presented. In Latin America, the distribution of *Phytophthora* is heterogeneous and several studies revealed the presence of species complexes representing high species diversity in contrast to African *Phytophtora* isolations. In this context, the comparison between Cuban and French Guiana strains, allowing a better understanding about the situation of *Phytophthora* in this area.

In this chapter, the methods used to identify the species of *Phytophthora* are described. Studies are focused on biological and molecular biology techniques.

Results of Chapter II were submitted for publication.

In Chapter III, the fungal endophytes isolated from healthy leaves of Cuban cocoa were identified. These fungi were collected for screening as a potential source of biocontrol agents for the pathogens of cocoa in Cuba in future studies. For molecular identification by DNA extraction and sequence analysis of nuclear ribosomal DNA (rDNA), the internal transcribed spacer (ITS) was chosen.

Results of Chapter III were submitted for publication.

In Chapter IV, yeasts from seven fermentations and their environments were isolated in east Cuba in September 2013 and September 2014. The basic fermentation parameters were recorded. Representative isolates were identified using the ITS, D1/D2 region of the large subunit rRNA gene, and partial actin gene sequences.

Results of Chapter IV were submitted for publication.

Chapter I

Context of the study

Context of the study

1. *Theobroma cacao* L

The cocoa tree (*Theobroma cacao* L.) belongs to the order Malvales, family Sterculiaceae. *Theobroma cacao* is one of 22 species of *Theobroma*. It grows in tropical and equatorial regions (Lachenaud et al. 2007) with its likely center of origin located in the Amazon basin (Laurent et al. 1993, Lachenaud et al. 2007). Processed cocoa beans are one of the main agricultural exports from these regions. However, more than two thirds of the cocoa world production comes from Africa (Cilas & Despréaux 2004, Hanak-Freud et al. 2000), especially from the West African countries Ivory Coast and Ghana, where cocoa has a high economic and social importance (Ruf 2011).

Cocoa trees thrive in tropical temperatures within the protective shadow of plants such as banana plants or palm trees. The ideal average temperature for cultivation is 26 °C, with a high relative humidity and regular rainfall throughout the year. These trees can grow in highly variable soil types and require a light shade (Cilas & Despréaux 2004).

1.1 *Theobroma cacao* cultivars and classification

There are three different major genetic types of *T. cacao*, each with their own particular characteristics: Forastero, Criollo and Trinitario (Laurent et al. 1993, Lanaud et al. 1999, Cilas & Despréaux 2004). Traditionally, the genetic groups have been defined within *T. cacao* based on morphological traits and geographical origins. Forastero is a vigorous, fast growing type, producing beans with strong flavor, and produce cocoa with a bitter taste. Forastero is most widely grown in West Africa (Ghana, Nigeria, Ivory Coast) and Brazil. The top producing countries grow primarily Forastero based on its disease resistance; Forastero adapts well to the environmental conditions (Lanaud et al. 1999, N'Goran et al. 1994). Forastero cocoa beans are usually processed together with Criollo beans to reduce their bitter taste. Criollo, native to Mexico and Central America, produces cocoa beans in small quantities with a delicate flavor. Criollo trees are less resistant to many cocoa diseases. Trinitario, obtained on the island of Trinidad as the results of a cross between Criollo and Forastero (N'Goran et al. 1994), has a strong but relatively refined aroma, is very easy to cultivate (Bekele 2004), and is more productive and disease resistant than Criollo. Trinitario replaced the old Criollo plantations in Ecuador (Soria 1970) and elsewhere in Central and South America (Cheesman 1944).

For other authors, Criollo and Trinitario should be considered as traditional cultivars rather than genetic groups (Motomayor et al. 2002). Also, other traditional cultivars have been described, named as Nacional and Amelonado (Motomayor et al. 2003).

However, the classification of *Theobroma cacao* L. populations, based on genetic data, is lacking for the breeding. A study, to understand the origin, classification, and population differentiation within *T. cacao* was performed with 1241 individuals from several collecting expeditions and different geographical origins. The individuals were genotyped with 106 microsatellite markers, allowing the identification of 10 clusters according to Bayesian statistics. These clusters were named according to the geographical location or traditional cultivar most represented in that particular cluster: Marañon, Curaray, Criollo, Iquitos, Nanay, Contamana, Amelonado, Purús, Nacional and Guiana (Motomayor et al. 2008). This classification differentiated populations within what was classified in the Forastero genetic group. The ten clusters are found in the South American forests while only individuals from the Criollo cluster are represented in Central American forests. The highest genetic diversity was found in the Upper Amazon region (Motomayor et al. 2008). This result is in concordance with the location of the putative center of origin of *T. cacao* L (Cheesman 1944). The genetic classification of Motomayor et al. (2008) explained the genetic diversity of *T. cacao* better than the traditional classification.

1.2 *Theobroma cacao* in Cuba

Theobroma cacao is widespread in eastern Cuba, specifically in Baracoa. The cultivated area is 6500 ha, of which 4303 ha are in production, with a yield of 0.33 $t \cdot ha^{-1}$ (Oficina Nacional de Estadísticas e Información 2014). In Baracoa the climatic conditions are very favorable to its culture with more than 2000 mm of rainfall per year an environmental temperature average of about 24-28 °C (Márquez Rivero & Aguirre Gómez 2008). There is a strong family tradition in the cultivation of cocoa.

There are two theories about the *T. cacao* introductions in Cuba. One theory claims an introduction to Cabaiguán, in the center of the Island, from Central America in 1540 as a result of the Spanish expansion in America, but there is no evidence (Hartmann & Larramendi 2011). The second theory raises the possibility of the introduction from Haiti during the 18th century, through the exodus of French colonists during the Haitian Revolution (Núñez González 2010). This theory is supported by a recent study that showed the similarity of cocoa germplasms between Cuba and the Dominican Republic (Bidot et al. 2015).

Several types of *T. cacao* are recognized in Cuba. Fifty nine percent of Cuban cocoa consists of hybrids introduced by the United Fruit Company (UF) from Costa Rica in 1955. These clonal varieties are produced at the germplasm bank of the Instituto de Investigaciones Agroforestales (IIAB), Baracoa, and are generally planted as a mixture of different clones. The most wide-spread clones are UF 650, UF 654 and UF 677. Thirty seven percent of Cuban cocoa consists of hybrids introduced in 1991 and reproduced by seeds of selected Trinitario and Forastero clones (Núñez González 2010). Four percent of Cuban cocoa belongs to the progeny of Trinidad Selected Hybrid (TSH) cacao. It was introduced to the country by

seeds in 1970 and is now distributed in the regions of Granma, Santiago de Cuba, Holguín and Guantánamo.

Recently, Bidot et al. (2015) conducted a study with traditional or ancient cocoa plants in Cuban cocoa plantations, following the classification proposed by Motomayor et al. (2008). According to their results, ancient Cuban cacao plants are likely Trinitario hybrids of the genetic groups Amelonado and Criollo.

2. *Theobroma cacao* diseases

Cocoa is affected by a range of diseases, caused by fungi, viruses, and nematodes, with a negative effect in the global production (Bowers 2001). Most of these diseases are only regionally important. Swollen shoot caused by Cocoa swollen shoot virus (CSSV), is reported to be destructive in West African and Asian countries (Benin, Ghana, Côte d'Ivoire, Nigeria, Indonesia) (Brunt et al. 1971). Vascular streak dieback, caused by *Oncobasidium theobromae*, is present only in Asia (Guest & Keane 2007). Ceratocystis wilt, caused by *Ceratocystis cacaofunesta* is present in Brazil, Colombia, Costa Rica, Ecuador (Engelbrecht & Harrington 2005).

The three most frequent fungal diseases that affect the cocoa tree are Frosty Pod Rot, Witches' Broom and Black Pod (Evans 2007) and this chapter will be restricted to them.

2.1 Frosty Pod Rot disease

Frosty Pod Rot disease is caused by the hemibiotrophic basidiomycete *Moniliophthora roreri* (Evans et al. 1978), a neotropical plant parasitic fungus (family Marasmiaceae), widely distributed in the Latin American region (Colombia, Ecuador, western Venezuela, Panama, Costa Rica, Peru, Nicaragua, Honduras, Guatemala, Belize, and Mexico), and responsible of significant losses in cocoa production. Caribbean countries, Africa and Asia are still free of this pathogen (Aime & Phillips-Mora 2005, Evans 2007, Parra et al. 2009, Phillips-Mora 2003). There are no official reports about the presence of Frosty Pod Rot disease in Cuba.

Moniliophthora roreri infects the developing pods, resulting in distorted pods by light yellow swellings and premature ripening. Inside the pods, the beans are necrotic. In advanced stages, it is possible to see brown lesions and the white to cream colored mycelium on the pod surface. If the infected pods remain attached to the trees, branches become necrotic and hard mummies consisting of dehydrated, spore-filled pods are formed. The spores spread quickly and spore densities reach values as high as 44 million conidia per square centimeter (Evans 1981, Fulton 1989). The spores are distributed to healthy fruits by wind or rain causing the proliferation of the disease. The best way to control the advance of this disease is the reduction of the inoculum sources through a good cocoa plantation management.

2.2 Witches' Broom disease

Witches' Broom disease is caused by the basidiomycete fungus *Moniliophthora perniciosa* (Aime & Philips-Mora 2005). This fungus, indigenous to tropical South America, is spreading along all of South America and the Caribbean, causing losses in cocoa production. In the region of Bahia, Brazil, this disease caused a decrease in production of almost 70% during a period of 10 years (Bowers et al. 2001).

The first detailed study about Witches' brooms was carried out by the German pathologist Gerald Stahel, during his work in Surinam and he named the causal agent *Marasmius perniciosus* (Stahel 1915).

The infection begins in *T. cacao* meristems with the appearance of multiple small shoots from flower clusters and branches in the host that are named witches brooms (Isaac et al. 1993). These cause the early death of cells and tissue. After the death of the broom tissue, the pathogen forms small pink mushrooms with abundant basidiospores that are spread by wind, allowing the dissemination of the disease within the humid cacao plantations. *Moniliophthora perniciosa* can also infect cocoa pods, penetrate the husk and destroy the seeds (Pereira 1999).

There are no official reports about the presence of Witches' Broom disease in Cuba but an alert system is necessary because the disease is known to exist on several Caribbean islands, e.g. Dominican Republic, Grenada, St Vincent and Grenadines, Trinidad and Tobago and Saint Lucia (Griffith et al. 1994, Kelly et al. 2009). The alert system is destined to prevent transport of potentially pathogen-contaminated material from diseased areas to disease-free areas.

2.3 Black pod disease

Black pod disease is a major constraint of cocoa cultivation in most of the cocoa-growing countries, especially in West and Central Africa and there are reports about the presence of this disease in Cuban cocoa plantations (Matos et al. 1998). Mainly four species of *Phytophthora* are known to cause black pod disease in cocoa. *Phytophtora megakarya* is the most aggressive in Africa and can cause between 60 and 100 % crop loss (Nyasse et al. 1999). *Phytophthora palmivora, P. citrophthora* and *P. capsici are* present in South America (Vos et al. 2003, Nyasse et al. 2007). *Phytophthora capsici* is often isolated from affected pods along with *P. palmivora* (Campelo & Luz 1981), although it is likely that the main causal pathogen is *P. palmivora*. *Phythophtora palmivora* is able to infect the pods at all stages of development, and the flowers and trunks at all stages of the growth cycle, sometimes causing the death of the tree (Gregory 1974, Lass & Wood 1985).

Other *Phytophthora* species have been reported causing black pod disease without producing significant damage [*P. botryosa* (Chee & Wastie 1970), *P.*

heveae (Lozano & Romero 1984), *P. katsurae* (Liyanage & Wheeler 1989) and *P. megasperma* (Zentmeyer 1988, Zadoks 1997)]. This contributes to the need to identify the cause of black pod disease at the species level.

The most notable symptom of this disease is black pods (Fig 1). The lesions start as small yellow spots on the pod that darken to brown or black color and grow fast covering the whole pod and internal tissues. Infected pods become reservoirs of inoculum for extended time periods. For example, *P. palmivora* spores on mummified pods remain active for at least 3 years (Dennis & Konam 1994).

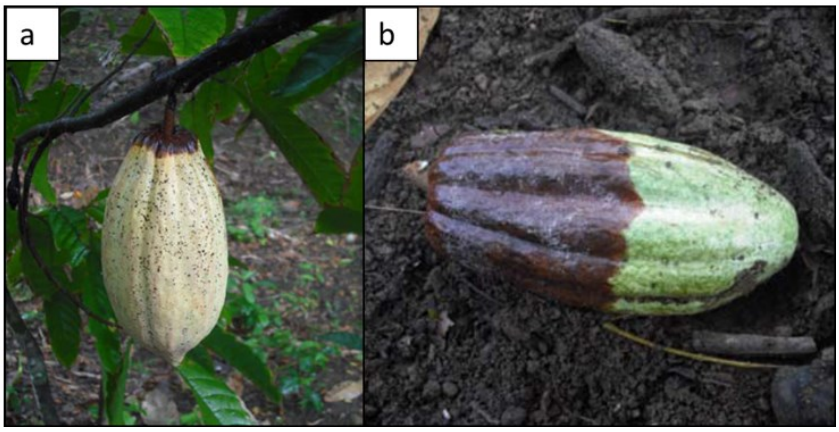

Fig 1 Cocoa pods affected by black pod disease caused by *Phytophthora*.

a) Beginning of the infection. b) Advanced state of infection.

3. Characteristics of *Phytophthora*

Phytophthora is a major genus of plant pathogens responsible for devastating diseases on a wide range of crops. The genus belongs to the Domain Eukaryota, Kingdom Chromalveolata, Phylum Heterokontophyta, Class Oomycetes, Order Peronosporales, Family Phythiaceae and Genus *Phytophthora* (Cavalier-Smith 1986). Oomycetes were previously considered to belong to the fungi because they have an osmotrophic nutrition and produce filamentous threads resembling fungal mycelium. Nowadays, the oomycetes are classified as a distinct group based on a number of unique characteristics showing that they have little affinity with filamentous fungi but are more closely related to brown algae. Such unique characters of

oomycetes are for example the production of diploid oospores, and a cell wall containing cellulose and β-glucans. True fungi do not produce oospores as a result of sexual reproduction, their rather produce zygospores, ascospores or basidiospores and their cell wall contains chitin and β-glucans. The vegetative cells of oomycetes are coenocytic hyphae, which contain diploid nuclei. In true fungi the mycelium is divided into cells by cross-walls and each cell contains one or more haploid nuclei (Kroon et al. 2012, Rossman & Palm 2006).

Traditionally, *Phytophthora* species were divided into six morphological groups considering the reproductive behavior, the features of the sporangium and the antheridium. Often these features do not allow a clear identification (Stamps et al. 1990). In recent years, the availability of DNA sequence data for multiple loci has led to an increase in the number of species descriptions. The first study on a large number of *Phytophthora* species was done using the internal transcribed spacer (ITS) region to analyze the phylogenetic relationships among 50 species obtaining ten primary clades (Cooke et al. 2000). Another study with the same *Phytophthora* species, using two mitochondrial (cytochrome c oxidase subunit I and NADH dehydrogenase subunit I) and two nuclear (translation elongation factor 1α, β-tubulin) genes, assigned some species to different clades (Kroon et al. 2004). In 2008, another study using seven of the most informative loci with approximately 8700 nucleotide sites for 82 *Phytophthora* spp., including newly discovered species, divided the genus into ten well-supported clades with the major *Phytophthora* cocoa pathogens *P. palmivora* and *P. megakarya* united in the same clade. The seven loci used in this study were the 28S ribosomal DNA, the 60S ribosomal protein L10, β-tubulin, translation elongation factor 1α, enolase, heat shock protein 90 and the TigA gene fusion protein (Blair et al. 2008).

A Latin American study showed the presence of species complexes in *Phytophthora* (Hérail et al. 2004). The identity, genetic diversity, geographic distribution, and impact of the comprised cryptic species are still under discussion. *Phytphthora palmivora* is present throughout the American continent and the Caribbean. *Phytophthora capsici* was divided into three entities: *P. capsici* sensu stricto, *P. capsici* "cocoa" and *P. tropicalis* (Hérail et al. 2004). The distribution of these taxa is heterogeneous; thus, for example, *P. capsici* "cocoa" appears to be the dominant taxon in Mexico.

4. Black pod disease situation in Cuba

The situation of *Phytophthora* in Cuba is controversial. Cuban plantations of small producers in the Eastern Province are variously affected by the black pod disease. The infection rate can reach up to 100% of the pods of some plants, but is erratic and there are no official reports. Although the disease is locally referred to as *Phytophthora*, no detailed study using modern techniques of genetic characterization has been undertaken to identify the etiological agents, their development and their impact. The actual incidence

of the disease and the influence of varieties and cultural practices are not known.

4.1 Cocoa disease control

There are no *T. cacao* cultivars completely resistant to any of the three main cocoa diseases, and the array of pathogens and their high genetic variability make breeding for resistance complicated. Different forms of crop protection are employed for cocoa such as cultural practices, chemical control with the use of pesticides and biological control agents.

Cultural control allows the establishment of conditions that avoid the spread and multiplication of pathogens. Pruning of cocoa, pod tripping, reduction of shade to the minimum required for good growth of the cocoa and removal of low branches, can contribute to the control of cocoa diseases. Other practices include regular harvesting of healthy ripe pods and the elimination of infected pods, including pod mummies that can remain sources of infection for long periods.

Chemical control, using copper-metalaxyl-based fungicides, was found to be effective in controlling the disease. However, these fungicides provide limited protection, particularly during the wet season when heavy rains are likely to wash away chemical treatments (Gockowski et al. 2010).

Biological control has been suggested as the most sustainable long-term solution in the context of integrated pest management (Holmes et al. 2004). Recent evidence shows that endophytic fungi restrict cocoa pathogen growth (Rubini et al. 2005, Tondje et al. 2006).

4.2 Fungal endophytes as potential biological control agents of *Theobroma cacao* pathogens

Endophytism is a phase in the life cycle, of numerous taxonomically very diverse fungal species that inhabit asymptomatically the various plant organs such as roots, trunks, branches, leaves, flowers and fruits, in which they form micro-colonies. They mostly belong to the Ascomycota, but encompass also several Basidiomycota. Endophytic fungi have been found in a wide variety of plants, covering most taxonomic lineages, and in fact might be present in all plants. They are thought to have co-evolved with the plants over time (Wilson 1995, Stone et al. 2004, Hyde & Soytong 2008).

Knowledge of the roles and biological interactions of endophytic fungi with and within their hosts is still incomplete. Nevertheless, it has been shown that they might confer pathogen resistance to their host (Arnold et al. 2003, Clay 2004, Crozier et al. 2006, Evans et al. 2003, Gond et al. 2010, Hanada et al. 2008, Holmes et al. 2004, Mejia et al. 2008, Rodríguez et al. 2009, Samuels et al. 2006).

The diversity of the fungal endophytic communities of cocoa has been assessed in many cultivation areas and several studies have been carried out on biological control of cocoa diseases using fungal endophytes (Arnold et al. 2003, Samuels et al. 2006, Mejía et al. 2008). Nevertheless, the majority of the studies concerned South and Central America (Amin et al. 2014, Arnold et al. 2003, Crozier et al. 2006, Evans et al. 2003, Hanada et al. 2008, Holmes et al. 2004, Mejia et al. 2008, Rubini et al. 2005, Samuels et al. 2006). They were mostly carried out in relation to the endophytes potential towards the biological control of Frosty pod, witches' broom, or Black pod disease, and mostly resulted in selecting strains of *Trichoderma* (e.g. De Souza et al. 2008, Krauss et al. 2006, 2010, Mbarga et al. 2014, Sriwati et al. 2015, Tondje et al. 2007). This selection includes *Trichoderma asperellum* (e.g. for control of black pod caused by *P. megakarya* in Africa, Mbarga et al. 2014, Tondje et al. 2007), *Trichoderma ovalisporum* (for control of frosty pod rot caused by *Moniliophthora roreri*, Krauss et al. 2010) and *Trichoderma stromaticum* (e.g. for control of witches' broom caused by *M. perniciosa*, Pomella et al. 2007). In Brazil, the development of a registered biological control product, called Trichovab® is based on *T. stromaticum* (Pomella et al. 2007, Samuels 2009).

Significant decrease in leaf necrosis and mortality were observed by Arnold et al. (2003), when endophyte-inoculated seedlings were challenged with a pathogenic strain of *Phytophthora. Trichoderma, Curvularia, Fusarium, Pestalotiopsis* and *Tolypocladium* species isolated from stems and branches of *T. cacao* and *T. grandiflorum* trees were tested against *P. palmivora,* resulting in a significant decrease in disease severity (Hanada et al. 2010).

The fungal endophyte *Gliocladium catenulatum* isolated from *T. cacao* in Brazil was tested for antagonism against *M. perniciosa*, the causal agent of witches broom disease, and showed the reduction of the symptoms (Rubini et al. 2005). There are evidences which show that several endophytes of tropical trees as *Acremonium*, *Clonostachys, Trichoderma* and *Fusarium* have potential for biological control (Burgess & Keane 1997, Driesche & Bellows 1996, Harman 2004, 2006, Odintsova et al. 2009, Papavizas 1985, Xue 2003). *Trichoderma* is the best example. *Trichoderma koningiopsis,* isolated in Brazil from the *T. cacao* tree was relatively effective against *M. roreri*, causal agent of frosty pod rot disease (Holmes et al. 2004). *Trichoderma theobromicola* and *T. paucisporum* were tested against *M. roreri in vitro* and in pod trials to evaluate possible mechanisms of their biological control potential. Both species produced a volatile and diffusible antibiotic that inhibits the development of *M. roreri* (Samuels et al. 2006). Another study found that several *Trichoderma* isolates produced inhibitory metabolites or were able to parasitize cultures of *M. roreri* (Bailey et al. 2008). In this context, cocoa trees have a big advantage with a highly diverse endophytic community (Melnick et al. 2011). Due to the complexity of the interactions among the biocontrol agents, pathogen, plant, microbial community and environment, it would be of advantage to use native isolates of fungal endophytes that are adapted to the local environmental conditions and microbial communities. Additionally, the utilization of native endophytes

would avoid a potential negative impact that introduced species or strains might have on native ecosystems (Emmert & Handelsman 1999).

Nowadays, fungal endophytes are a good alternative for a proper control of the black pod disease that would strengthen cocoa plantations, reduce crop losses and ensure healthy pods as raw material for the post-harvest processing. The evaluation of the fungal endophyte *Colletotrichum gloesporioides* against black pod disease showed a significantly decreased pod loss in four farms in Panama (Mejía et al. 2008). These results support the use of fungal endophytes as biological control agents to reduce the losses caused by black pod disease in cocoa. However, there are limitations for the field application of fungal endophytes. Careful evaluation of side effects needs to precede the commercialization of fungal endophytes as biocontrol agents (Alabouvette et al. 2006, Merriman & Russell 1990). Upon release the endophyte may not only colonize the plants targeted for biocontrol and may cause adverse effects or behave as pathogen in non-target plants (Hyde & Soytong 2008). Several publications give information about the positive effects of fungal endophytes against black pod disease using *in vitro* inoculation test systems under constant environmental conditions (Hanada et al. 2010, Mbarga et al. 2014, Sriwati et al. 2015). An important step is the translation and realization of those tests to plants growing under field conditions. Another challenge is the development of suitable formulations to apply fungal endophytes effectively in the field (Nakkeran et al. 2005).

5. Cocoa bean fermentation process

Theobroma cacao is a crop of the humid tropics, often produced by small-scale farmers and is grown for its fruit pods that contain the seeds or beans. Cocoa beans are the raw material for chocolate production and their quality depends on various factors such as the planting material, the harvest and the post-harvest processes fermentation, drying, and roasting. The fermentation of the cocoa pulp-bean mass is the most important process and can be carried out in different ways. Two practices are the most widely used: fermentations in heaps or in boxes. For both techniques the beans are removed from the pods and placed in heaps or boxes to be fermented during 5 to 7 days (Schwan et al. 1995). Diseased and damaged pods should be removed. It is also important not to use immature pods in which the beans are not ready for fermentation, because the pulp contains less free sugars and less ethanol is produced during yeast fermentation (Packiyasothy et al. 1981). The maturation process influences cocoa characteristics by the evolution of sugars, carboxylic acids, fatty acid composition, aspartic, endoproteinase activity, theobromine as the dominant purine alkaloid, and caffeine that is produced towards the end of maturation (Bucheli & McCarthy 2001).

During cocoa fermentation a microbial succession of yeasts, lactic acid bacteria and acetic acid bacteria takes place. These microorganisms induce

biochemical transformations inside the beans, allowing the formation of important precursors of the chocolate flavor (De Vuyst et al. 2010).

The yeasts start an alcoholic fermentation of the pulp sugars, generating ethanol under low oxygen conditions. Yeast also contribute to the pectin degradation in the pulp reducing pulp viscosity and allowing bean aeration, which creates suitable conditions for the growth of lactic acid bacteria and acetic acid bacteria in an acidic environment (pH < 4.0). Lactic acid bacteria transform citric acid and carbohydrates in the pulp into lactic acid, acetic acid and mannitol, contributing to the balance of the pH. The ethanol produced by yeasts is oxidized by acetic acid bacteria to produce acetic acid, which increases the temperature to 45-50°C. Ethanol and acetic acid diffuse into the beans, inactivate the seed embryo, causing the breakdown of the cell walls, allowing endogenous enzymes, such as invertase, glycosidases, proteases and polyphenol oxidase, to come into contact with their substrates (Camu et al. 2007, 2008, Daniel et al. 2009, Nielsen et al. 2007).The action of two proteases, aspartic endopeptidase and serine carboxy (exo) peptidase, produce the enzymatic degradation of globulin storage protein. These biochemical reactions in the cocoa beans generate various flavor precursors such as reducing sugars, peptides, and amino acids. Roasting of these precursors produced significant cocoa flavor (Voigt et al. 1994).

5.1 Cocoa fermentation practices in Cuba

Theobroma cacao grows during the whole year with usually two harvesting periods. In Baracoa, the small harvest, with low production, takes place from September to December and the big harvest from April to July when the highest production levels are reached. The agricultural system is organized in production cooperatives in which the landowner is the government and the farmers are either salaried workers or tenants. The farmers deliver the harvest to government-owned cooperatives that centralize the fermentation and drying processes, and control the production chain.

The harvest and fermentation practice can be described as follows (Fig. 2): two or three days before the delivery day, the farmer selects mature cocoa pods without disease symptoms to be harvested. These pods are cut from the trees and collected on the ground. On the delivery day, pods are opened with a knife and the pulp-bean mass is extracted from the pods by hand into a container named yagua made from a dead palm leaf (Fig. 3). Jute or synthetic fabric bags are filled with 40 to 50 kg pulp-bean mass each from the yagua and transported by horse to the cooperatives collection points and from there to the fermentation centers. At the fermentation centers, the cocoa pulp-bean mass is assembled into heaps on a concrete platform exposed to the sun. The heaps are covered by black plastic. From day 3 onwards the heaps are turned daily in the morning.

Problems are the duration of the transport and the variable waiting periods in bags from several hours up to two days, which depends on the availability of transport. The fermentation starts already with the removal of the pulp-bean

mass from the pods. The conditions under which the fermenting mass is kept in bags are highly variable, for example due to exposure to sun, rain, or bag material. The fermentation in mini-batches (per bag) influences the further fermentation in the later assembled heaps.



Fig 2 Harvest and fermentation practices in Cuba. a) collected pods. b) removal of the pulp-bean mass from the pods and collection in a yagua. c, d) fermentation heaps at the fermentation center Jamal in Baracoa.



Fig 3 Yagua: container made from a dead palm leaf to collect the bean-pulp mass of cocoa in cocoa plantations in Baracoa, Cuba.

5.2 Yeast diversity in cocoa fermentation

Fermentative yeasts are typical colonizers of carbohydrate-rich, acidic substrates such as fruits. Their predominantly unicellular growth allows fast multiplication in suitable environments, but does not permit them to easily reach fresh substrate sources and yeasts rely on vectors for transfer to fresh substrates. The cocoa environment provides both, rich substrate sources and insects attracted by the ripe, opened cocoa pods. Numerous studies revealed an abundant yeast diversity in cocoa bean fermentations (Fig. 4, Table 1, Annexes). The most frequently isolated species were *S. cerevisiae*, *Pichia kudriavzevii, Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae* (Arana-Sanchez et al. 2015, Daniel et al. 2009, Hamdouche et al. 2014, Ho et al. 2014, Lagunes-Gálvez et al. 2007, Meersman et al. 2013, Papalexandratou et al. 2011, 2013). Determination of yeasts diversity is a key step towards the industrialization of cocoa bean fermentation (Ho et al. 2014). The inoculation of selected yeast strains allows the control of fermentation processes to obtain chocolate with desirable characteristics (de Melo-Pereira et al. 2012).

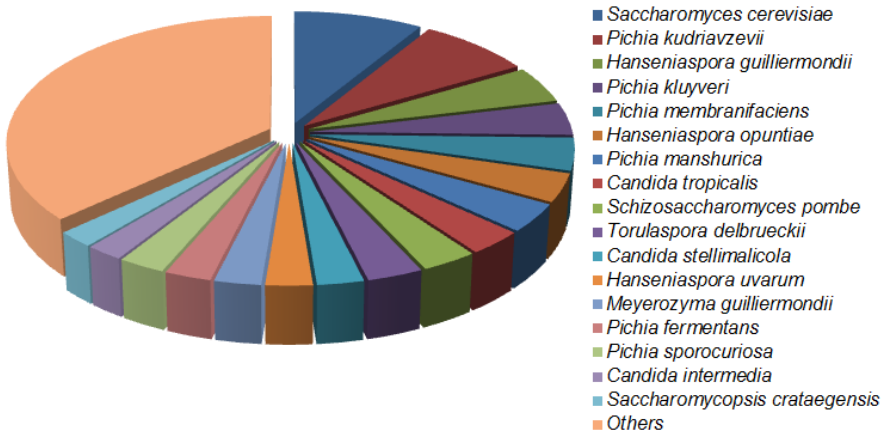

Fig 4 Yeasts species that have been reported from cocoa fermentations in at least four references, according to the literature cited in Table 1 (Annexes).

Yeasts have an impact on the sensory qualities of chocolate characteristics (Lefeber et al. 2012, Ho et al. 2014). Cocoa beans fermented in the absence of yeasts produced chocolates of variable characteristics, often lacking typical chocolate aromas. Yeasts are responsible for the production of ethanol, the secretion of pulp degrading enzymes and produce esters and higher alcohols that contribute to the mixture of volatile aroma compounds (Schwan & Wheals 2004).

Crafack et al. (2013) reported the influence of aromatic and pectinolytic yeasts on cocoa flavor using *Pichia kluyveri* and *Kluveromyces marxianus* in defined mixed starter cultures for cocoa fermentation. Sensory analysis showed that the two starter cultures incorporating different yeast species (*P. kluyveri* / *Lb. fermentum* / *A. pasteurianus* and *K. marxianus* / *Lb. fermentum* / *A. pasteurianus*) modified the flavor profile of un-conched chocolate differentially. The chocolate produced from fermentations inoculated with the *P. kluyveri* / *Lb. fermentum* / *A. pasteurianus* starter showed the highest intensity of fruitiness, sweetness and cocoa aroma compared with chocolates produced using the *K. marxianus* / *Lb. fermentum* / *A. pasteurianus* starter and from the spontaneously fermented control. Cocoa bean fermentation inoculated with the *K. marxianus* / *Lb. fermentum* / *A. pasteurianus* starter produced the most bitter, astringent and sour chocolate in the same comparison.

Diversity assessments rely on precise identification techniques. The development of genotype-based approaches has provided a powerful identification tool that has surpassed the inherent variability of phenotypic tests (Kurtzman 1984). The genes of the ribosomal gene complex (rRNA) are used to identify yeasts, especially the 5' region of the large subunit gene (LSU) that contains the variable D1/D2 regions (Kurtzman & Robnett 1998, Fell et al. 2000). Nowadays, a large database of yeast D1/D2 LSU sequences exists, for which reason this region is most commonly used for sequence-based yeast identification. However, there are reports of closely related taxa in which D1/D2 LSU sequences do not show sufficient divergence (Daniel & Meyer 2003). For example, this is the case for the species *H. opuntiae* and *H. guilliermondii*, *Candida carpophila* and *Meyerozyma guilliermondi* that are impossible to separate using D1/D2 LSU sequences (Daniel et al. 2009). Identification can be complemented by fingerprint methods based on PCR amplification that are more reliable in the recognition of conspecific isolates, because they address the entire genome without a preselection of a particular genetic marker. The PCR-fingerprinting technique allows to unambiguously discriminate between different yeast species; in the case of *Saccharomyces cerevisiae*, it also allows the distinction of strains. This method combines the relative ease and speed of the PCR technique with the hybridization-based DNA fingerprinting, using a single primer specific to hypervariable genomic DNA sequences (Lieckfeldt 1993). PCR-fingerprinting is a comparative technique and either requires the inclusion of type strains of the species to be identified or the identification of isolates representing similar fingerprint profiles. In case the standard sequencing target D1/D2 LSU is not sufficient to distinguish certain closely related species, sequencing of additional genes such as the internal transcribed spacer (ITS) region of the rRNA gene cluster (Scorzetti 2002) or protein-coding genes such as the actin gene (ACT1) (Daniel & Meyer 2003) is necessary.

Chapter II

Characterization of *Phytophthora*, etiological agent of cocoa black pod disease in Cuba and French Guiana

Submitted

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Abstract

Black pod is one of the major like-fungal diseases of *Theobroma cacao* in all its cultivation areas. It is caused by several species of *Phytophthora*. In Latin America, the etiological agents of the black pod disease are more likely diverse and, in a taxonomic perspective, incompletely known, especially in small producing countries, such as Cuba and French Guiana. In the present work, we studied two sets of *Phytophthora* strains isolated from diseased pods and originating from Cuba and French Guiana, combining morphological, biological and molecular approaches to identify the species involved. Phylogenetic analyses were performed based on DNA sequence data from three loci, ribosomal internal transcribed spacer, partial β-tubulin and translation elongation factor-1α genes. Two species were identified in Cuba, *viz*. *P. palmivora* and *P. tropicalis*, and two species in French Guiana, *viz*. *P. tropicalis / capsici* complex and a potential novel *Phytophthora* sp. In Cuba, *P. palmivora* was by far the dominant species (98% of the isolates) and the strains studied belonged to the Mating Type A2 (88 out of 90 isolates). In French Guiana, *P. tropicalis / capsici* complex was the dominant species in our sampling (7 out of 8 isolates).

Keywords: *Phytophthora*, cocoa, internal transcribed spacer, Translation elongation factor 1α, β-Tubulin.

Introduction

Cocoa (*Theobroma cacao* L.) is affected by many fungal diseases and pests in all its areas of cultivation. Among the numerous diseases, black pod disease is one of the major constraints. The disease is caused by several species of *Phytophthora* (Oomycota).

The taxonomy of *Phytophthora* species associated with cocoa black pod disease has been controversial for a long time. Historically, the strains associated with black pod disease were identified as a single species, *P. palmivora* (Waterhouse 1963), including possible morphological variants (Griffin 1977, Waterhouse 1963). Brasier& Griffin (1979) first recognized the presence of a complex of species causing the black pod disease; four main species were then distinguished *viz*. *P. palmivora*, *P. megakarya*, *P. capsici*, and *P. citrophthora*. *Phytophthora palmivora* is more likely the most damaging species worldwide, occurring in all cultivation areas; including South America (Erwin & Ribeiro 1996). *Phytophthora megakarya* for instance is an overly aggressive species, endemic in West Africa, where it causes important damage (Nyasse et al. 1999).

The situation of *Phytophthora* causing black pod disease is likely the most complex in the Neotropics; several taxa are involved, whose identities, genetic diversity, geographic distribution, and impact are still under discussion (Hérail et al. 2004).

This might be linked to the fact that South America is the native area of *T. cacao* with wild populations widespread over the Amazonian basin. Its pathogens might be diverse in its natural environment. *Theobroma cacao* also has a long story of human-originating dispersion in the Neotropics.

Phytophthora palmivora has been identified throughout the Neotropics, from South- to Mesoamerica and the Caribbean (Thevenin et al. 2012). *Phytophthora citrophthora*, *P. capsici*, *P. tropicalis*, and at a lesser degree, *P. collocasiae*, have been reported, e.g. in Brazil (Vos et al. 2003, Nyasse et al. 2007). However, *P. capsici* or *P. citrophthora* in Latin America, on cocoa, might encompass distinct taxonomic entities whose rank remains uncertain. *Phytophthora capsici* could include several taxa *viz*. *Phytophthora capsici* s. s., *Phytophthora capsici* "cocoa" and *Phytophthora tropicalis* (Hérail et al. 2004). *Phytophthora tropicalis* was recognized as a species distinct from *P. capsici* in 2001 and is reported on numerous hosts, including *T. cacao* (Aragaki & Uchida 2001). Nevertheless, *P. tropicalis* and *P. capsici* are able to hybridize in *in vitro* crosses (Donahoo & Lamour 2008) and the distinction between both taxa remains critical. The distribution of these three taxa also is incompletely known; for instance, *P. capsici* "cocoa" would be the dominant taxon in cocoa plantations in Mexico.

The situation has been studied in the major growing areas, such as Brazil, Mexico, or Ecuador (Ducamp et al. 2004, Lawrence et al. 1982). However, in

many regions of lesser importance and in wild populations of *T. cacao*, the *Phytophthora* species causing black pod disease are still poorly known.

Cuba is a small cocoa producer, mostly for local consumption. The cocoa production is located almost exclusively in the easternmost region, in the area of Baracoa. This area is the most suitable for cocoa plantation owing to the climatic factors, including adequate rainfall, humidity, and temperature (Hartmann & Larramendi 2011). Cuban cocoa plantations are severely affected by black pod disease, mainly because of lack of resources and poor management of the plantation. As well, French Guiana is a very small producer and plantations mostly old, from the 19th century relic plantations, are scattered. Nevertheless, native, wild populations of *T. cacao* are present, scattered in the southern half of the country, and local *Phytophthora* species might be expected. Previously, a set of *Phytophthora* strains originating from old remnants of cocoa plantations was tentatively identified as *P. capsici* (Lachenaud et al. 2015). However, to date no detailed study, using modern techniques of characterization has been undertaken to identify the *Phytophthora* species affecting cocoa in Cuba.

The objective of the current study was to identify and to characterize two sets of *Phytophthora* strains isolated from black pod disease, originating from Cuba and French Guiana. The strains from French Guiana were useful to clarify the complex situation of *Phytophthora* species in Latin America.

Materials and methods

Collection of isolates

Ninety isolates of *Phytophthora* were isolated from pod lesions during different field surveys in the different plantations in the area of Baracoa between 2009 and 2010 (Table 1). Eight isolates were obtained from French Guiana (Table 1), all originating from areas upstream of Régina, along the bank of the Approuage. Isolates were maintained on V8 agar medium at 24-29°C.

Table 1 Strains of *Phytophthora* collected in Baracoa, Cuba and French Guiana.

Zone	Location	Strains	Country	Date
Poa, El Chocolate	20°17'19,39"N 74°27'56,10"W	PHYTO15 PHYTO16 PHYTO17 PHYTO18 PHYTO19 PHYTO20 Poa1 Poa2 Poa3 Poa5	Cuba	April, 2009 April, 2010
Boris Miraflores, El Porvenir	20°13'47,20"N 74°26'45,90"W	PHYTO25 PHYTO26 PHYTO27 PHYTO28 PHYTO29 PHYTO30 PHYTO31 PHYTO32 PHYTO33 PHYTO34	Cuba	April, 2009
Lomo de Camaguay	20°30'41,12"N 74°42'34,90"W	CU/09-41 CU/09-PL6812 CU/09-PL6813 CU/09-42 CU/09-43 CU44 CU0944 CU/0944c CU/09-44d	Cuba	November, 2009
Juan Romero	20°12'25,86"N 74°23'17,08"W	CU/09-50 CU/09-60 CU/09-63 CU/09-71A CU/09-71B CU/09-73 CU/09-74 CU/09-75 CU/09-76 CU/09-77 CU/09JR06 CU09JR08 CU/09JR10B CU/09JR11A	Cuba	November, 2009

Zone	Location	Strains	Country	Date
Naranjal del Toa	20°23'49,69"N 74°32'55,62"W	PHYTO36 PHYTO39 PHYTO40	Cuba	April, 2009
Capiro, Nueva Vista	20°13'06,24"N 74°23'29,34"W	CU/09-45A CU/09-45B CU/09-47 CU/09-48	Cuba	November, 2009
Arroyo de la vieja finca	20°12'29,52"N 75°38'27,42"W	CU/09-56 CU/0957 CU/09-61 CU/09-62	Cuba	November, 2009
Capiro, Nueva vista	20°13'06,24"N 74°23'29,34"W	CU0958 CU/09-59	Cuba	November, 2009
Esteban Silot	20°13'34,49"N 74°30'21,89"W	CU/09-SILOT3 CU/09-SILOT5	Cuba	November, 2009
Los hoyos de Sabanilla	20°16'51,32"N 74°27'52,87"W	Est1 Est2 Est3 Est4 Est5 Est6 Est7	Cuba	April, 2010
Cayayal	20°15'44,02"N 74°27'51,28"W	Cay1 Cay2	Cuba	April, 2010
Paso de Cuba	20°20'03,72"N 74°28'05,66"W	P-C1 P-C2 P-C3	Cuba	April, 2010
Santa Rosa	20°21'20,47"N 74°31'58,65"W	S-R1 S-R2	Cuba	April, 2010
Mabujabo, Silina	20°19'06,92"N 74°26'49,33"W	Mab1 Mab2	Cuba	April, 2010
Guayabo	20°26'24,98"N 74°36'12,74"W	Gua1 Gua2	Cuba	April, 2010
Saibá	20°26'02,89"N 74°36'27,70"W	Sai1	Cuba	April, 2010
San Luis, Victor Prieto	20°18'11,43"N 74°25'56,35"W	S-L2	Cuba	April, 2010
Jamal	20°16'34,65"N 74°25'32,35"W	Jam1	Cuba	April, 2010
Jobo Dulce	20°19'22,55"N 74°26'46,31"W	Job2	Cuba	April, 2010
Sabanilla	20°16'42,11"N 74°27'52,86"W	Hur1	Cuba	April, 2010

Zone	Location	Strains	Country	Date
Régina, Crique Courouaïe, right bank	4°16'07,24"N 51°57'52,13"W	Reg 2-1 Reg 2-3 Reg 2-4 Reg 2-6 Reg 2-7 Reg 2-8 Reg 2-10	French Guiana	March 2009-2014
Régina, Approuague River, left bank	4°20'43,95"N 52°02'01,35"W	Reg 3-1	French Guiana	March 2014

Morphological characterization

The different morphological characters (mycelium, sporangia, oospores, chlamydospores) of the strains were observed under the microscope Olympus BX50. Pictures of these fungal structures were taken via the microscope, using the program Ulead Photo explore SE (V 7.0). Growth of the mycelium was observed in Petri dish for each individual. Fungal structures were observed microscopically. Measures of the latter were carried out on each specimens collected in Cuba.

Pairing test

The mating type of *P. palmivora* strains was determined by pairing with references MT A1 and A2 strains of *P. palmivora* (MUCL 52539 and MUCL 52540) on V8 juice agar. The isolate to be tested was placed on one side of the Petri dish, the mating type reference isolate on the other side. The Petri dishes were incubated at 25°C in the dark for about 14 days. On the basis of the appearance of oospores, a tested isolate was classified as mating type A1 or A2 (Tooley et al. 1989).

DNA sequence determination and phylogenetic analyses

DNA was extracted from freshly collected mycelium grown in liquid malt extract at 25°C in the dark. Extractions were carried out using the QIAGEN Dneasy plant Mini Kit (QIAGEN Inc., Hilden, Germany), and later purified with Geneclean® III kit (Q-Biogene, USA), following the manufacturer's recommendations. The primer pairs TUBUR1-TUBUF2 (Kroon et al. 2004), ELONGF1-ELONGR1 (Kroon et al. 2004, Martin et al. 2014) and NS7-ITS4 (White et al. 1990) were used to amplify the β-tubulin, TEF1-α and ITS, respectively. Successful PCR assays resulted in a single band observed on a 0.8%, m/v, agarose gel, corresponding to approximately 900 bps (ITS) and

500 bps. PCR products were cleaned using the QIAquick® PCR purification kit (250) (QIAGEN Inc., Hilden, Germany), following the manufacturer's protocol. Sequencing reactions were performed using CEQ DTCS Quick Start Kit® (Beckman Coulter Inc), according to the manufacturer's recommendations, with the primers ITS2, ITS3, and ITS4 (White et al. 1990) and Bt2a and Bt2b. Nucleotide sequences were determined with a CEQ 2000 XL capillary automated sequencer (Beckman Coulter Inc). Initially, nucleotide sequences were automatically aligned with Clustal X for MacIntosh (version 1.5b), then manually adjusted as necessary by the editor in PAUP* (version 4.0b10).

Phylogenetic analysis of the aligned sequences was performed using the maximum parsimony method of PAUP* version 4.0b10 (Swofford 2002) with gaps treated as fifth base. The strains used in this study are listed in Table 2. The most parsimonious trees were identified using heuristic searches with random addition sequence (1000), max tree set to 100, and further evaluated by bootstrap analysis, retaining clades compatible with the 50% majority-rules in the bootstrap consensus tree. Analysis conditions were: tree bisection addition branch swapping (TBR), starting tree obtained via stepwise addition, steepest descent not in effect, MulTrees effective. *Phytophthora multivora* strain (CBS 124094) was used as an outgroup.

The ITS, β-tubulin and TEF1-α sequences were compared to those encoded in Genbank using the BLAST search option at the National Center for Biotechnology Information (NCBI)

Table 2 Isolates used in the phylogenetic studies

Phytophthora species	Sequences identification in Genbank					
	β-tubulin	Elongation factor 1-α	ITS	International identification[a]	Host	Country
P. tropicalis	EU080306	EU080307		PD00020		United states
P. tropicalis	EU079994	EU079995		PD00152		
P. tropicalis	EF617446					United States
P. tropicalis	EF617443				*Theobroma cacao*	Mexico
P. tropicalis	EU080859	EU080860		PD01307		United States
P. tropicalis	EF6174449				*Rhododendron sp*	United States
P. tropicalis	EF617441				*Theobroma cacao*	Mexico
P. tropicalis	EF617442				*Theobroma cacao*	Mexico
P. tropicalis	EF617444				*Rhododendron sp*	United States
P. capsici	EU080620	EU080621		PD00052		United States
P. tropicalis	AY564046	AY564103				
P. capsici	EU080852	EU080853		PD00009		Mexico
P. capsici	EF617436				*Theobroma cacao*	Cameroon
P. capsici	EF617428				*Theobroma cacao*	El Salvador
P. capsici	EF617429				*Theobroma cacao*	Mexico
Phytophthora sp	EU079576	EU079577		PD00095		United States
P. mexicana	EU080703	EU080704		PD00061		Mexico
P. glovera	FJ008021	FJ008029		PD01617	*Nicotianatabacum*	Brazil
P. siskiyouensis				PD01635	*stream*	United States
P. siskiyouensis				PD01636		United States

Phytophthora species	Sequences identification in Genbank					
	β-tubulin	Elongation factor 1-α	ITS	International identification[a]	Host	Country
P. brasiliensis	EF617445				*Theobroma cacao*	Brazil
P. brasiliensis	EF617447				*Theobroma cacao*	Brazil
P. brasiliensis	EF617448				*Theobroma cacao*	Brazil
P. mengei	GU191332	GU191203		PD02698		Mexico
Phytophthora sp	EU080095	EU080096		PD00170		Australia
Phytophthora sp	EU080392			PD00002		Costa Rica
Phytopthora sp	JN605841				*citrus*	South Africa
P. multivora	FJ665260				*Eucalyptus*	
P. tropicalis		AM 040497			*Artocapusaltilis*	Brazil
P. tropicalis		EU080159				United States
P. capsici		KF318774			Black pepper	India
P. capsici		KF318773			Black pepper	India
P. capsici		KY318775			Black pepper	India
P. mengei		GQ247669			Avocado	United States
P. mengei		GQ247670			Avocado	United States
P. citrophthora		AY564112				
P. plurivora		KC855387				
P. multivora		GU191183			nursey	South Africa
P. multivora		GU191194		PD01226		
P. palmivora		EU080340		PD00022	*Theobroma cacao*	Costa Rica

Phytophthora species	Sequences identification in Genbank					
	β-tubulin	Elongation factor 1-α	ITS	International identification[a]	Host	Country
P. palmivora			AY208127			
P. palmivora			AY208126			
P. palmivora		EU080466	GU259121	PD00032	*Papaye*	Hawaii
P. palmivora				PD01611	*Theobroma cacao*	Costa Rica
P. palmivora				PD01612	*Theobroma cacao*	Costa Rica
P. palmivora				PD01614		United States
P. palmivora				PD01624		Canada
P. palmivora				PD01625		United States
P. palmivora				PD01621	*Duriosp*	Malaysia
P. palmivora				PD01623		Malaysia
P. palmivora				PD01622	*Cocos nucifera*	Indonesia
P. tropicalis			AY207010			
P. palmivora		*PHYTO19	*PHYTO19		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO29	*PHYTO29		*Theobroma cacao*	Cuba
P. palmivora			*CU0944		*Theobroma cacao*	Cuba
P. palmivora		*CU0955	*CU0955		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO15	*PHYTO15		*Theobroma cacao*	Cuba
P. palmivora		*MAB1	*MAB1		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO39	*PHYTO39		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO33	*PHYTO33		*Theobroma cacao*	Cuba

Phytophthora species	Sequences identification in Genbank					
	β-tubulin	Elongation factor 1-α	ITS	International identification[a]	Host	Country
P. palmivora		*MAB2	*MAB2		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO16	*PHYTO16		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO18	*PHYTO18		*Theobroma cacao*	Cuba
P. palmivora		*PHYTO34	*PHYTO34		*Theobroma cacao*	Cuba
P. tropicalis	*PHYTO40	*PHYTO40	*PHYTO40		*Theobroma cacao*	Cuba
P. tropicalis	*CU58	*CU58	*CU58		*Theobroma cacao*	Cuba
Phytophthora sp	*Reg2-1	*Reg2-1	*Reg2-1		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg2-3	*Reg2-3	*Reg2-3		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg2-4	*Reg2-4	*Reg2-4		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg2-6	*Reg2-6	*Reg2-6		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg2-7	*Reg2-7	*Reg2-7		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg2-8	*Reg2-8	*Reg2-8		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg2-10	*Reg2-10	*Reg2-10		*Theobroma cacao*	French Guiana
Phytophthora sp	*Reg3-1	*Reg3-1	*Reg3-1		*Theobroma cacao*	French Guiana

[a] Identification of *Phytophthora* database

*Sequences obtained in the current study

Results

Morphological characterization

The microscopic observations showed that 88 Cuban strains have a similar, petaloide growing pattern on V8 medium. Two Cuban strains (CU58 and PHYTO40) and the 8 strains from French Guiana showed a chrysanthemum growing pattern on V8 medium (Fig. 1).

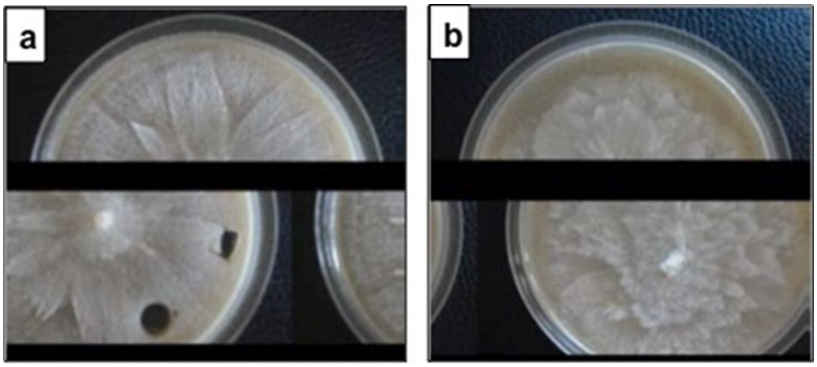

Fig 1 Characteristic colony morphology of *Phytophthora* species isolated from cocoa on V8 agar medium after 4 days of growth at 24-29°C in the dark: a: petaloide shape (strain MAB1). b: chrysanthemum shape (strain CU58).

Microscopically, they produce abundant ovoid to ellipsoidal, papillate, deciduous, mostly sessile or shortly stalked (pedicel≤ 4 µm long) sporangia, whose size ranges 38-60 µm in length and 27-40 µm in wide, with a ratio L / B (length / breadth) from 1.25-1.92 (Fig 2a). Chlamydospores also are present, and are a diameter of 32.5-43.5 µm (Fig 2c). These morphological characteristics observed in 88 of Cuban isolates, correspond to those observed in *P. palmivora* (Appiah et al. 2003, Bush et al. 2006).

Microscopically, in two Cuban isolates (CU58 and PHYTO40) and in all the isolates from French Guiana, sporangia measure 37-50 µm in length and 22-32 µm in wide, and a ratio L / B ranging from 1.8-2.4. These sporangia are deciduous but with a long hyphal-like pedicel (longer than 50 µm) (Fig 2d). According to these characters the strains belong to the complex *P. capsici* - *P. tropicalis* within which morphological characters do not allow further distinction (Aragaki & Uchida 2001).

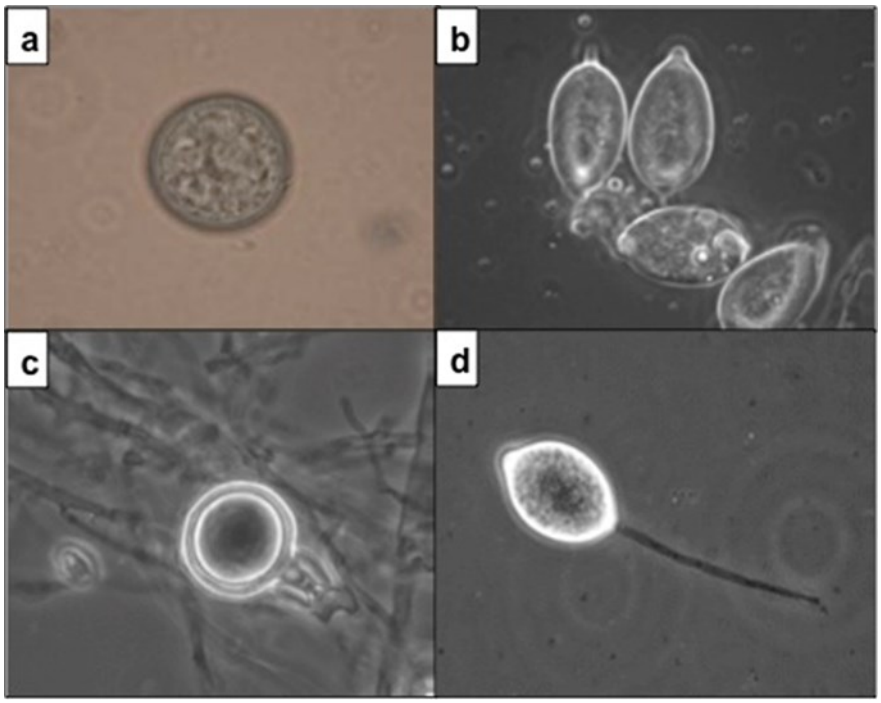

Fig 2 Morphological structures of *P. palmivora* and *P. tropicalis* strains from Cuba. a: ovoid-ellipsoidal sporangia of *P. palmivora* (strain MAB2) b: oospores of *P. palmivora* (strain MAB2) c: chlamydospores (strain MAB2). d: sporangia of *P. tropicalis* (strain PHYTO40).

Pairings

Crosses were carried out between the 88 Cuban strains assumed to belong to *P. palmivora*, and both references MT A1 and A2 strains (MUCL 52539 and MUCL 52540). These 88 Cuban strains were found compatible with the mating type A1 strain, resulting in the formation of oospores. Oospores are globose, and have an average size of 25 µm diameter (Fig 2b).

Two Cuban strains, CU58 (MUCL 55783) and PHYTO40 (MUCL 55782), were found to be sterile in the presence of each of the reference mating type of *P. palmivora*.

Phylogenetic analysis

Phytophthora palmivora

The *P. palmivora* strains compared showed a high degree of similarity (Figs 3, 4); there was very little intraspecific variation in the DNA regions analysed. Based on that, a selection of strains was used in the phylogenetic trees of ITS region and TEF1-α gene. A maximum of 5 bp differences were observed within the 700 base pairs long ITS region, which corresponded to a proportion of variable sites of about 0.01. The 1000 base pairs long TEF1-α region was more variable with a maximum of 20 differences, which corresponded to a proportion of variable sites of 0.02bp.

P. tropicalis / *capsici* complex

β-tubulin data set

The phylogenetic analyses for β-tubulin included 40 taxa and *P. multivora* (CBS 124094) as an outgroup taxon. The data set comprised a total of 865 bp, of which 67 were parsimony informative. 1000 Heuristic searches resulted in 981 most parsimonious trees (MPT) (140 steps in length, CI = 0.771, RI = 0.904), of which two concurrent topologies are shown in Fig 5.1 and Fig 5.2. These two concurrent topologies were selected because the strains from French Guiana formed a well-supported clade, but their affinities are not well supported.

TEF1-α data set

The phylogenetic analyses for TEF1-α included 34 taxa, including *P. multivora* (CBS 124094) as an outgroup taxa. The data set comprised a total of 880 bp, of which 60 were parsimony informative. One thousand heuristic searches resulted in 492 MPTs (161 steps in length, CI = 0.694, RI = 0.829), of which two concurrent topologies are shown in Fig 6.1 and Fig 6.2.

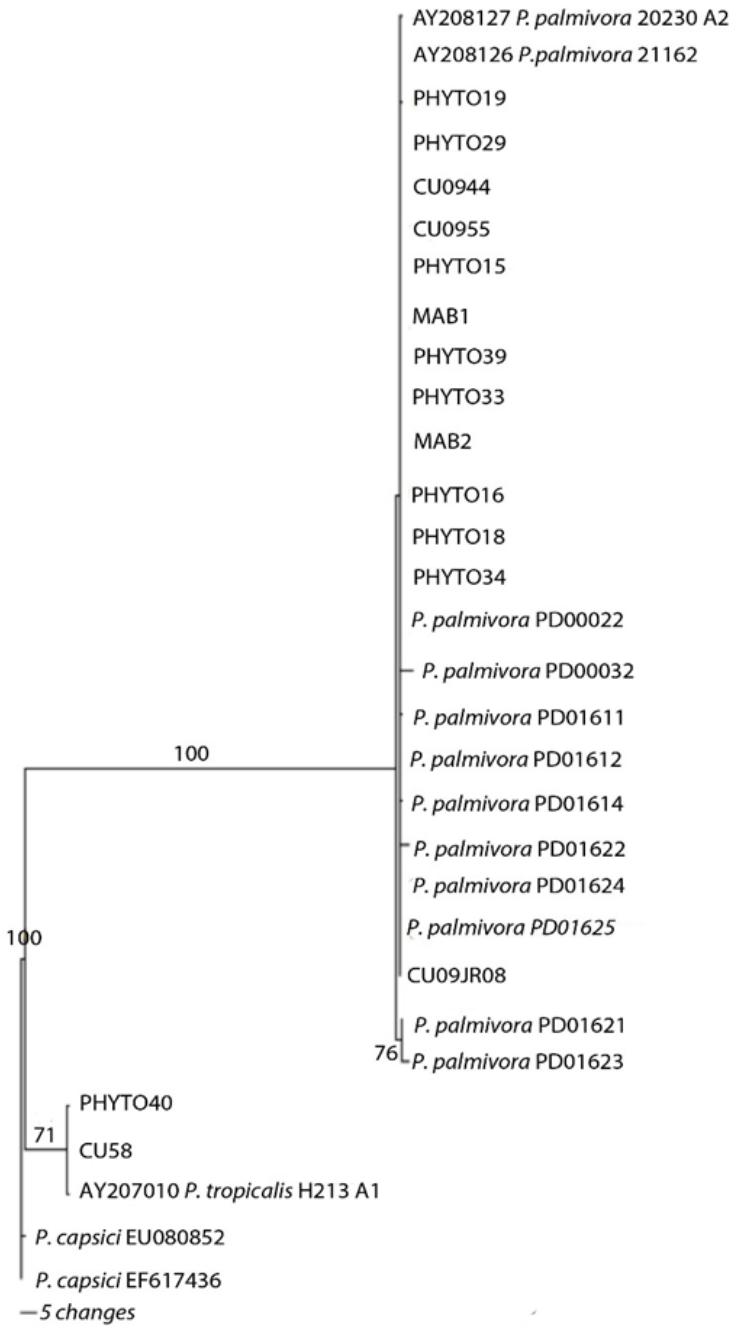

Fig 3 Phylogenetic tree showing the relationship of Cuban *Phytophthora* strains based on the sequences of the ITS region, inferred by maximum parsimony analysis. Numbers above nodes indicate bootstrap support values.

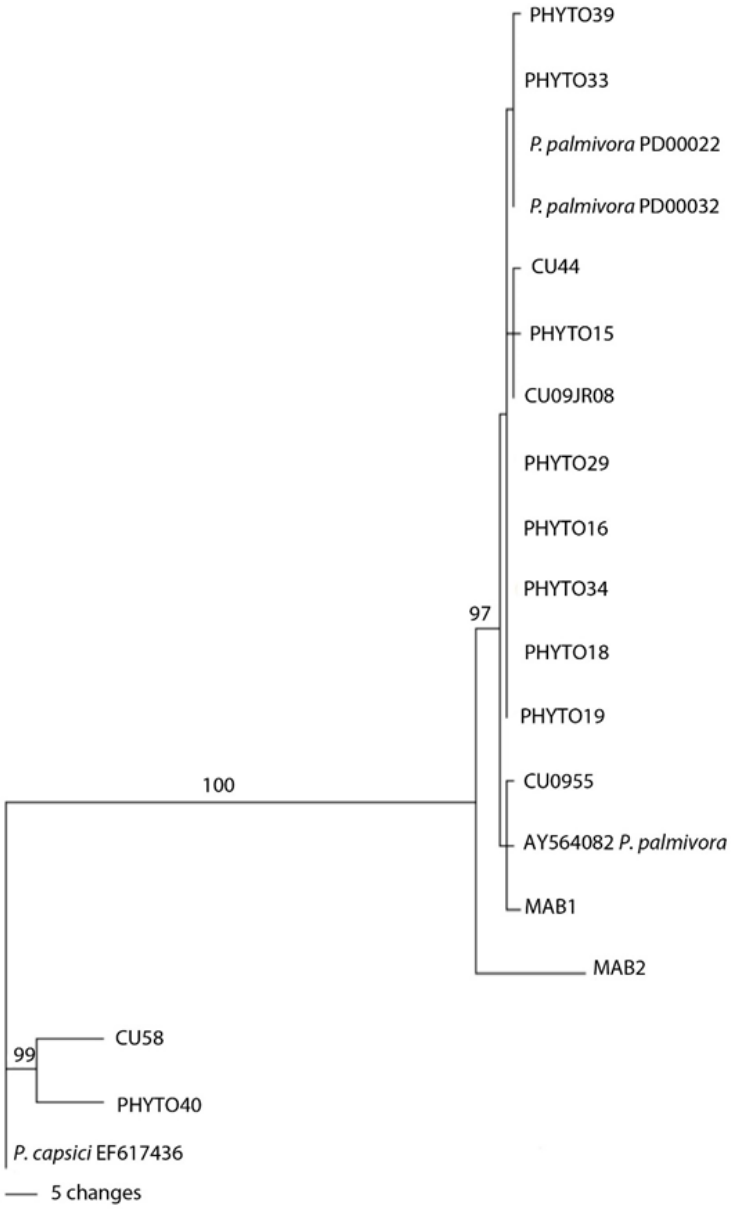

Fig 4 Phylogenetic tree showing the relationship of Cuban *Phytophthora* strains based on the sequences of the elongation factor 1α gene, inferred by maximum parsimony analysis. Numbers above nodes indicate bootstrap support values.

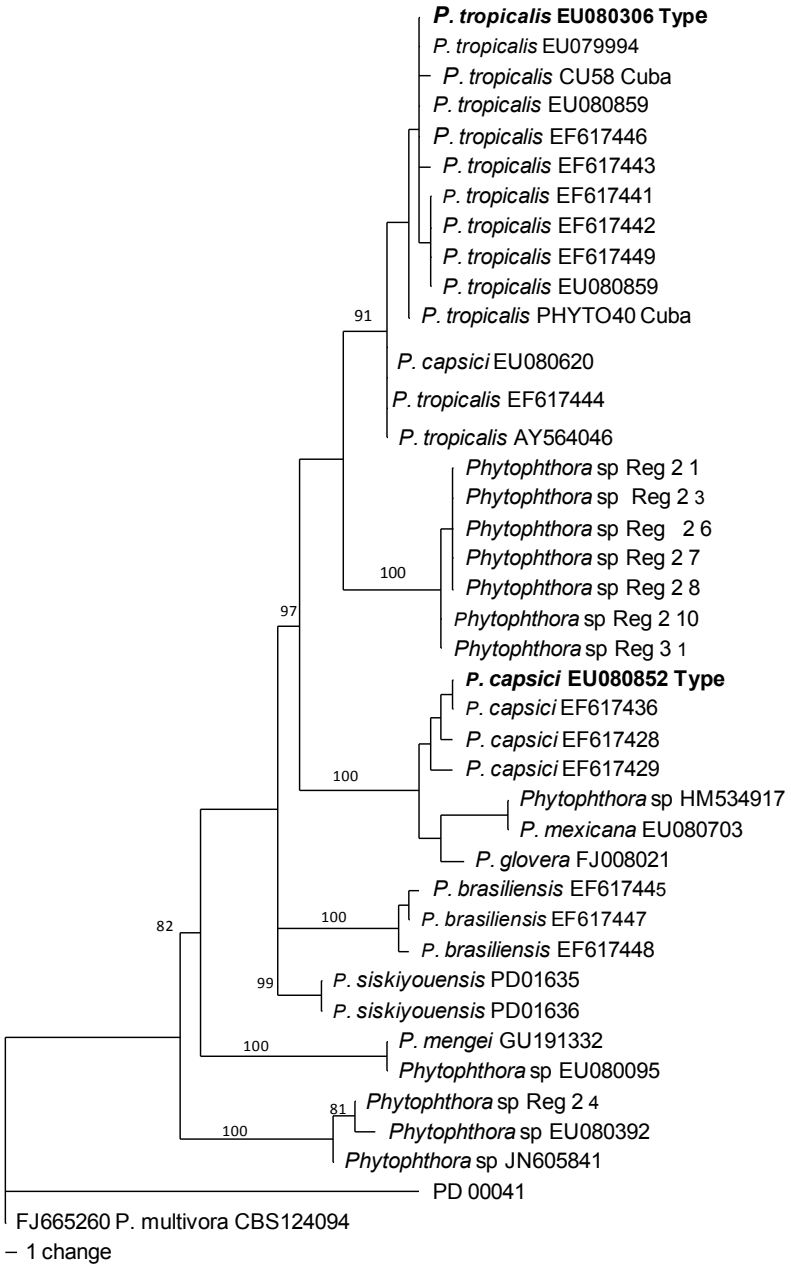

Fig 5.1 Phylogenetic tree showing the relationship of *Phytophthora* strains based on the sequences of the β-tubulin gene, inferred by maximum parsimony analysis (most frequent topology). Numbers above nodes indicate bootstrap support values. In bold the type strains of *P. tropicalis* and *P. capsici.*

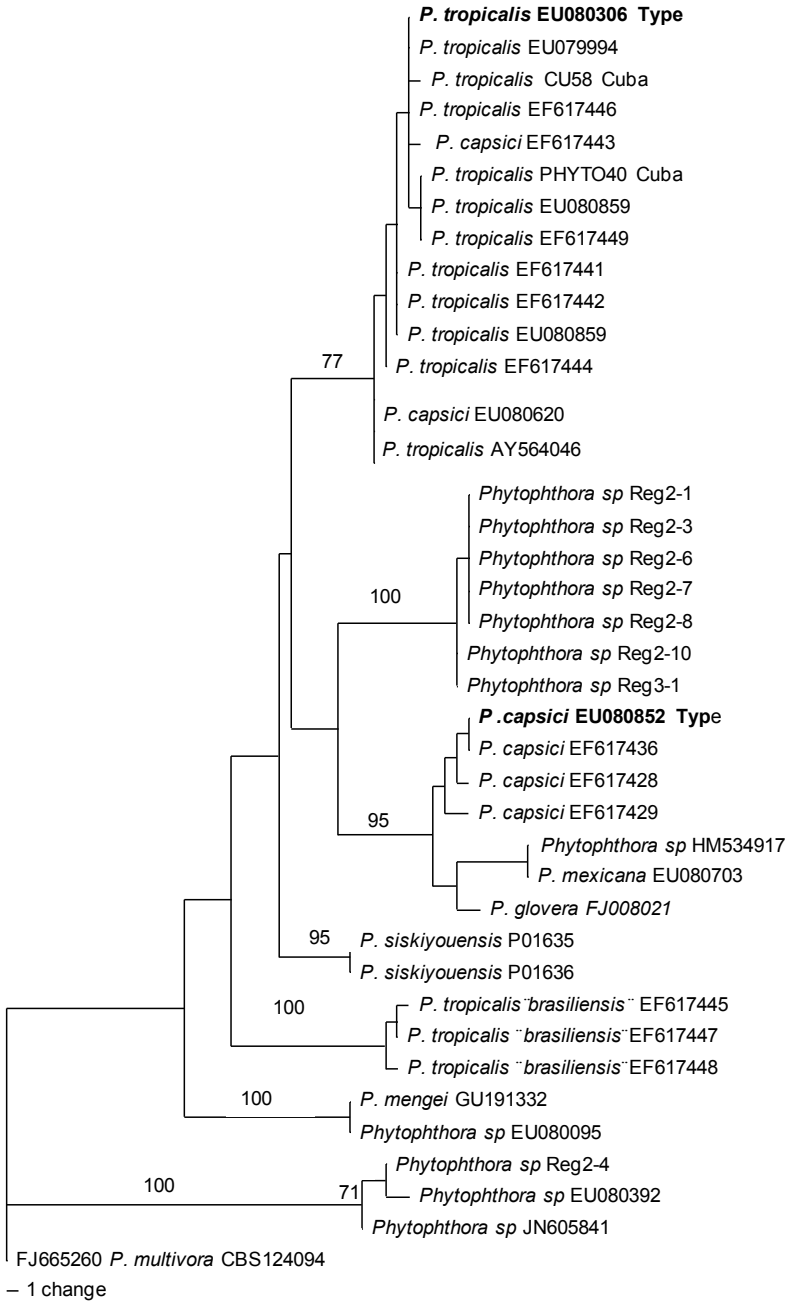

Fig 5.2 Phylogenetic tree showing the relationship of *Phytophthora* strains based on the sequences of the β-tubulin gene, inferred by maximum parsimony analysis. Numbers above nodes indicate bootstrap support values. In bold the type strains of *P. tropicalis* and *P. capsici.*

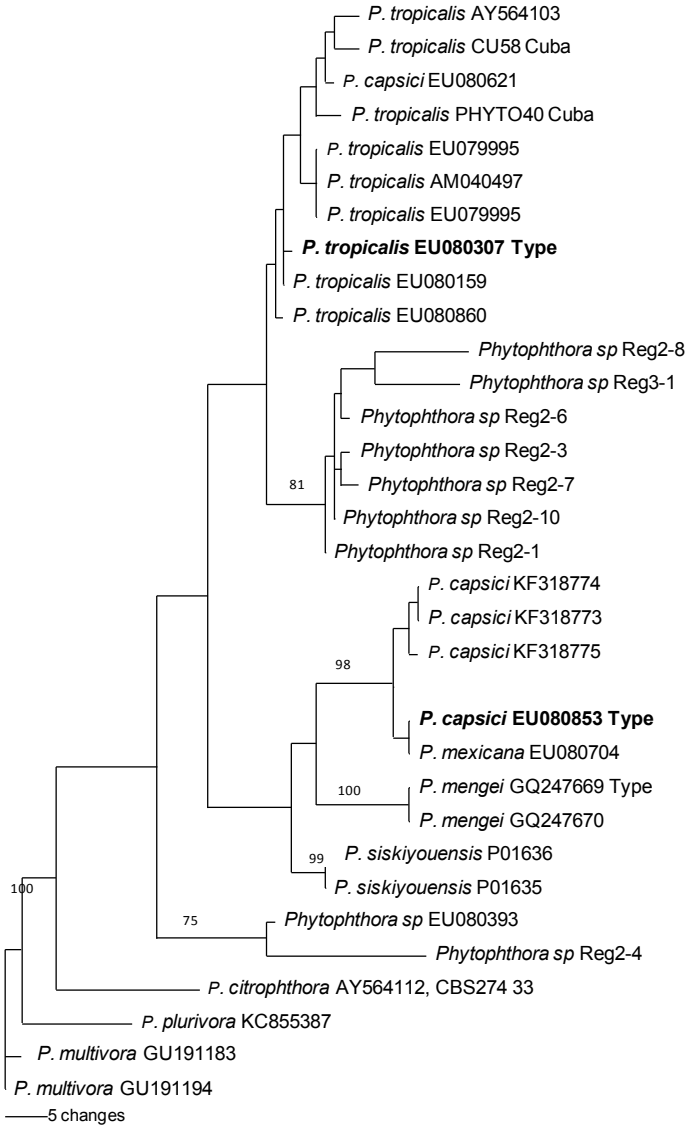

Fig 6.1 Phylogenetic tree showing the relationship of *Phytophthora* strains based on the sequences of the elongation factor gene, inferred by maximum parsimony analysis (most frequent topology). Numbers above nodes indicate bootstrap support values. In bold the type strains of *P. tropicalis* and *P. capsici.*

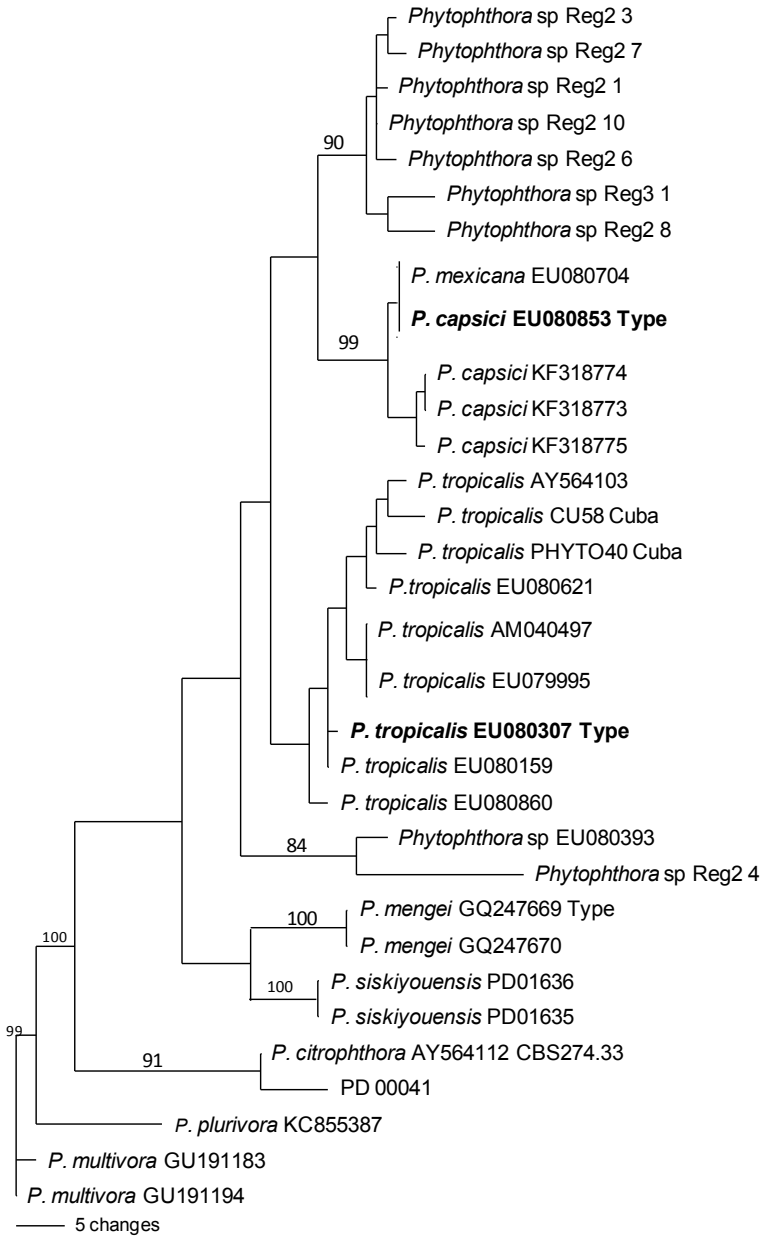

Fig 6.2 Phylogenetic tree showing the relationship of *Phytophthora* strains based on the sequences of the elongation factor gene, inferred by maximum parsimony analysis. Numbers above nodes indicate bootstrap support values. In bold the type strains of *P. tropicalis* and *P. capsici.*

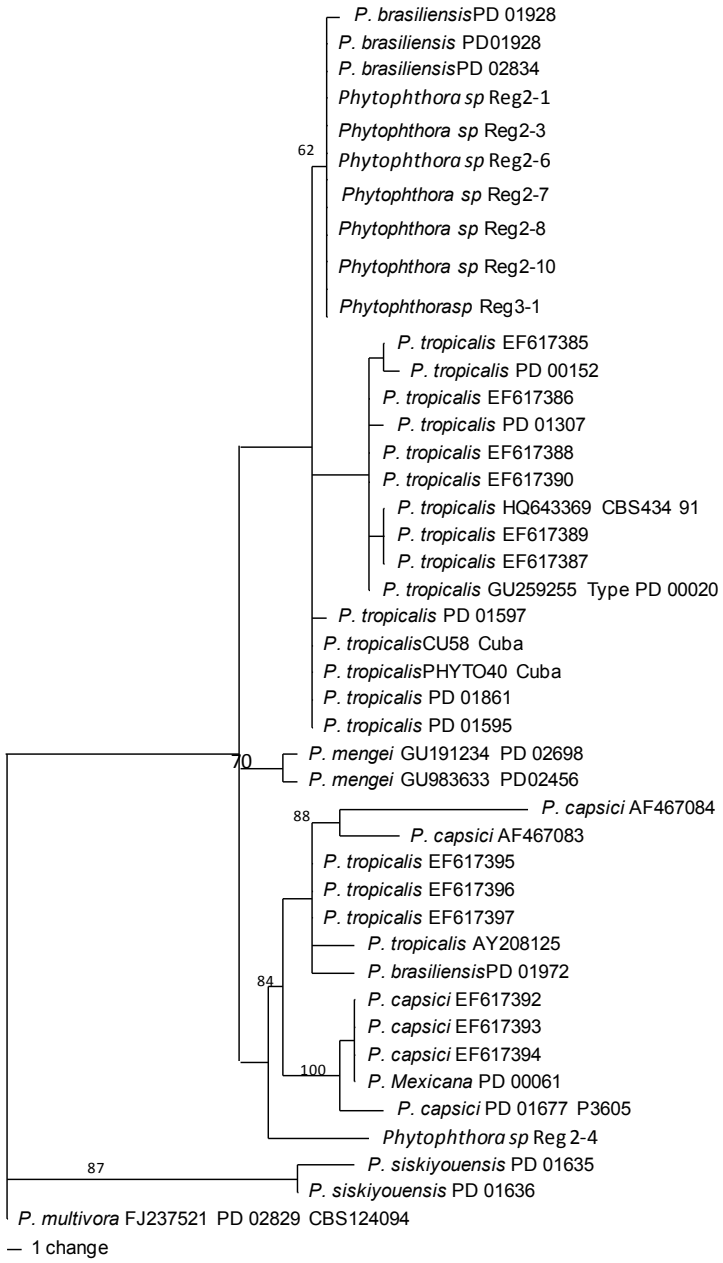

Fig 7 Phylogenetic tree showing the relationship of *Phytophthora* strains based on the sequences of the internal transcribed spacer, inferred by maximum parsimony analysis. Numbers above nodes indicate bootstrap support values.

In the phylogenetic trees, whatever the sequences used (ITS, TEF1-α, β-tubulin), the two Cuban isolates (CU58, PHYTO40) tentatively identified to the *P. tropicalis / capsici* complex clustered in the *P. tropicalis* s.s. clade; this clade contains the type strain of *P. tropicalis* (CBS434.91), additional strains of various origins and hosts, and is well supported. The Cuban strains differ from *P. tropicalis* (type) in 4 to 5 bp, 6 to 7bp, 3 to 5bp, in ITS, TEF1-α and β-tubulin, respectively.

The isolates from French Guiana tentatively identified as *P. capsici* (Lachenaud et al. 2015) clustered in all phylogenies in two different lineages, which correlated with their morphological differences.

The first clade comprised 7 strains whereas the second clade contained one strain (Reg 2-4). The Reg 2-4 lineage was, in all phylogenetic inferences, distantly related from both the *P. tropicalis* and the *P. capsici* lineage. The 7 other strains formed together a well-supported lineage in the three phylogenetic analyses, hereafter referred to as the “Guiana lineage”.

The ITS-based phylogenetic analysis placed the Guiana strains in a sublineage of the *P. tropicalis* type lineage. The ITS sequences from the Guiana strains were very similar to sequences of strains labelled as *Phytophthora brasiliensis*.

In the β-tubulin and TEF1-α-based phylogenetic inferences, the Guiana lineage was well supported (Bootstrap 100) and has, in both inferences, an isolated position. However, its affinities were unresolved. Concurrent topologies emerged from these individual genes analyses; in the dominant topology (Fig 5.1, 6.1), the Guiana clade was associated with the *P. tropicalis* (type) lineage. In the second, less frequent topology (Fig 5.2, 6.2), this lineage was associated with the *P. capsici* lineage. However, none of these positions were supported. The TEF1-α sequences of the Guiana strains differed from the sequence of *P. tropicalis* (type) by 7 to 72 positions, and from the *P. capsici* reference sequence used (EU080853) by 19 to 89 positions. The β-tubulin sequence from the Guiana strains differed from the *P. tropicalis* (EU080306) and the *P. capsici* reference (EU080852) by 21 to 42 and 24 to 38 positions, respectively.

Discussion

Black pod disease caused by *Phytophthora* species is a major constraint in all cocoa- producing areas. It has been shown that each growing area has a complex of species that can induce this disease. The situation in Latin America is perhaps the most complex and uncertain (Hérail et al. 2004). *Phytophthora palmivora, P. citrophthora, P. capsici* and its variant (*P. capsici* s.s., *P. capsici* "cocoa", and *P. tropicalis*, are mentioned in Brazil, French

Guiana, or Mexico (Lachenaud et al. 2015, Nyasse et al. 2007, Vos et al. 2003).

In the present study, we characterized a set of *Phytophthora* strains that were isolated from black pods and originating from Cuba and French Guiana. The strains from French Guiana were previously tentatively identified as *P. capsici* (Lachenaud et al. 2015). Four lineages were resolved.

The vast majority of the strains from Cuba (88 out of 90) obtained from diseased cocoa pods from the whole cocoa plantation range in the region of Baracoa were shown to be genetically homogeneous and belonging to *P. palmivora*. Furthermore, all these strains were shown to belong to the MT A2. This suggested an absence, or an extremely low occurrence, of the opposite MT A1. The presence of the single MT A2 in *P. palmivora* strains affecting cocoa in Baracoa was in accordance to the situation observed in other areas. For instance, in Africa and Indonesia, the MT A1 was found in very small proportion compared to MT A2, with a ratio A1/A2 of 2:9 (Appiah et al 2003, McMahon et al. 2004). In Latin America, Thevenin et al. (2012) also reported occurrence of the sole MT A2 within a set of strains of *P. palmivora* isolated from diseased cocoa pods in the Paracou-Combi (French Guiana) collection. Nevertheless, the reverse situation also occurs. In Jamaica, the MT A1 was shown to be the dominant type on cocoa (Fagan 1988).

The presence of a single mating type in Cuban and French Guiana cocoa plantations prevent sexual recombination and an intra-specific genetic mixing (Appiah et al. 2003, McMahon et al. 2004).

However, the presence of the opposite mating type in distinct *P. palmivora* populations on different hosts should not be excluded. Furthermore, and as far as Cuba is concerned, the occurrence of the MT A1 in Jamaica could be a source of introduction, and possibly an additional threat for the nearby Cuban plantations.

The phylogenetic analyses based on ITS, TEF1-α and β-tubulin DNA sequence data indicate also that two strains from Cuba would belong to *P. tropicalis*. They are genetically closely related to the type strain of *P. tropicalis*.

The identity of the strains from French Guiana remains uncertain. Phylogenetic analyses showed that the strains were distributed into two distinct lineages. One lineage was represented by a single isolate, Reg 2-4. This isolate remains unidentified and of uncertain affinities. As far as the β-tubulin DNA sequence is concerned, this strain was closely related to two strains, P10417 (EU080392) and P1200 (JN605841) with 3 and 8 different positions, respectively. P10417 originates from Costa Rica but the original substrate/host is unknown. The strain P1200 was isolated from tree canker on *T. cacao*, in Brazil. It was first reported as *P. citrophthora* (Forster et al.

1999) and is currently accessed (*Phytophthora* database) under *P. siskiyouensis*. However, it is distinct from two other *P. siskiyouensis* strains (PD01635 and PD01636, *Phytophthora* database). The phylogenetic analyses based on ITS placed the seven other strains in a sublineage of the *P. tropicalis* lineage. These strains could enter within the range of variation of *P. tropicalis.* From a morphological perspective, the sporangia of French Guiana strains were similar to those of *P. tropicalis*, and therefore, within the range of *P. capsici* too.

In Cuban strains, we observed a percentage identity of about 99% of the individuals present in each case and encoded in Genbank, which corresponded on average with a difference of 5 nucleotides. There was a higher level of conservation within the ITS sequences with a variation of one nucleotide on average. The alignment of these sequences ITS and TEF1-α revealed a general uniformity of these regions within the species *P. palmivora*. This uniformity was also found among the strains of *P. tropicalis* analysed.

For a long time, identification of *Phytophthora* species relied on phenotypical features, including morphological characters and host relationships (Erwin & Ribeiro 1996). These included the colony pattern, dimensions and shape of the sporangia and of the oospores, the production of chlamydospores, and several colony characteristics. Criteria for the recognition of biological – interfertility tests – were also proposed to recognize species. However, these results were consistent with those observed in previous studies on the differentiation of different species of *Phytophthora* based on their ITS and TEF1-α regions (Ivors et al. 2004, Blair et al. 2008). These ITS and TEF1-α regions are obviously an important tool for the identification at the species level. The growth pattern may be variable and the analysis of the mycelium does not prove to be a criterion for identifying species (Appiah et al. 2003). As well, size and shape of sporangia may largely overlap, as in the pair *P. tropicalis / capsici*.

From these studies, we conclude that two species of *Phytophthora* are damaging cocoa pods in Cuba, *viz*. *P. palmivora*, which was predominant in our sampling, and *P. tropicalis*, detected at a very low occurrence. By contrast, in French Guiana, where *P. palmivora* is reported on cocoa (Thevenin et al. 2012), two additional species were identified occurring concomitantly in old plantations on the bank of the Approuague. They are *P. tropicalis / capsici* complex, which was dominant in our sampling, and a potential novel specie, known by a single isolate.

It could be noted that, in French Guiana, *P. palmivora* is known from the Paracou-Combi collections, that contains non-native, introduced cocoa varieties / genotypes.

In the area of Régina, plantations represent remnants of some 18th century plantations for sometimes recolonized and still surrounded by native, primary forest. Native *Phytophthora* strains / genotypes / species may exist locally.

More surveys in these remnants of old plantations and on wild cocoa genotypes occurring naturally in southern French Guiana are necessary. Crossing experiments with both mating type *P. tropicalis* and *P. capsici* would be necessary to clarify the species identity.

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Chapter III

Molecular characterization of fungal endophytes isolated from leaves of *Theobroma cacao* in Cuba

Submitted

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Abstract

Cuban cocoa plantations are variously affected by black pod disease, caused by several species of *Phytophthora.* Different forms of crop protection are employed to reduce the impact of this disease, cultural practices such as pod stripping and the use of pesticides. However, biological control is an important alternative. Nowadays, fungal endophytes seem to be a serious option for black pod disease control. This study was undertaken with the objective to identify fungal endophytes present in cocoa to use them in the context of integrated management systems for disease control.

During extensive field surveys in Baracoa, Cuba, endophytic fungi were isolated from healthy leaves of *Theobroma cacao* following the method of triple sterilization. M-13 fingerprinting of genomic DNA was used to select samples with different profiles. One-hundred-ten isolates were finally selected for molecular identification by DNA extraction and sequence analysis. The internal transcribed spacer of nuclear ribosomal DNA was chosen for sequencing since, as the fungal barcode, this locus has the highest probability of successful identification for the broadest range of fungi. The most common species were *Colletotrichum gloesporioides* followed by *Xylaria* spp. Further studies are required to screen selected strains for their potential as biocontrol agents of *Phytophthora palmivora*, causal agent of black pod disease in Cuba.

Keywords: Endophytic fungi, *Theobroma cacao,* Internal transcribed spacer (ITS)

Introduction

The main biological constraints to cocoa (*Theobroma cacao* L.) production worldwide are diseases (Bowers et al. 2001). Cacao black pod disease is the most important disease of cocoa, as it occurs in all cocoa-producing countries (Bowers et al. 2001, Evans 2007, Guest 2007). This disease is caused by several species of the straminipile (oomycete) genus *Phytophthora*, with losses sometimes reaching 90% of pod production in the absence of control measures and depending on climatic conditions (Bowers et al. 2001). *Phytophthora* can survive in soil and infected plant parts and for this reason it is nearly impossible to eliminate this pathogen and control demands long-term plans (Evans & Prior 1987). Cultural, chemical and genetic practices have been used to control this disease. Phytosanitation is the most important cultural control method of cocoa pests and diseases but is very labor-intensive (Ndoumbé-Nkeng et al. 2004, Opoku et al. 2000). In Cuba, this basic farming practice has been applied for several years. Nevertheless, there are reports that phytosanitation is not very effective against black pod caused by *Phytophthora megakarya* in Cameroon (Ndoumbe Nkeng et al. 2004). Chemical control, using correct application methods, are effective (Deberdt et al. 2008, Gockowski et al. 2010), but has major negative impacts such as reductions in the populations of beneficial organisms, the occurrence of secondary diseases, or pathogen resistance to pesticides (Avelino et al. 2011). Also, cost constraints are often too high for small, extensive farms. Genetic control, or disease resistance of the host plant, is likely to be the best option for economic and sustainable control of cocoa diseases on the long-term, yet to date no complete resistance against black pod disease exists (Guest 2007, Efombagn et al. 2013, Lachenaud et al. 2015). Thus, we are compelled to explore alternative options. One of these options is biological control of cocoa diseases (Holmes et al. 2004).

Recent studies give evidence that fungal endophytes play an important role in the outcome of plant–pathogen interactions. Diverse mechanisms by which endophytes may neutralize pathogen development have been found. It has been shown that endophytes produce antibiotic substances that inhibit pathogen growth (Aneja et al. 2005, Herre et al. 2007); and induce plant defense mechanisms that could reduce the impact of pathogen attacks (Mejía et al. 2014); and compete for space and resources; and act as mycoparasites (Wang et al. 2007, West et al. 1988). Several studies tested the biocontrol potential of fungal endophytes on black pod disease of *T. cacao*, finding a decrease in disease severity (Arnold et al. 2003, Hanada et al. 2009).

As the overall goal is to provide sustainable solutions to Cuban cocoa farmers, this project focused on the isolation and identification of fungal endophytes of *T. cacao* that could provide a source of novel biocontrol agents for *P. palmivora,* the causal agent of black pod disease in Cuba.

Materials and methods

Isolation of fungal endophytes

Cocoa leaves were obtained from 10 *T. cacao* plantations in Baracoa, Cuba. One hundred fifty leaves from 50 Trinitario trees (3 leaves per tree) were selected randomly from foliage that was not visible damaged by herbivores or pathogens (Arnold & Herre 2003). They were kept at 4°C and transported to the Centro para el Desarrollo de la Montaña in Guantánamo, Cuba. Leaves were washed, dried using tissue paper, and cut into 2 cm^2 pieces. The cut leaves were sterilized using the following agents subsequently: 95% ethanol for 30 s, 3% sodium hypochlorite for 10 min and 75% ethanol for 2 min. They were rinsed three times with sterile distilled water and dried with sterile tissue paper (Ishak et al. 2004). This method of surface-sterilization has been shown to effectively eliminate bacteria, yeasts, fast-growing *Zygomycetes*, and other putatively epiphyllous organisms from endophyte cultures (Arnold et al. 2000, Schulz et al. 1993).

The treated leaves were then cut into discs of Ø 5 mm using a sterile scalpel. Five such cocoa discs from each leaf were placed in a single petri dish containing 2% malt extract agar (MEA), a medium commonly used in endophyte studies (Sherwood-Pike 1985, Schulz et al. 1993), which was then sealed immediately with parafilm. Petri dishes were incubated at 25-30°C, under 12 h/12 h dark/light cycles to induce sporulation, during 3 to 15 days, with daylight-type fluorescent bulbs (40 W) placed at 60 cm above the plates. Plates were incubated until hyphal tips appeared more than 5 mm in length, yet were incubated no longer than two weeks. When hyphal tips reached a sufficient length, a 5 mm long hyphal fragment was excised and transferred onto PDA medium. Samples were then incubated at 30°C to promote sporulation at the same light cycles as described above.

DNA extraction and PCR-fingerprinting analyses

The isolates were grown on PDA medium. The mycelium was lyophilized and pulverized with fine sand and 200 µl of water in a MagNa Lyser (Roche, France), at 6000 rpm during 30 s. The suspension was incubated with 400 µl lysis buffer SLS and 20 µl proteinase K solution during 60 min at 65°C. The samples were then centrifuged at 13000 rpm for 5 min and genomic DNA was extracted using the Invisorb Spin Plant minikit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions.

PCR-based fingerprinting with the single primer M13 was performed (Groenewald et al. 2008) and the resulting PCR-fingerprinting profiles were grouped visually to select strains with different profiles for DNA sequencing.

DNA amplification and sequencing

The internal transcribed spacer (ITS) were amplified with primers ITS4/ITS5 (White et al. 1990). PCR assays were performed using the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 90 s, annealing at 55°C for 90 s, extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min.

PCR products were sequenced at Macrogen, Inc, Korea. The sequences obtained were performed with Sequencer 4.6 and compared to GenBank (http://www.ncbi.nlm.nih.gov) by full BLAST searches to predict the family and/or order for each isolate.

Results

A total of 345 culturable endophytic fungi were isolated from healthy cocoa leaves. The ITS of 110 isolates was analysed (Table 1). These 110 isolates were selected by grouping the strains with different M13-fingerprinting profiles of genomic DNA. Among them a total of 27 different species were found. *Colletotrichum gloesporioides* and *Xylaria sp.* were the most prevalent species with 36 (32.7%) and 13 (11.8%) isolates, respectively. Six genera found were represented by a single isolate (Table 1). We excluded isolates of *Aspergillus* and *Penicillium* species following the suggestions of Hyde & Soytong (2008), because we assumed that they were contaminants, based on their presence in the environment. A selection of 23 isolates belonging to the genus *Colletotrichum* and *Xylaria* (the most frequent species identified) was deposited at the Mycothèque de l'Université catholique de Louvain (MUCL) under the accession numbers MUCL 55635 to MUCL 55657 to make them available for future studies to evaluate their potential as biocontrol agents against *Phytophthora.*

Table 1 Identification of fungal endophyte isolates based on internal transcribed spacer (ITS) DNA sequence data.

ID based on ITS sequence	Number of isolates
Colletotrichum gloesporioides	36
Xylaria sp	13
Trametes maxima	9
Colletotrichum tropicale	6
Phomopsis sp	5
Diaporthe phaseolorum	4
Colletotrichum siamense	4
Phlebiopsis cf. flavidoalba	4
Cylindrocladium sp	3
Colletotrichum sp	3
Cylindrocladium insulare	2
Hypoxylon monticulosum	2
Phlebiopsis sp	2
Phlebiopsis gigantea	2
Daldinia clavata	2
Colletotrichum fructicola	2
Earliella scabrosa	1
Colletotrichum theobromicola	1
Cochliobolus sp	1
Corynespora sp	1
Daldinia eschsholzii	1
Fusarium solani	1
Mycosphaerella sp	1
Phanerochaete sordida	1
Xylaria globosa	1
Xylaria hypoxylon	1
Xylaria feejeensis	1

Discussion

This study found at least 27 foliar endophytic fungi inhabiting *T. cacao* plants from Baracoa, Cuba. Tropical plants have a large diversity of endophytic microorganisms (Strobel & Daisy 2003), including cacao (Crozier et al. 2006, Thomas et al. 2008). The explanation for this high endophytic diversity could be the subsistence-farming type agroforestry system. These agroforestry systems generally harbor a large diversity of associated plant species, which in turn may favor endophytic diversity (Redman et al. 2001).

The finding that *Colletotrichum* and *Xylaria* were the dominating genera in *T. cacao* in Cuba were consistent with previous findings that reported *Colletotrichum, Fusarium* and *Xylaria* as the three genera most frequently found in previous endophytic surveys in cacao in South and Central America, (Strobel & Daisy 2003, Arnold & Here 2003, Rubini et al. 2005, Tejesvi et al. 2007). There are reports that show *Colletotrichum tropicale* as the dominant species isolated from healthy cocoa leaves (Rojas et al. 2010). *Xylaria* species are the most commonly isolated endophytes in tropical regions (Rodrigues & Petrini 1997, Guo et al. 2003). Yet, in this study, *Fusarium* was somewhat underrepresented (0.9%).

The cocoa endophytic community is under study for biological control of plant pathogens and the production of compounds with pharmacological properties (Peixoto-Neto 2002). *Trichoderma* species are amongst the best known and most promising biological control agents. Many species of *Trichoderma*, e.g. *T. stromaticum, T. koningiopsis* (Samuels & Druzhinina IS 2006), *T. theobromicola* and *T. paucisporum* (Samuels & Evans 2006), *T. evansii* (Samuels & Ismaiel 2009), and *T. asperellum (*Tondje et al. 2007) have been found endophytically in cocoa. Many of these endophytic species exhibit antagonistic capacities towards cocoa pathogens. The endophyte *Trichoderma ovalisporum* was used in controlling frosty pod rot disease in cocoa, caused by *Moniliophthora roreri* (Crozier et al. 2015, Krauss et al. 2010). Another species, *Trichodema martiale*, limited black pod disease (Hanada et al. 2008). However, within the species identified in this study, *Trichoderma* was not found. One possible explanation could be that *Trichoderma* species are frequently considered soil organisms colonizing plant roots, and we isolated the fungal endophytes from leaves. *Colletotrichum* and *Pestalotiopsis* species were reported as prolific producers of bioactive substances, many of them showing antifungal activity against plant pathogens (Ding et al. 2008, Inacio et al. 2006, Ishak et al. 2004, Li et al. 2008, Tejesvi et al. 2007). The current survey provides a large number of *Colletotrichum* isolates, but no *Pestalotiopsis* species were detected. Pod treatment with *C. gloesporioides* significantly reduced the symptoms of black pod disease under field conditions in Panama (Mejía et al. 2008) and during greenhouse experiments (Arnold & Here 2003). In a recent study, Mejía et al (2014), inoculated *Colletotrichum tropicale* to cocoa leaves and found increased resistance to pathogen damage from increased expression of a host gene.

Further studies are required to determine the biocontrol potential of the *C. gloesporioides* and *Xylaria* isolates from Cuba. Although the strains of *C. gloesporioides* were isolated from healthy leaves, one cannot rule out the possibility that they may be pathogenic to other members of the cocoa agrosystem (e.g., banana, coco, citrus). *Colletotrichum gloesporioides* has been reported as one of the most important pathogens and is the principal cause of anthracnose, which constrains the production (Giblin et al. 2010, Hindorf 2000, Hyde et al. 2009, Sreenivasaprasad & Talhinhas 2005). After confirming their biocontrol effect, isolates should be tested for pathogenic effects on other plant species that are part of agrosystems.

This study contributes to the understanding of the endophyte diversity in *Theobroma cacao* and provides a riche source of endophytic fungal cultures in the public culture collection BCCM/MUCL for the exploration of their biocontrol and pharmacological potential.

Chapter IV

The environmental and intrinsic yeast diversity of Cuban cocoa bean fermentation heaps

Submitted

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Abstract

The environmental yeast diversity in relation with spontaneous cocoa bean fermentations in east Cuba was investigated. Seven fermentations, 25 equipment- and handling-related samples, and more than 100 environmental samples, such as surfaces, tools, insects and plants, were analysed. The basic fermentation parameters temperature and pH were recorded during five fermentations for at least six days. A total of 435 yeast isolates were identified by a combination of PCR-fingerprinting of genomic DNA with the M13 primer and sequence analysis of DNA from representative isolates, using the internal transcribed spacer (ITS) region, the D1/D2 region of the large subunit rRNA gene, and an actin gene-encoding fragment, as required. Among 65 yeast species detected, *Hanseniaspora opuntiae, Pichia manshurica*, and *Pichia kudriavzevii* were the most frequent overall and in particular from fermentation-related samples. *Saccharomyces cerevisiae* was isolated occasionally. The yeast diversity was larger in the environmental samples compared to the fermentations and the most frequently isolated environmental yeasts were *Candida carpophila*, *Candida conglobata*, and *Candida quercitrusa*. Potential selective advantages of the most frequently isolated species were only partly explained by their physiological traits; for instance, ethanol tolerance and assimilation potentially conferred a selective advantage to *Pichia* spp., but *H. opuntiae* was not the only species that tolerated elevated glucose concentrations.

Keywords: cocoa bean heap fermentation, yeast diversity, Cuba

Introduction

Cocoa trees (*Theobroma cacao* L.) are assumed to have been introduced into Cuba by the Spanish after the colonization of Mexico in the mid-16th century (Hartmann & Larramendi, 2011). Today, Cuba is a small-scale cocoa-producing country and strives to offer high-quality fermented dry cocoa beans to the world market. Although cocoa-growing was widespread throughout Cuba from the 17th till 19th century, the current production is centred around Baracoa, surrounded by tropical rainforest-covered mountains, and located in the easternmost province of Cuba, Guantánamo. More than 70% of the national cocoa production takes place in this region (Oficina Nacional de Estadísticas e Información, 2012). Farming and harvest are performed by individual farmers with familial transmission of the techniques (Márquez Rivero & Aguirre Gómez, 2010). Most cocoa plantations contain a variety of crops and other plants, such as banana, coconut, and the endemic royal palm *Roystonea regia*. The farmers deliver the largest part of the harvest to government-owned cooperatives that centralise the fermentation and drying processes. Small domestic-scale fermentations are carried out by farmers with increasing rarity. Traditionally, they use a yagua, a container made from a dry leaf of the Cuban royal palm to collect the cocoa pulp-bean mass at the harvest sites and to carry out fermentations of 10-20 kg close to their farm houses. Empirically, smaller fermentations are said to result in better quality of fermented cocoa beans. Detailed practices, such as mixing, leaf covers, and exposure to sun, differ among farms.

Cocoa pulp contains mostly fructose and glucose in concentrations of 40-80 mg/g, which are fermented to alcohol largely by yeasts and metabolised by bacteria to lactic acid, acetic acid, and mannitol (e.g., Camu et al. 2007, 2008, Papalexandratou et al. 2011b, Ho et al. 2014). The initial low pH of the cocoa pulp-bean mass of 3.3 to 4.0, mainly due to 5-40 mg/g citric acid (Ho et al. 2014 and references therein), favours growth of acid-tolerant yeast strains of the genera *Hanseniaspora* and *Pichia* in the first phase of the fermentation process (Daniel et al. 2009). The pH then rises due to the metabolic conversion of citric acid by lactic acid bacteria (Camu et al. 2007, 2008, Ho et al. 2014). The draining of pulp compounds caused by pectin degradation by the yeasts facilitates oxygen access, allowing acetic acid bacteria to aerobically oxidize ethanol into acetic acid in an exothermic reaction that causes a temperature increase from ambient temperature (25-32°C) to up to 50°C (Schwan & Wheals 2004, Camu et al. 2007, 2008). Increasing temperatures together with rising ethanol concentrations of up to 20 mg/g in spontaneous fermentations (Camu et al. 2007, 2008) limit the yeast activity, depending on the yeast species. From major cocoa bean fermentation species isolated in Ghana, *Saccharomyces cerevisiae* isolates were ethanol- but not thermotolerant, while certain *Pichia kudriazevii* isolates united ethanol, temperature, and acid tolerance (Daniel et al. 2009). The high temperatures and various metabolites, including organic acids produced during cocoa bean fermentation, loosen the seed coat, kill the germ, reduce bitter taste compounds, and provide precursors for aroma

formation during the subsequent roasting process. The study of bacterial starter cultures without yeasts shows highly variable fermentation results (Lefeber et al. 2011). Cocoa bean fermentations inoculated with *S. cerevisiae*, *Pichia kluyveri*, and *Kluyveromyces marxianus* demonstrated the differential influence of these yeast species on the flavour profile of the resulting chocolates compared to spontaneous fermentation (Lefeber et al. 2011, Crafack et al. 2013). By comparing yeast-free fermentations with common spontaneous cocoa bean fermentations, the essential role of yeasts for flavour development has been shown and a likely link to the lack of ethanol in such fermentations has been established (Ho et al. 2014).

A considerable diversity of more than one hundred yeast species has been isolated from cocoa bean fermentations and the tools involved (Ho et al. 2014, Meersman et al. 2013, Papalexandratou et al. 2011a, 2013, Nielsen et al. 2010, Daniel et al. 2009 and references therein). The most frequently encountered species are *S. cerevisiae*, *P. kudriavzevii*, and *Hanseniaspora guilliermondii* (12, 10, and 8 out of 15 reports, respectively), although other species such as *Pichia membranifaciens*, *P. kluyveri*, *Candida tropicalis*, *Hanseniaspora opuntiae*, *Pichia manshurica*, *Torulaspora delbrueckii*, and *Meyerozyma guilliermondii* have been found frequently (7 to 4 reports). While the yeast diversity of cocoa bean fermentations, including equipment and cocoa handling-related samples has been studied repeatedly, neither the habitat in which cocoa bean fermentation yeasts reside between the harvest and fermentation campaigns nor the vectors that inoculate spontaneous cocoa bean fermentations have yet been explored. Therefore, the linkage of environmental and fermentation yeast diversity from the same locality had to be studied, which will lead to the first insights into the spontaneous inoculation of cocoa bean fermentations by environmental yeasts. The spontaneous nature of the fermentations may be the source of microbial variation and hence of variable end-product quality.

This study aimed to detect the yeast diversity in habitats that may harbour yeasts that could initiate cocoa bean fermentations. No studies of the microbiology and biochemistry of cocoa bean fermentations have yet been performed in Cuba and, globally, there are no reports about the isolation of yeasts from the environments of cocoa bean fermentations, other than from tools used during the fermentations.

Materials and methods

Harvest and fermentation

Field experiments were carried out in 2013 and 2014. Selected cocoa pods were cut from the trees using a 2 to 3 m long stick with an attached knife. They were piled up on the ground close to the harvest site. On the delivery day, pods were opened with a knife and the cocoa pulp-bean mass scooped out from the pods by hand, sometimes protected by a glove, into a yagua. Fabric bags were filled with 40 to 50 kg of cocoa pulp-bean mass each from

the yagua and transported by horse to either a collection point or the cooperative. The duration of the transport and waiting period in bags varied between several hours up to two days. At the cooperative, cocoa pulp-bean mass from different farms were used to form long fermentation heaps (several m) of about 70 cm height on a concrete platform exposed to the sun (further referred to as industrial-scale fermentations). The heaps were covered by a black plastic seal. From day 3 onwards, the heaps were turned daily in the morning by manual shovelling. Fermentation was considered as finished when the inner colour of the beans was reddish, which usually required seven days. Also, two small domestic-scale fermentations performed in yaguas close to the respective harvest sites were sampled.

Sampling sites

The sampling sites and their specifications are listed in Table 1. Cocoa-related sites located within a radius of 2 km were contrasted with rural sites without cocoa trees or cocoa bean fermentation activities. These rural sites were located within a radius of about 40 km.

Fermentation sampling

The cocoa pulp-bean mass was transported on the harvest day (2013 field experiment) or within three days after the harvest (2014 field experiment) to the cooperative and was set up into large fermentation heaps together with cocoa pulp-bean mass from other plantations. Five cocoa bean fermentations representing four industrial-scale fermentation heaps and one small domestic-scale fermentation (Table 2) were followed over their entire duration by three to four samples on day 1 and 2, two samples on day 3, and then daily samples until yeast growth ceased (between 80-166 h) or until the beans were spread out for drying. Two fermentations were only partially sampled.

Sampling methods

Sampling of cocoa pulp-bean mass was started 10-20 min after opening the cocoa pods. Initial samples were taken from the yagua, followed by samples from the fabric bags used by the farmers to transport the cocoa pulp-bean mass, and finally from the assembled fermentation heaps to which the content of the transport bags had been added. One sample of 50 to 100 g from about 20-30 cm below the surface was collected in sterile plastic sample bags per sampling site and time point. The samples were transported to the laboratory on ice and processed on the same day or kept refrigerated for one night. Flowers and tree exudates were either collected in sterile tubes and later submerged in saline (0.85% NaCl, m/v) or liquid was taken from the flower by 10 µl microcapillaries and transferred into tubes containing saline. Insects were either enclosed in tubes and later submerged in saline or enclosed on agar plates. Samples from surfaces, such as leafs and tools, were taken by swabbing a 5x5 cm surface with saline-moistened cotton swabs.

Table 1 Sampling sites. All sites are located in the province of Guantanamo, Cuba, and samplings were performed in September of the respective year.

	Latitude, longitude*	Distance to cooperative*	Cocoa type and approximate age of the trees	Samples	Year
Cooperative, El Jamal	20.284313, -74.429720	n.a.	n.a.	Fermentations	2013, 2014
Farm 1, El Jamal	20.281879, -74.426227	450 m	Tradicional, about 50 years	Cocoa, equipment, plants, insects	2013
Farm 2, El Jamal	20.280938, -74.428794	400 m	Unknown	Plants	2014
Farm 3, Saint Luis	20.291132, -74.437839	1.1 km	Trinitario, UF clones, 4 to 20 years	Cocoa, fermentation, equipment, plants, insects	2014
Farm 4, Palo Gordo	20.281597, -74.460794	3.3 km	Trinitario, 40 years	Cocoa, equipment, plants	2014
Farm 5, road to Mata	Unknown	Unknown	Unknown	Fermentation	2014
Yumuri river	20.295187, -74.290377	15 km	n.a.	Plants, insects	2014
Taco Bay National Park station	20.510832, -74.671696	36 km	n.a.	Plants	2014
Zoologico de Piedras, Alto de Boqueron	20.298194, -74.386859	67 km	n.a.	Plants	2014
Cajuajo Beach	20.298194, -74.386859	4.7 km	n.a.	Plants, insects	2014

*Derived from Google Maps (www.google.be/maps/) on which the sampling locations were determined as precisely as possible
n.a., not applicable

Table 2 Sampled cocoa bean fermentation processess. All samplings were performed in September of the respective year.

	Year	Location	Approximate length and content	Farms from which cocoa was included	Mixing	Sampling duration (h)	Remarks
Industrial-scale heap 1	2013	Cooperative	27 m, 18 t	1	Turned daily, starting at 48 h	168	Sampled at sites A and B, 10 m apart
Industrial-scale heap 2	2014	Cooperative	22 m, 12 t	3	Turned daily, starting at 48 h	128	
Industrial-scale heap 3	2014	Cooperative	22 m, 12 t	3	Turned daily, starting at 48 h	128	
Industrial-scale heap 4	2014	Cooperative	4 m, 2 t	4	Turned daily, starting at 48 h	104	
Industrial-scale heap 5	2014	Cooperative	22 m, 12 t	Unknown	Turned daily, starting at 48 h	57	Yeast growth below detection limit
Domestic-scale heap D1	2014	Farm 5	20 kg	5	Unknown	4	No further access was permitted
Domestic-scale heap D2	2014	Farm 3	10 kg	3	Never mixed	128	Protected from direct sun

Temperature and pH measurements

Temperature and pH of the 2013 fermentations were measured continuously in the bags and heap sites with a portable pH 340i sensor (WTW GmbH, Weilheim, Germany). Temperatures of the 2014 fermentations were measured with a digital thermometer (Sunartis, Mingle Instrument GmbH, Willich, Germany). The pH of the 2014 fermentations was measured in the laboratory with a benchtop PHSJ-4A pH meter (Shanghai precision, Shanghai, China).

Yeast isolation

In the case of fermentation samples, 20 g of cocoa pulp-bean mass were transferred into a stomacher bag, homogenized by hand during 1 min, and serially diluted in saline up to 10-7. Aliquots of 100 µl were spread on dextrose-yeast extract-peptone-agar (DYPA) medium (20 g l-1 of dextrose, 10 g l-1 of yeast extract; 5 g l-1 of peptone, 20 g l-1 of agar), supplemented with 200 mg l-1 of chloramphenicol. Incubation was carried out at 30-35ºC for 48 h, and 10 to 20 yeast colonies representing all morphotypes in their observed proportions were transferred into 2 ml cryo-tubes containing DYPA medium with chloramphenicol. After transport to Belgium, the isolates were purified by at least two streak inoculations before identification. Representative isolates are preserved at the BCCM/MUCL culture collection (Louvain-la-Neuve, Belgium).

DNA extraction and PCR-fingerprinting analyses

DNA was extracted using the InnuPREP Plant DNA kit (Westburg, Leusden, The Netherlands). From a culture grown on DYPA medium at 25°C for 72 h, three 10 µl loops of cells were resuspended in 650 µl of sorbitol buffer (1 M sorbitol, 100 mM sodium-EDTA, 14 mM β-mercaptoethanol) and 200 U of lyticase (Sigma, Diegem, Belgium). The suspension was shaken (100 rpm) at 30°C overnight, microcentrifuged at 13,000 rpm for 2 min, and the supernatant was discarded. Four hundred µl of lysis buffer P and 25 µl of proteinase K solution (20 mg ml-1) were added, the mix was homogenised with a pipette tip, and incubated at 50°C for 60 min. The suspension was transferred onto a spin filter and microcentrifuged at 13,000 rpm for 5 min. Ten µl of RNAse A (100 g l-1 in molecular biology-grade water) were added to the filtrate, which was vortexed briefly, and incubated at room temperature for 45 min. Two hundred µl of binding buffer P were added, the sample was vortexed briefly, transferred to a fresh spin filter, and microcentrifuged at 12,000 rpm for 1 min. Five hundred µl of wash buffer were added to the spin filter, the filter was centrifuged as before, and the filtrate was discarded. A similar treatment using 750 µl of wash buffer II and microcentrifugation at 12,000 rpm for 2 min concluded the DNA purification. The DNA was then eluted with 100 µl of molecular biology-grade water and microcentrifuged at 8000 rpm for 1 min.

PCR-based fingerprinting with the single M13 primer was performed to dereplicate isolates at the species level (Groenewald et al. 2008) and the resulting PCR-fingerprinting profiles were grouped.

Amplification and sequencing of the ITS, D1/D2 LSU rDNA regions and partial *ACT1* gene sequences

The primers ITS5/ITS4 (White et al. 1990) were used to amplify and sequence the internal transcribed spacer (ITS) region. The primers LR0R/LR6 were used for amplification and the primers LR0R/LR3 for sequencing of the D1/D2 domain of the large subunit ribosomal DNA (Moncalvo et al. 2000). PCR assays were performed using the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 90 s, annealing at 55°C for 90 s, extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. PCR products were sequenced using the amplification primers by the Macrogen facility (Seoul, South Korea). Sequence assembly and editing was performed in Sequencher (https://www.genecodes.com/). Most similar species were determined by BLAST searches in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Type strain sequences of those species were compared to query sequences by alignments in BioEdit 7.5.2 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) and MEGA6 (http://www.megasoftware.net/).

Partial *ACT1* gene sequences were determined if species determination was uncertain by the ribosomal gene complex sequences. The primers CA1, CA5R, CA21, and CA22R were used for amplification (Daniel & Meyer 2003). The amplification conditions were: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. Sequence assembly, editing, and comparisons were performed as described above. DNA sequences judged to be unique from BLAST searches and therefore representing potentially new species were deposited in the EMBL database (http://www.ebi.ac.uk/ena, Hinxton, UK), with the accession numbers LN871014-LN871030 (D1/D2 LSU), LN871031-LN871039 (ITS), and LN871040-LN871041 (*ACT1*).

Physiological characterisation

Physiological profiles of selected strains supplemented by two environmental *S. cerevisiae* strains (MUCL 45750 and MUCL 45754), previously obtained in Cuba, were determined with the automated microplate method Allev/Biolomics (BioAware SA, Hannut, Belgium) of Robert et al. (1997), based on standard taxonomic criteria and including 12 carbon fermentation, 56 carbon assimilation, and 12 nitrogen assimilation test substrates, 10 vitamin requirements, and 16 other tests (Kreger-van Rij 1987; Van der Walt & Yarrow 1984).

To investigate the physiological adaptation of the yeast communities to the cocoa bean fermentation environment, growth of selected isolates was tested in tubes containing 5 ml of growth medium, agitated periodically, and incubated at 25°C (unless mentioned otherwise) during one week, as to their citrate and ethanol assimilation, pH tolerance, ethanol tolerance, and heat tolerance. The test medium was composed of 6.2 g l-1 of yeast nitrogen base (Difco, Basingstoke, UK) and 5 g l-1 of carbohydrate source (citrate, ethanol, or sucrose). Yeast nitrogen base without added carbon source was used as a negative control. To test pH tolerance, test medium with 5 g l-1 of glucose was adjusted to pH 2.5, 3.5, and 5.0 with 1 M HCl. To test ethanol tolerance, test medium with 5 g l-1 of glucose was supplemented with 5%, 10%, or 15% (vol/vol) ethanol. To test heat tolerance, test medium with 5 g l-1 of glucose, adjusted to pH 5.5, was incubated at 25°C, 35°C, and 45°C. Growth, assessed by medium turbidity, was interpreted as tolerance.

Results

Fermentation parameters

Viable yeast counts and pH and temperature courses of the sampling sites are shown in Fig. 1. Samples from nine cocoa pods obtained from different harvest sites and opened aseptically in the laboratory did not result in yeast growth. Therefore, the manual removal of the cocoa pulp-bean mass from the pods at the harvest sites was considered as the starting point of the cocoa bean fermentations. After a rapid yeast count increase from this starting point, the highest counts were reached after about 8 (industrial-scale heaps 2, 3, and 4) to 26 h (domestic-scale heap 2) (Fig. 1). This timing of most intense yeast multiplication coincided in most industrial-scale fermentations with the transfer of the fermenting cocoa pulp-bean mass from the transport bags to the fermentation heaps. The CFU peak was followed by a slow decrease of the yeast counts.

The pH measurements of seven cocoa pods opened in the laboratory varied between 2.8 and 3.7, depending on their ripeness. The average initial pH in five samples of cocoa pulp-bean mass obtained within 30 min after opening the pods was 3.3. The continuously recorded pH data at sites A and B of the industrial-scale fermentation 1 showed an increase to 4.4 after 22 h and 4.6 after 30 h of fermentation, respectively. After a decrease to pH 4.0, the readings increased again to 4.4 and 4.7. The manually recorded data were similar; only industrial-scale heap 4 did not allow the recognition of the slight temporary increase in pH.

Although the cocoa pulp-bean mass started fermenting already in the transport bags, the temperatures in the bags did not raise above 34°C, despite partially higher environmental temperatures (25-37°C). Temperatures increased fast after the transfer of the cocoa pulp-bean mass from the transport bags to the fermentation heaps, about 24 h after the fermentation started. With values of 48 to 52°C, temperatures reached their

maxima after 80 h (industrial-scale heaps 2 and 3) to 156 h (industrial-scale heap 1, site A, data not shown). The automated temperature recording in heap 1 showed a wave-like temperature profile, with peaks occurring at night time, suddenly lowered by mixing the fermentation heaps in the morning. Industrial-scale heap 5 showed already 38°C after 26 h, 48°C after 57 h (data not shown), and only marginal yeast growth.

The parameters of the domestic-scale heap 2 differed in several aspects from all other fermentations. Yeast counts decreased only slightly after a relatively late peak and then remained high throughout the observed period, which was limited by spreading the beans to dry them. The pH rose steeply to above 7.0 after 48 h and continued to rise thereafter. The temperature increased steeply 24 h after the start of the fermentation to reach 37°C, a far lower maximum than in the industrial-scale fermentations, then decreased slowly and continuously until the liquids had drained away and the beans became dry after about 100 h, after which the temperature increased further.

It was not possible to obtain the results of a routinely foreseen quality assessment by the cooperative; however, low-quality fermented dry cocoa bean batches were noticed by the downstream processing units in recent years. The domestic fermentation was judged to be good according to a typical cocoa smell when adding saline during the laboratory treatment of samples of the advanced fermentation and according to the farmers' own experience.

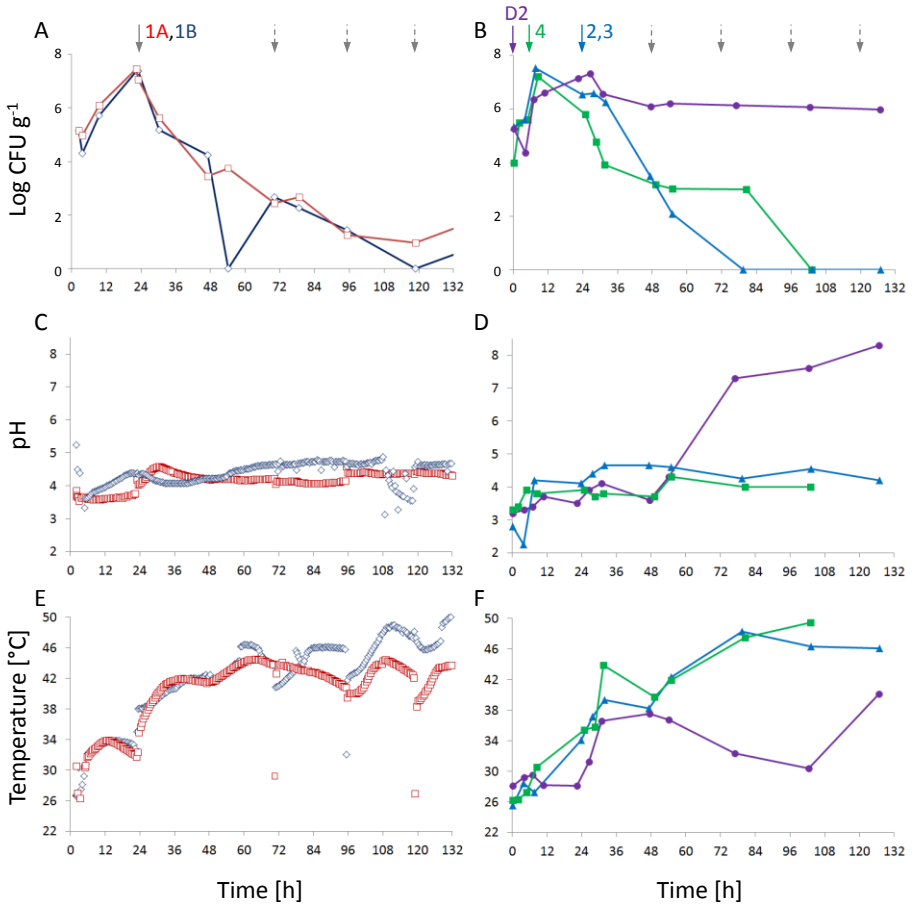

Fig 1 Course of the colony forming units (A, B), pH (C, D), and temperature (E, F) in the Cuban cocoa bean industrial-scale heaps 1A (□), 1B (◊); 2 and 3 (averages, ▲); 4 (■); and domestic-scale heap D2 (●). Temperature and pH measurements in sites 1A and 1B were automatically performed every 20 min; no connecting lines were used for clarity. Time scales start about 10 min after the emptying of the cocoa pods. Solid line arrows mark the first samples from the assembled heaps, dashed line arrows mark mixing of the heaps. The domestic-scale heap D2 was formed directly after harvest and never mixed.

Yeast diversity

Yeasts from 115 environmental, 25 equipment- and production-related, as well as 69 fermentation samples were isolated and identified to the species level (Supplementary Table S1). The identification methods were not adapted to convey reliable information on intra-species variability. Nevertheless, a representative M13-fingerprint gel of *H. opuntiae* isolates

showed variation in the profiles of independent isolates (Fig. 2). Fig. 3 provides an overview of all 65 yeast species with their frequency of isolation. Multiple isolates of the same species from the same sample were counted only as one occurrence. Supplementary Tables S2 and S3 list the distribution of 30 yeast species identified during the course of the cocoa bean fermentations and 54 species with their environmental and equipment sources, respectively. The most frequently occurring species from all seven fermentations were *P. manshurica* (23), *H. opuntiae* (20), and *P. kudriavzevii* (16). All other species occurred only in eight and fewer fermentation samples. *Pichia manshurica* was encountered in all, *H. opuntiae* in four, and *P. kudriavzevii* in only two of the six fermentations that were sampled during at least 104 h (Table 3).

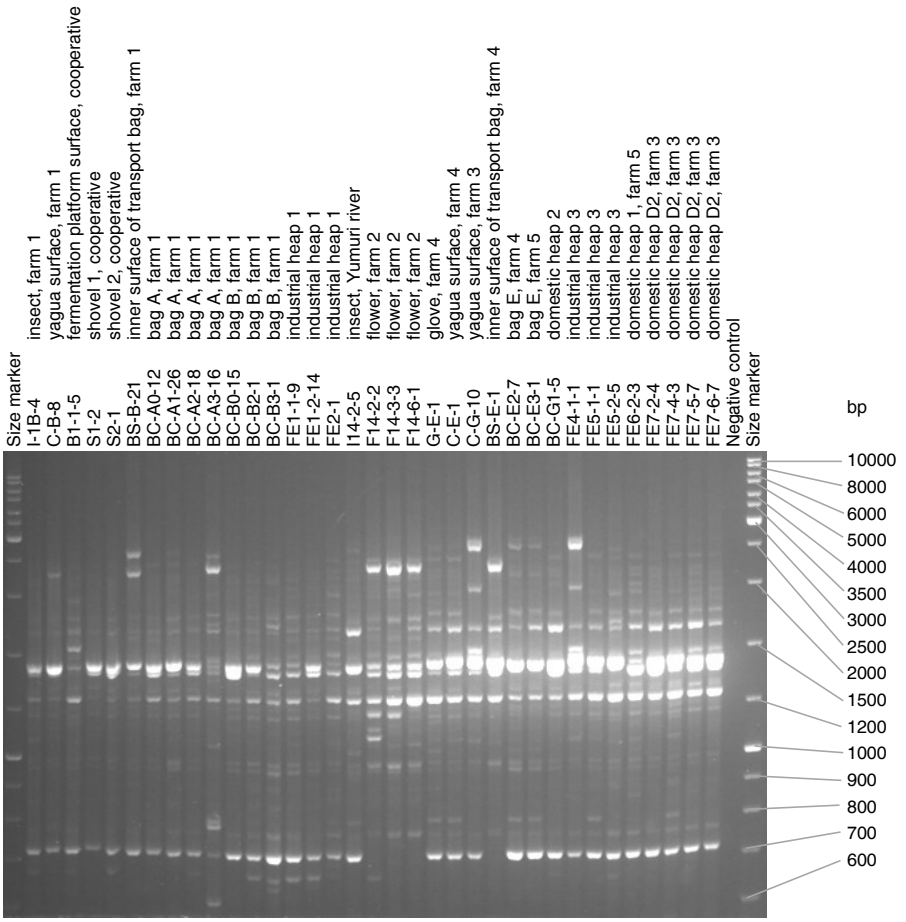

Fig 2 M13-fingerprint profiles of genomic DNA from *Hanseniaspora opuntiae* isolates, representing all 35 samples from which this species was obtained.

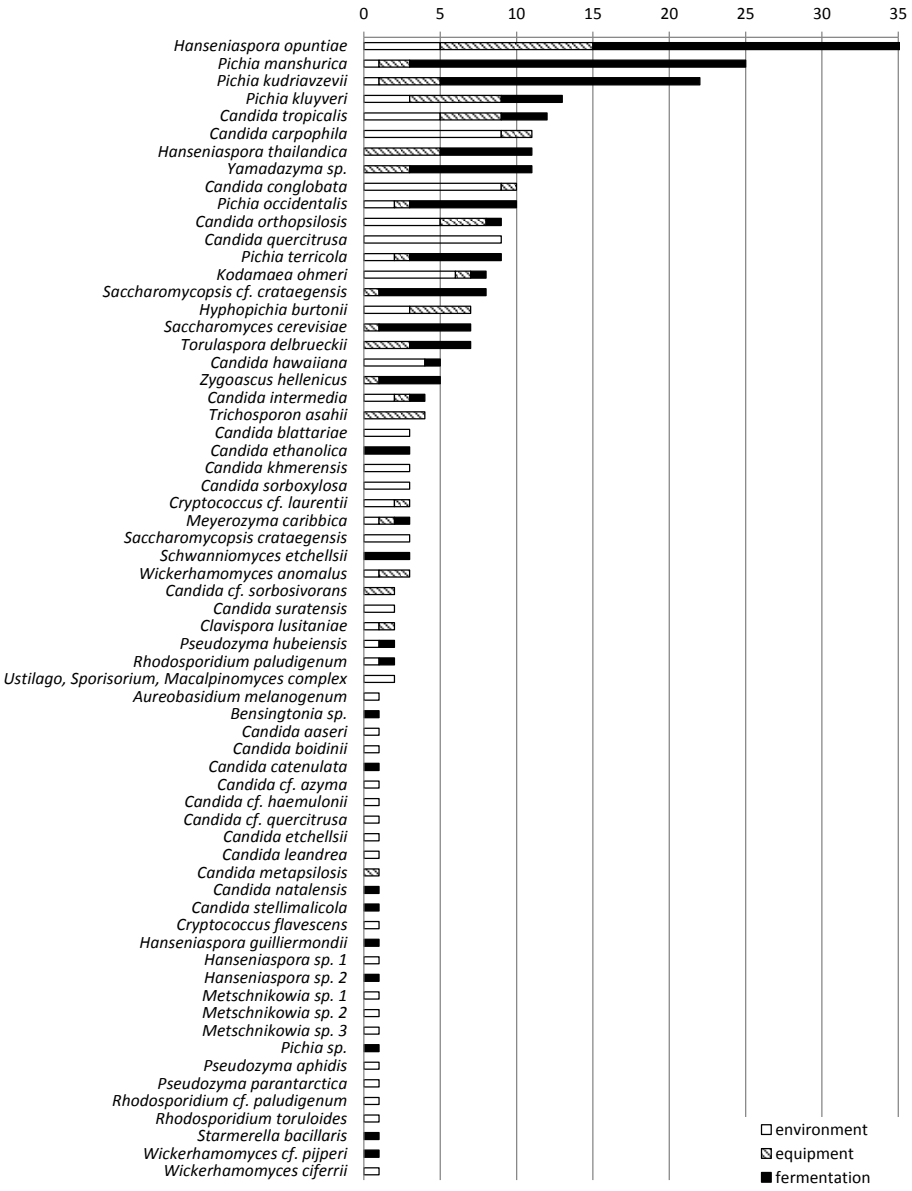

Fig 3 Number of occurrences of yeast species in 115 environmental, 25 equipment- and production-related, and 69 cocoa bean heap fermentation samples from Cuba.

Table 3 Yeast species repeatedly isolated from Cuban cocoa bean heap fermentations and their environments. It is indicated whether the species was isolated from transport bags (B), or from the heap (H) to which they were added when building the heap. Numbers represent the occurrence of the species in different samples, summarised also by sample types.

	Candida ethanolica	*Candida tropicalis*	*Hanseniaspora opuntiae*	*Hanseniaspora thailandica*	*Pichia kluyveri*	*Pichia kudriavzevii*	*Pichia mandshurica*	*Pichia occidentalis*	*Pichia terricola*	*Saccharomyces cerevisiae*	*Saccharomycopsis cf. crataegensis*	*Schwanniomyces etchellsii*	*Torulaspora delbrueckii*	*Yamadazyma sp.*	*Zygoascus hellenicus*
Industrial-scale heap 1A		1H	4B, 2H	2B, 1H		3B, 3H	1H		2H	2H		1H	2H		
Industrial-scale heap 1B	1H		3B, 1H	2B		1B, 3H	1H		3B	3H		2H	1B, 1H		
Industrial-scale heap 2		1B, 1H			1B, 2H		2H				1H			1B, 5H	2B
Industrial-scale heap 3*			1H				3H							2H	
Industrial-scale heap 4			2B, 2H			6H	4H	1B, 3H	1B						2B
Industrial-scale heap 5*						1H				1H					
Domestic-scale heap 1*			1H					2H			1H				
Domestic-scale heap 2*	2H		5H	1H	1H		12H	1H			5H				
Fermentations	3	3	20	6	4	16	23	7	6	6	7	3	4	8	4
Equipment	0	4	10	5	6	5	2	1	1	1	1	0	3	3	1
Environment	0	5	5	0	3	1	1	2	2	0	0	0	0	0	0

*No bag was available for sampling

The three most frequently isolated fermentation yeast species were also obtained from the environment, namely from ground-collected *R. regia* flowers at farm 2 (but not from such flowers collected at Taco Bay, Supplementary Table S1), from an *Drosophila*-like insect attracted to the heap of collected cocoa pods on farm 1, and from *Drosophila*-like insects on rotting fruit at the Yumuri river. The same three yeast species were also obtained from all sampled equipment types: knife, glove, yagua, transport bag, fermentation surface, plastic cover, and shovel.

Seventeen potentially novel yeast species were discovered, of which two, referred to as *Yamadazyma* sp. and *Saccharomycopsis cf. crataegensis*, were isolated repeatedly from fermentations and equipment, but not from the environment. *Saccharomyces cerevisiae* was only obtained from the industrial-scale heap 1, industrial-scale heap 5, and a knife used to open the cocoa pods during the 2014 field experiment (Table 3). Four to eleven species were encountered per completely sampled fermentation site, while up to five additional species were detected per bag of cocoa pulp-bean mass before being assembled into fermentation heaps. Three species that were not isolated from cocoa bean fermentations were the most frequently encountered in environmental samples, each with nine isolates: *Candida carpophila*, *Candida conglobata* and *Candida quercitrusa*.

Yeast physiology

Physiological testing of nine yeast species, selected based on their occurrence during cocoa bean fermentations, resulted in profiles typical for the genus to which they belonged (Supplementary Table S4). One to ten isolates originating from fermentations, and/or equipment, and/or the environment were tested per species. Tests in standardised microplate arrays were complemented by growth in tubes to evaluate the growth potential of the isolates under stresses relevant during cocoa bean fermentations. With respect to the isolates tested, 45°C prevented growth and growth at 37°C was variable with widespread tolerance for *S. cerevisiae*, *Pichia* spp., and *C. tropicalis*. Ethanol tolerance was variable at 5% and 10%, while negative at 15%. Growth at 10% ethanol was detected for fermentation-derived isolates of *S. cerevisiae* and most *Pichia* spp., but not for their environmental isolates. All tested pH levels were tolerated. Growth in the presence of 50% glucose was frequent among *T. delbrueckii*, *Hanseniaspora* spp., and *C. tropicalis* isolates, while none of the *Pichia* spp. isolates were capable of growth. Ethanol assimilation was frequent among isolates of *Pichia* spp., *C. tropicalis*, and *T. delbrueckii*, while rarely detected for H*. opuntiae*. All isolates tested were citrate-negative. Although intra-species variation in physiological characteristics was found for all species for which multiple isolates were tested, the only tendency to increased tolerance towards fermentation stresses was seen for *S. cerevisiae* and *P. kudriavzevii* strains from cocoa bean fermentations and equipment, which grew well at 37°C and tolerated 10% ethanol in contrast to environmental strains of the same species.

Discussion

A yeast species diversity assessment of Cuban cocoa bean fermentation heaps and their environment was performed in the search for natural habitats and vectors of cocoa fermentation yeasts. This study was accompanied by the monitoring of the fermentations regarding viable yeast counts, pH, and temperature development, to allow a comparison of the so far unstudied Cuban cocoa bean fermentations with those known from other countries.

The maximal yeast counts occurring within the first 26 h of cocoa bean fermentation, accompanied by a moderate temperature increase, were similar for most fermentations monitored and corresponded to those reported from cocoa bean fermentations before (e.g., Ardhana & Fleet 2003, Schwan & Wheals 2004, Jespersen et al. 2005, Camu et al. 2007, 2008). Only industrial-scale heap 5, considered a failed fermentation and not monitored till the end, yielded yeast counts below the detection limit. Also the pH development in the industrial-scale fermentations, with a slow continuous increase over the entire duration and a slight temporary increase at the height of the yeast growth; commonly interpreted to be due to the consumption of citrate by yeasts and lactic acid bacteria, corresponded to data reported previously (Camu et al. 2007, 2008, Papalexandratou et al. 2013). Similar durations of yeast growth until 80-100 h were found among the industrial-scale fermentations, when considering the subsequent low counts in industrial-scale heap 1 as re-infections and when disregarding the failed fermentation in industrial-scale heap 5. The local practices to transport the cocoa pulp-bean mass in fabric bags allowed liquidised pulp to drain away before setting up the fermentation heaps. The transfer of the already fermenting cocoa pulp-bean mass from the bags to large fermentation heaps, occurring at different times during the first 24 h of fermentation, was followed by a decrease in yeast counts corresponding to previous reports (e.g., Ardhana & Fleet 2003, Schwan & Wheals 2004, Jespersen et al. 2005, Camu et al. 2007), although yeast growth has also been found to remain at its highest numbers during longer time periods (Camu et al. 2008, Papalexandratou et al. 2013, Meersman et al. 2013). The continuous yeast growth in the domestic-scale heap 2 may be explained by temperatures never exceeding 38°C, possibly due to the placement of the yagua in the shade. Potential explanations for the marked pH increase in this small domestic-scale fermentation could be a lower bacterial production of organic acids, a consumption of organic acids, for instance by yeast, or a higher release of alkaline compounds such as ammonia by bacilli, which often develop upon prolonged cocoa bean fermentations (Schwan & Wheals, 2004). As bacterial growth and metabolite concentrations could not be monitored during the present study, these assumptions remain to be tested during similarly performed cocoa bean fermentations. Alkaline pH values have been reported for cocoa bean fermentations in Ivory Coast (Samagaci et al. 2014). The practice of transporting the cocoa pulp-bean mass instead of entire pods to the fermentation site seems to shorten the yeast-dominated fermentation phase and could result in a lower ethanol production compared

to fermentations in which yeast activity proceeds longer. Ethanol production is an important characteristic of the cocoa bean fermentation process in driving the exothermic aerobic oxidation of ethanol into acetic acid by acetic acid bacteria leading to high temperatures of 45 to 50°C that inhibit further yeast growth, and by its influence on the diffusion of flavour-effective compounds and precursors into the cocoa beans (Schwan & Wheals 2004, Camu et al. 2007, 2008).

The most important species in the Cuban cocoa bean heap fermentations studied were *P. manshurica* and *H. opuntiae*, when considering their presence in all and almost all fully monitored fermentations, respectively. This result differs from previous studies that found *S. cerevisiae* as a dominating cocoa bean heap and box fermentation yeast at different locations (e.g., Mexico: Arana-Sánchez et al. 2015, Malaysia: Meersman et al. 2013 and Papalexandratou et al. 2013, Brazil: Pereira et al. 2012, and Ghana: Daniel et al. 2009). *Pichia manshurica* and *H. opuntiae* have been reported from cocoa bean fermentation processes before (Ivory Coast: Hamdouche et al. 2014, Malaysia: Meersman et al. 2013 and Papalexandratou et al. 2013, Ecuador: Papalexandratou & De Vuyst 2011, and Ghana: Crafack et al. 2013, Daniel et al. 2009, and Jespersen et al. 2005). The infrequent isolation of *S. cerevisiae* suggests an only temporary presence of this yeast in the environments investigated. Its presence mostly in the fermentation heap sampled during the 2013 field experiment may also indicate seasonal differences as possible cause for its weak presence in the 2014 fermentations. The domestic-scale heap 2 with yeast remaining at high counts during the entire six-day fermentation, a pH rise, and a low temperature did not show a different yeast species composition; only the dominance of *P. manshurica* was noted throughout the entire fermentation process. This species was described from alcoholic fermentation of sorghum in Manshurica, China (Saito 1914) and is synonymous with *Pichia galeiformis* (Ueda-Nishimura & Mikata 2001). *Pichia manshurica* has been reported in natural fermentations of plant material (Kurtzman 2011). It is detrimental in pickled cucumbers because of a loss of preservation capacity due to lactic acid consumption by the yeast (Franco & Pérez-Díaz 2012), and in olive processing due to reduction of olive firmness assigned to its polygalacturonase activity (Golomb et al. 2013), while it does not contribute to lipid degradation in olive processing (Rodríguez-Gómez et al. 2010). All three properties are relevant for cocoa bean fermentations too, as lactic acid is abundant, polygalacturonase activity would contribute to the desired degradation of the cocoa pulp, and lipids in the cocoa seeds should not be affected. *Hanseniaspora opuntiae* has been described based on strains isolated from rotting cactus parts (*Opuntia ficus-indica*) in Hawaii, USA (Cadez et al. 2003) and has subsequently been reported most frequently from cocoa bean fermentations as listed above, but also from other fermenting substrates, such as grape marcs and wine (Garofalo et al. 2015, Wang et al. 2013, Bovo et al. 2009), oranges (Liu et al. 2015), coffee beans (de Melo Pereira et al. 2014), and pineapple juice (Chanprasartsuk et al. 2013). Decaying plant material that harbour yeasts serves as mating, oviposition, and feeding site for drosophilids (e.g.; Markow & O'Grady 2008,

Gilbert 1980, Phaff et al. 1956). Attraction tests of *Drosophila melanogaster* to yeast–inoculated grape juice recovers *P. kluyveri* and *Hanseniaspora uvarum* among the most attractive yeasts in a panel dominated by *Saccharomyces* spp. strains (Palanca et al. 2013). Although drosophilid sample numbers in the present study were restricted, *P. kluyveri* and closely related *Pichia* and *Hanseniaspora* species were recovered from these samples, suggesting drosophilids as possible vectors of these yeasts to the opened cocoa pods.

The large number of yeast species isolated from the environment confirmed that the sampling performed accessed a large part of the yeast diversity that might potentially enter fermenting cocoa pulp-bean mass. Flowers were sampled extensively and insects to a lesser degree according to their availability, as both constitute niches that often harbour specialised yeast communities based on the substrate they provide and the vectors that visit them (e.g., Lachance et al. 2001). *Candida carpophila* and *C. quercitrusa*, two of the three most frequent species exclusively obtained from the environment, are associated with the insect-plant interface, which corresponds to the in this study sampled substrates (Lachance et al. 2011). The third most frequent environmental species, *C. conglobata*, rarely reported before (Lachance et al. 2011), seems to occupy a similar habitat, judged from its isolation, which is mostly from fallen *R. regia* flowers in a cocoa-unrelated location, but which is also a flower and a leaf in a cocoa plantation. All three species were isolated more often from the environment than the most frequent cocoa fermentation yeast species. While *H. opuntiae* and *P. kudriavzevii* were detected also in the farm environment, *P. manshurica*, the most frequently encountered species in the current cocoa bean fermentations, was isolated only from a site distant to the cocoa farming place. The sampling performed was rather broad and explorative, discovered only few isolates of the major cocoa bean fermentation yeasts in the environment, but indicated flowers of *R. regia* palms in cocoa farms as a promising starting point for further searches.

The three most frequent cocoa bean fermentation yeasts were also isolated from all equipment types sampled. Among them, yaguas, cocoa bean collection containers made from the Cuban royal palm leafs, and the cocoa bean transport bags were the most frequent isolation sources. These containers might play a role in the transmission of yeasts from one cocoa bean fermentation to another, as they are used in the harvest collection process and are partially reused in subsequent seasons.

The broad physiological characterisation of selected yeast species and strains based on a miniaturised microplate array was confirmed and refined by tests more specific to conditions relevant to cocoa fermentations in larger volumes (Daniel et al. 2009). Although the most extreme test conditions might never be reached during average fermentations, stress tolerances tested covered this large range and helped to explain the yeast diversity detected in cocoa bean fermentations. Tolerance to high osmotic stress,

occurring in fresh cocoa pulp and simulated by growth at 50% glucose, favoured growth of early-stage cocoa fermentation yeasts, such as H. *opuntiae*, *T. delbrueckii*, *S. cerevisiae*, *C. tropicalis*, but also a rather fortuitous member of the communities, *Candida hawaiiana*. *Candida tropicalis* was isolated from the environment at the same frequency as *H. opuntiae*, had thereby a similar chance to enter the fermentations, and even united ethanol assimilation and consistent growth at 37°C as additional advantageous characters. Nevertheless, *C. tropicalis* was not a dominating species during Cuban cocoa bean fermentations, possibly because of its low ethanol tolerance. Even the lowest tested pH of 2.5 was not restricting yeast growth, emphasising yeasts well suited to be active early in the degradation process of the initially citric acid-rich, fermenting cocoa pulp-bean mass. The perception of citrate consumption by yeasts (Schwan & Wheals 2004) should be reconsidered, because certain species of lactic acid bacteria are able to metabolise this compound during cocoa bean fermentation processes (Camu et al., 2007, 2008; Ho et al., 2014). The current observation that none of the yeast isolates selected for physiology testing showed citrate assimilation underlined this possibility. Strong ethanol tolerance, ethanol assimilation, in combination with temperature tolerance, were factors that favoured particularly *P. kudriavzevii*, but also other *Pichia* spp., including *Candida ethanolica*, which belongs to *Pichia* phylogenetically (Daniel et al. 2014). In contrast, *H. opuntiae*, presumably due to its lack of ethanol tolerance and only rare temperature tolerance, was not isolated in later stages of the fermentation process. *Pichia* spp. and *S. cerevisiae* isolated from cocoa bean fermentations, in contrast to those isolated from the environment, were mostly tolerant to high ethanol concentrations, indicating selective or adaptive effects on strain level.

Conclusions

This first comparative study of the yeast diversity during Cuban cocoa bean heap fermentations and their environment indicates inoculation of the fermentation heaps from the environment and equipment. Furthermore, this study first points towards insects as natural vectors that initiate cocoa bean fermentations, followed by the selection of those species that thrive in the sugar-laden, acidic and, at later stages, ethanol-enriched raw material. While *S. cerevisiae* appears to be the most universally adaptable species, it was not isolated from the environment during the current study, nor did it occur in the majority of the Cuban cocoa bean fermentation processes studied. In contrast, *Hanseniaspora* spp. and *Pichia* spp., easily detected also in the environment, were present during most fermentations. Given the similar yeast species profiles and process parameters during industrial-scale Cuban cocoa bean fermentations compared to those from other geographical regions, the inoculation principles of the fermentations may also be similar. The relatively short phase of high yeast activity during industrial-scale fermentations may be due to the local practices to transport the already fermenting cocoa pulp-bean mass in small batches before setting up the final fermentation heaps. It appears of interest to analyse additional

domestic-scale cocoa bean fermentations to access their variability and to more precisely link fermentations to specific farm environments.

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General Discussion and Conclusions

General Discussion and Conclusions

Cocoa production depends on the availability of healthy, undamaged pods, which contain the beans that, after fermentation, roasting and further processing, form the raw material to produce cocoa powder and chocolate. Cocoa pods are susceptible to a range of fungal (e.g. Black Pod disease, Frosty Pod Rot caused by many *Phytophthora* spp. and *Moniliophthora roreri*, respectively) and viral diseases (e.g. Cocoa swollen-shoot virus) as well as by pests (e.g. Cocoa pod borer *Conopomorpha cramerella*). Some of these pests and diseases are increasing their geographical distribution and, under the current global warming, may be considered as invasive. Cocoa producing countries all over the world are challenged to fight cocoa yield decrease and to satisfy the ever increasing demand for cocoa beans (Guest 2007).

Cuba is seeking to build a low-pesticide, sustainable cocoa-production chain to assure a high cocoa quality. Its well-educated human resources are one prerequisite for such labor-intensive production approach. To improve production, it is of prime importance to map the diversity of major pathogens, identify their potential microbial antagonists and describe the beneficial flora involved in cocoa bean fermentation. With this in view, three major groups of fungi or fungal-like organisms were investigated: the *Phytophthora* species infecting pods, the endophytes colonizing plants and surroundings and the fermentative cocoa yeasts.

In a first part, the study was focused on the identification of the *Phytophthora* species responsible for Black Pod disease in *T. cacao* in Cuba. In a second step, fungal endophytes present in *T. cacao* were identified as a source of potential biocontrol agents against the causal agent of black pod disease. Finally, in a third step, the diversity of yeasts in cocoa bean fermentations and their environment was investigated. The spontaneous character of the fermentations may be the responsible of microbial variation and hence of variation in product quality.

Black pod disease, caused by several species of *Phytophthora*, is a major constraint of cocoa production worldwide (Erwin & Ribeiro 1996, Kroon et al. 2012). The number of *Phytophthora* species described in the literature is around 124 (Bertier et al. 2013, Martin et al. 2012, Reeser et al. 2013, Yang et al. 2013) and several of these species are causal agents of black pod disease in *T. cacao* (Akrofi 2015, Erwin & Ribeiro 1996, Lachenaud et al. 2015, Thevenin et al. 2012). The diversity of *Phytophthora* has seldom been explored in Cuba, even though the potential host plants are grown since the 18th century in this country. In the present study, we noticed the prevalence of *P. palmivora,* which was detected in nearly all sites explored. The presence of *P. tropicalis* was also detected but at a very low occurrence. *P. palmivora* is thus the dominant species responsible for black pod disease in cocoa in Cuba. These results are useful to increase the on the species of *Phytophthora* causing black pod disease in Latin America. In this region, several taxa are have been reported, but their distribution remains

poorly known (Hérail et al. 2004). There are reports about the presence of *P. palmivora, P. citrophthora,* and *P. capsici* in South America (Vos et al. 2003, Nyasse et al. 2007). *Phytophthora palmivora* is present throughout the American continent and the Caribbean. *Phytophthora capsici* was divided into three entities: *P. capsici* sensu stricto, *P. capsici* "cocoa", which appears to be the dominant taxon in Mexico, and *P. tropicalis* (Hérail et al. 2004).

Decreasing the pressure caused by *Phytophthora,* as well as other pathogens, is central to increase yield and improve the quality of cocoa production. An appropriate control strategy thus needs to be established to reduce the incidence or eliminate the disease. Black pod disease is very difficult to control due to the ability of *P. palmivora* to reproduce sexually, forming long-lived oospores. It has been reported that oospores can persist up to 13 years in the field and resist harsh (e.g. drought) environmental conditions (Erwin & Ribeiro 1996, Gevens et al. 2007, Konam & Guest 2002).

Nowaday, the control of *Phytophthora* spp. is most often done by chemicals such as copper and metalaxyl-based fungicides. These pesticides are expensive for the farmers, needs to be applied repetitively and may impact the environment and health of practitioners (Opoku et al. 2007). These conventional treatments may potentially also affect non-target fungi such as endophytes that are beneficial for plant health and yeasts that are essential for the fermentation of cocoa pods. It is common practice of the Cuban cocoa producers to start fermentation in the plantations by opening the pods before their delivery to the centralizing cooperatives. A fungicide reduction or even suppression in cocoa plantations could well favor the presence of *Drosophila*-like insects that depend on environmental yeasts as nutritional resources (Anagnostou et al. 2010) and vector them successfully to cocoa fermentations. The current study provided data that allow hypothesizing that the insects play a role in the transfer of yeasts from habitats such as palm flowers and decaying plant material to the cocoa fermentation. Decaying plant material constitutes a niche for yeasts by serving as oviposition and feeding site for drosophilids (Markow & O'Grady 2008). In our study, *P. kluyveri,* and closely related *Pichia* and *Hanseniaspora* species have been recovered from these samples, suggesting drosophilids as possible vectors of these yeasts to the opened cocoa pods. Hence, the role of *Drosophila*-like insects as part of the established plantation ecosystem is worth to be mentioned in view of the insecticide use in the area due to recent outbreaks of mosquito-transmitted Dengue fever. Further explorations should address also potentially negative roles *Drosophila* spp. may play in fruit damage and pathogen transfer. One example is the spotted wing *Drosophila*, (*D. suzukii*, that was found in several Californian counties infesting ripening cherries, and in coastal areas infecting ripening raspberry, blackberry, blueberry, and strawberry (Walsh et al. 2001).

The considerable numbers of endophytic fungi in the Cuban cocoa agro-system provide a rich source for further studies on the biological control. Carefully developed biological control approaches offer the possibility to act long after their release, a requirement for successful management of fungal

plant pathogens. Fungal endophytes have demonstrated their effectiveness in the reduction of pod loss due to *Phytophthora* spp. (Avelino & DeClerck 2011, Evans & Thomas 2003, Holmes et al. 2004, Holmes et al. 2006, Mejía et al. 2008, Rubini et al. 2005, Tondje et al. 2007). In the present study 27 endophytic fungal species were isolated from healthy leaves of *T. cacao*. The most frequent species were *Colletotrichum gloesporioides* and a potentially novel *Xylaria* species. These results are consistent with other reports on endophyte diversity (Rubini et al. 2005, Tejesvi et al. 2007). The biocontrol potential of *C. gloesporioides* against black pod disease (Mejía et al. 2008) is encouraging towards the further study of the newly obtained Cuban isolates of this species.

The combination of cultural control methods such as regular harvesting, elimination of infected pods, pod tripping and pruning of cocoa with fungal endophytes as biological control agents should enable Cuban farmers to reduce their dependence on chemicals and increase sustainability. This will result in improved disease management. For example, agricultural practices alone, such as regular removal of diseased pods, reduced *P. palmivora* occurrence by 35–66% in Peruvian cocoa plantations (Soberanis & Krauss 1999) and was 32% more profitable than control with fungicides in Ghana (Dormon 2007). The recommended strategy is based on consequent good agricultural practices that reduce *Phytophthora* occurrence enough to allow the still present beneficial mycoflora to exert its natural biocontrol potential. The development of endophytes as potential biocontrol agents should be envisaged to enhance their beneficial effects.

Ecological networks of cocoa culture and fermentation may be severely disturbed by the application of agro- and other chemicals. The integration of cultural principles that consider the local fungal diversity might confer yet unexpected advantages to cocoa production in Cuba and worldwide.

Future perspectives

Future perspectives

The results obtained in this study, represented by the datasets developed in Chapters 2, 3 and 4 will serve as a starting point for further research. Below, an overview of the potential future research topics related to these data is given.

The control of plant diseases is required to maintain the quality and abundance of food and fiber produced by growers worldwide. Different approaches may be used to prevent or control plant diseases. Plant pathologists describe the components of disease as a 'disease triangle' in which, the components are the host, pathogen and environment. For the occurrence of the disease, the host has to be susceptible, the pathogen has to be virulent and the environmental conditions need to be favorable (Agrios 1988).

Phytophthora palmivora causes serious production losses in all cocoa-growing countries (Bowers et al. 2001, Drenth & Guest 2004, Erwin & Ribeiro 1996). The major losses result from infection of pods that begin as small, hard, dark spots at any stage of pod development. Lesions develop quickly covering the entire pod surface and internal tissues, including the beans (Guest 2007, Opoku & Owusu 2000). *Phytophthora* can infect flowers, young leaves, roots, and cause cankers on trunks and branches, producing indirect losses (Gregory & Maddison 1981). As genetic control method, the use of resistant cocoa varieties has a positive effect (Thevenin et al. 2012). Cocoa genetic improvement programs have been performed with the aim to identify cocoa varieties resistant to *Phytophthora* (Lachenaud et al. 2007, 2015, Paulin et al. 2008, 2010, Tahi et al. 2006).

As result of this study, *P. palmivora* was identified as the main causal agent of black pod disease in Cuba. Laboratory tests on the Cuban cocoa varieties held in the Instituto de Investigaciones Agroforestales, Baracoa, using the local strains of *P. palmivora* are needed to select resistant varieties that could be used directly or as parents in breeding programs. Inoculation of cocoa leaves or leaf discs with zoospores of *Phytophthora* spp. is a routine method of screening for resistance to Black Pod disease. This method was well described by several researchers (e.g. Akaza et al. 2009, Nyasse et al. 1995, Tahi et al. 2006) and explained the inoculum concentration, light intensity during incubation incubation temperature and duration.

Biological control is an additional method to manage plant diseases (Krauss & Hebbar 1999), reducing the disease and the utilization of chemicals at the same time (Bajwa & Kogan, 2004). In face of the growing concern about the presence of pesticide residues in food products and soil-damaging effects of fungicides, the implementation of a sustainable method for disease control in the cocoa crop is of high importance (Krauss et al. 2006). Studies on the potential of fungal endophytes as biological control against *Phytophthora* in cocoa-producing regions are promising. Previous studies have

demonstrated a clear biocontrol potential of several fungal endophytes against *P. palmivora,* by the reduction of leaf and pod damage (Avelino et al. 2011, Deberdt et al. 2008, Hanada et al. 2009, Mejía et al. 2008, Ten Hoopen et al. 2003, Tondje et al. 2007).

In the present thesis, 27 fungal endophyte species were found in healthy cocoa leaves. Further investigations should follow to assess those which may have potential as classical biocontrol agents of black pod disease in the Cuban context. Inhibition of *P. palmivora* by these fungal endophytes isolates can be tested by *in vitro* studies using a dual culture technique. *In vitro* fungal interactions may lead to one of the following observations: production of an inhibition zone, contact inhibition or no inhibition (Adebola & Amadi 2010).

An integrated pest management program for the control of black pod disease in Cuba is likely to increase the number of healthy pods and to reinforce and enlarge the cocoa production with the resulting economic benefits. In the area of Baracoa, Cuba, cocoa production is the principal economic base. In this light, it is important to recognize the important role that farmers play in the adoption of best practices. During this study, problems were identified in the cocoa bean fermentation process, potentially influencing the product quality. The further study of the yeast niche-vector system should lead to recommendations how to preserve the natural system that provides the base to initiate cocoa bean heap fermentations. The Universidad de Guantánamo and its experts are dedicated to make the best known practices of cocoa culture and fermentation available to the cocoa-producing community in the Province of Guantánamo in a practical format such as manuals and guidelines. The international exchange is increasingly recognized as an essential component of this work and is on the way to be improved.

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Annexes

Table 1 Yeast diversity found in different cocoa fermentation studies. Countries of origin are abbreviated as follows: Au, Australia; Be, Belize; Br, Brazil; DR, Dominican Republic; Ec, Ecuador; Gh, Ghana; IC, Ivory Coast; In, Indonesia; Ma, Malaysia; Me, Mexico

Species	Number of occurrences	Me	IC	Au	Br	Ma	Ma	Br	Br	Ec	Gh	Gh	Gh	DR	Gh	Gh	In	Be	Br	IC	Gh, Ma	IC
		Arana-Sánchez et al. 2015	Hamdouche et al. 2014	Ho et al 2014	Moreira 2013	Meersman et al. 2013	Papalexandratou et al. 2012	de Melo Pereira et al. 2012	Illeghems et al. 2012	Papalexandratou et al. 2011	Daniel et al. 2009	Nielsen et al. 2008	Nielsen et al. 2007	Lagunes Gálvez et al. 2007	Nielsen et al. 2005	Jespersen et al. 2005	Ardhana & Fleet 2003	Thompson et al. 1997	Board 1995	Schwan, Rose,	Ravelomanana et al. 1985	Carr et al. 1979	Gauthier et al. 1977
Candida albicans	1								1													
Candida aff. neodendra/diddensiae	1						1															
Candida asiatica	1					1																
Candida awuaii	1												1									
Candida boidinii	1																	1				
Candida bombi	1																		1			
Candida carpophila	1										1											
Candida castellii	2																			1		1
Candida cylindraceae	1												1									
Candida diversa	1												1									
Candida ethanolica	2										1		1									
Candida friederichii	1														1							
Candida fructus	1					1																

Species	Number of occurrences	Arana-Sánchez et al. 2015	Me	Hamdouche et al. 2014	IC	Ho et al 2014	Au	Moreira 2013	Br	Meersman et al. 2013	Ma	Papalexandratou et al. 2012	Ma	de Melo Pereira et al. 2012	Br	Illeghems et al. 2012	Br	Papalexandratou et al. 2011	Ec	Daniel et al. 2009	Gh	Nielsen et al. 2008	Gh	Nielsen et al. 2007	Gh	Lagunes Gálvez et al. 2007	DR	Nielsen et al. 2005	Gh	Jespersen et al. 2005	Gh	Ardhana & Fleet 2003	In	Thompson et al. 1997	Be	Board 1995	Br	Schwan, Rose,	Br	Ravelomanana et al. 1985	IC	Carr et al. 1979	Gh, Ma IC	Gauthier et al. 1977	IC
Candida glabrata	2															1										1																	
Candida halmia	1																							1																			
Candida humicola	1																															1											
Candida humilis	2		1											1																													
Candida inconspicua	1																									1																	
Candida insectorum	1			1																																						
Candida intermedia	4				1									1																1			1									
Candida jaroonii	1											1																														
Candida metapsilosis	1									1																																
Candida michaelii	1																							1																			
Candida orthopsilosis	3									1				1						1																							
Candida parapsilosis	2									1																				1												
Candida quercitrusa	3									1														1						1												
Candida rugopelliculosa	1																																			1					
Candida rugosa	1																																			1					
*Candida saitoana (*syn. *Torulopsis candida)*	1																																								1
Candida sake	1																																						1		

Species	Number of occurrences	Arana-Sánchez et al. 2015	Hamdouche et al. 2014	Ho et al 2014	Moreira 2013	Meersman et al. 2013	Papalexandratou et al. 2012	de Melo Pereira et al. 2012	Illeghems et al. 2011	Papalexandratou et al. 2011	Daniel et al. 2009	Nielsen et al. 2008	Nielsen et al. 2007	Lagunes Gálvez et al. 2007	Nielsen et al. 2005	Jespersen et al. 2005	Ardhana & Fleet 2003	Thompson et al. 1997	Board 1995	Schwan, Rose,	Ravelomanana et al. 1985	Carr et al. 1979	Gauthier et al. 1977
		Me	IC	Au	Br	Ma	Ma	Br	Br	Ec	Gh	Gh	Gh	DR	Gh	Gh	In	Be	Br		IC	Gh, Ma	IC
Candida silvae	1												1										
Candida sorbosivorans-like	1									1													
Candida sorboxylosa	2					1							1										
Candida sp. (Torulopsis sp.)	3		1															1				1	
Candida stellimalicola	5	1							1		1				1	1							
Candida tropicalis	6	1				1	1			1	1						1						
Candida zemplinina	3											1	1		1								
Candida zeylanoides	3													1							1		
Cryptococcus flavescens	1			1																			
Cryptococcus laurentii	2					1										1							
Cryptococcus humicolus	2															1					1		
*Debaryomyces hansenii (*syn. *Candida famata)*	1																				1		
Debaryomyces sp.	1																					1	
Dekkera anomala	2	1																1					
Geotrichum ghanense	1												1										
Guehomyces pullulans	1																				1		

Species	Number of occurrences	Arana-Sánchez et al. 2015	Hamdouche et al. 2014	Ho et al 2014	Moreira 2013	Meersman et al. 2013	Papalexandratou et al. 2012	de Melo Pereira et al. 2012	Illeghems et al. 2011	Papalexandratou et al. 2011	Daniel et al. 2009	Nielsen et al. 2008	Nielsen et al. 2007	Lagunes Gálvez et al. 2007	Nielsen et al. 2005	Jespersen et al. 2005	Ardhana & Fleet 2003	Thompson et al. 1997	Board 1995	Schwan, Rose,	Ravelomanana et al. 1985	Carr et al. 1979	Gauthier et al. 1977
		Me	IC	Au	Br	Ma	Ma	Br	Br	Ec	Gh	Gh	Gh	DR	Gh	Gh	In	Be	Br		IC	Gh, Ma	IC
Hanseniaspora sp.	2	1																				1	
Hanseniaspora guilliermondii	9			1			1					1	1	1	1	1	1	1					
Hanseniaspora occidentalis	2																1				1		
Hanseniaspora opuntiae	7		1			1	1		1	1	1					1							
Hanseniaspora osmophila	1																				1		
Hanseniaspora thailandica	2					1	1																
Hanseniaspora uvarum	5				1			1	1											1	1		
Hanseniaspora valbyensis	1													1									
Hanseniaspora vineae	1																1						
Hansenula sp.	1																					1	
Kazachstania exigua	2																				1		1
Kluyveromyces marxianus	3			1						1										1			
Kluyveromyces wickerhamii	1																				1		
Kodamaea ohmeri	2					1					1												
Lachancea thermotolerans	1																			1			
Lodderomyces elongisporus	1																			1			

Species	Number of occurrences	Arana-Sánchez et al. 2015	Hamdouche et al. 2014	Ho et al 2014	Moreira 2013	Meersman et al. 2013	Papalexandratou et al. 2012	de Melo Pereira et al. 2012	Illeghems et al. 2012	Papalexandratou et al. 2011	Daniel et al. 2009	Nielsen et al. 2008	Nielsen et al. 2007	Lagunes Gálvez et al. 2007	Nielsen et al. 2005	Jespersen et al. 2005	Ardhana & Fleet 2003	Thompson et al. 1997	Board 1995	Schwan, Rose,	Ravelomanana et al. 1985	Carr et al. 1979	Gauthier et al. 1977
		Me	IC	Au	Br	Ma	Ma	Br	Br	Ec	Gh	Gh	Gh	DR	Gh	Gh	In	Be	Br		IC	Gh, Ma	IC
Metschnikowia reukaufii	1																	1					
Meyerozyma caribbica	1										1												
Meyerozyma guilliermondii	5	1		1		1										1		1					
Nakazawae holstii	1																				1		
Pichia aff. fermentans	1						1																
Pichia holstii	3		1															1				1	
Pichia kluyveri	5	1							1		1				1	1							
Pichia kudriavzevii	6	1				1	1			1	1						1						
Pichia manshurica	3											1	1		1								
Pichia membranifaciens	3													1							1		
Pichia occidentalis	1			1																			
Pichia pijperi	2					1										1							
Pichia sp.	2															1					1		
Pichia sporocuriosa	1																				1		
Pichia terricola	1																					1	
Rhodotorula glutinis	2	1																1					
Rhodotorula minuta	1												1										
Rhodotorula mucilaginosa	9			1			1					1	1	1	1	1	1	1					

Species	Number of occurrences	Arana-Sánchez et al. 2015	Me	Hamdouche et al. 2014	IC	Ho et al 2014	Au	Moreira 2013	Br	Meersman et al. 2013	Ma	Papalexandratou et al. 2012	Ma	de Melo Pereira et al. 2012	Br	Illeghems et al. 2011	Br	Papalexandratou et al. 2011	Ec	Daniel et al. 2009	Gh	Nielsen et al. 2008	Gh	Nielsen et al. 2007	Gh	Lagunes Gálvez et al. 2007	DR	Nielsen et al. 2005	Gh	Jespersen et al. 2005	Gh	Ardhana & Fleet 2003	In	Thompson et al. 1997	Be	Board 1995	Br	Schwan, Rose,	Ravelomanana et al. 1985	IC	Carr et al. 1979	Gh, Ma	Gauthier et al. 1977	IC
Rhodotorula rubra	2																															1						1				
Rhodotorula sp.	1																																						1			
Saccharomyces cerevisiae	17		1					1		1		1		1		1		1		1	1	1	1	1				1		1		1		1		1			1				
Saccharomyces sp.	1																																						1			
Saccharomycodes ludwigii	1																				1																						
Saccharomycopsis (Candida) amapae	1																											1															
Saccharomycopsis crataegensis	4		1							1												1	1																				
Saccharomycopsis fibuligera	1																																										
Saccharomycopsis sp.	2																																		1						1		
Schizosaccharomyces pombe	6		1									1		1		1								1											1								
Schizosaccharomyces sp.	2																																		1						1		
Sporopachydermia lactativora	1																																							1			
Torulaspora delbrueckii	6		1							1		1						1						1															1			

Species	Number of occurrences	Arana-Sánchez et al. 2015 Me	Hamdouche et al. 2014 IC	Ho et al 2014 Au	Moreira 2013 Br	Meersman et al. 2013 Ma	Papalexandratou et al. 2012 Ma	de Melo Pereira et al. 2012 Br	Illeghems et al. 2012 Br	Papalexandratou et al. 2011 Ec	Daniel et al. 2009 Gh	Nielsen et al. 2008 Gh	Nielsen et al. 2007 Gh	Lagunes Gálvez et al. 2007 DR	Nielsen et al. 2005 Gh	Jespersen et al. 2005 Gh	Ardhana & Fleet 2003 In	Thompson et al. 1997 Be	Board 1995 Br	Schwan, Rose, Board 1995 Br	Ravelomanana et al. 1985 IC	Carr et al. 1979 Gh, Ma IC	Gauthier et al. 1977 IC
Torulaspora globosa	1					1																	
Torulaspora pretoriensis	1																			1			
Trichosporon asahii	2														1	1							
Wickerhamomyces anomalus	3					1											1			1			
Yamadazyma mexicana	1										1												
Yarrowia lipolytica	1													1									
Zygosaccharomyces bailii	1																				1		
Zygosaccharomyces rouxii	1																				1		

Supplementary Table S1. Overview of samples, their categories (cat.: Env, environmental; Eq, equipment and handling; F, fermentation), sample type, description, date, location, number of in Cuba obtained isolates versus the in Belgium recovered isolates, and the yeast species identified from the sample.

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
PS-A1	Pod surface	Env	Swab	Cocoa pod surface	13.09.2013	Farm 1, El Jamal	0/0	
PS-A2	Pod surface	Env	Swab	Cocoa pod surface	13.09.2013	Farm 1, El Jamal	0/0	
PS-B1	Pod surface	Env	Swab	Cocoa pod surface	13.09.2013	Farm 1, El Jamal	0/0	
PS-B2	Pod surface	Env	Swab	Cocoa pod surface	13.09.2013	Farm 1, El Jamal	0/0	
PS-C1	Pod surface	Env	Swab	Cocoa pod surface on heap	12.09.2014	Farm 3, Saint Luis	10/10	*Candida carpophila, Candida orthopsilosis*
PS-G2	Pod surface	Env	Swab	Cocoa pod surface on heap	15.09.2014	Farm 3, Saint Luis	10/10	*Candida carpophila, Candida orthopsilosis, M. caribbica*
OP-A1	Opened Pod	Env	Bag	Cocoa pod, opened aseptically	13.09.2013	Farm 1, El Jamal	0/0	
OP-A2	Opened Pod	Env	Bag	Cocoa pod, opened	13.09.2013	Farm 1, El Jamal	0/0	

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
				aseptically				
OP-B1	Opened Pod	Env	Bag	Cocoa pod, opened aseptically	13.09.2013	Farm 1, El Jamal	0/0	
OP-B2	Opened Pod	Env	Bag	Cocoa pod, opened aseptically	13.09.2013	Farm 1, El Jamal	0/0	
OP-C1	Opened Pod	Env	Bag	Cocoa pod, opened aseptically	9.09.2014	Farm 3, Saint Luis	0/0	
OP-C2	Opened Pod	Env	Bag	Cocoa pod, opened	9.09.2014	Farm 3, Saint Luis	0/0	
OP-C3	Opened Pod	Env	Bag	Cocoa pod, opened aseptically	9.09.2014	Farm 3, Saint Luis	0/0	
OP-G	Opened Pod	Env	Swab	Freshly opened pod at the harvest site	15.09.2014	Farm 3, Saint Luis	0/0	
F-A1	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
F-A2	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
F-A3	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	2/2	*Rhodosporidium cf.*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
								paludigenum
F-A4	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
F-A5	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	10/12	*Pseudozyma aphidis, Rhodosporidium paludigenum, Ustilago/Sporisorium/Macalpinomyces complex*
F-A6	Flower	Env	Tube	*Widelia triloba*	20.09.2013	Farm 1, El Jamal	0/0	
F-A7	Flower	Env	Tube	Issara red	20.09.2013	Farm 1, El Jamal	0/0	
F-A9	Flower	Env	Tube	*Roystonea regia*	20.09.2013	Farm 1, El Jamal	10/9	*Candida sorboxylosa, Kodamaea ohmeri*
F-B1	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
F-B4	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
F-B5	Flower	Env	Tube	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
F-B7	Flower	Env	Tube	*Widelia triloba*	13.09.2013	Farm 1, El Jamal	10/9	*Candida hawaiiana*
F-B8	Flower	Env	Capillary	Banana	20.09.2013	Farm 1, El Jamal	3/0	
F-B10	Flower	Env	Capillary	Unidenified	20.09.2013	Farm 1, El Jamal	0/0	
F-B11	Flower	Env	Capillary	Banana	20.09.2013	Farm 1, El Jamal	0/0	
F2	Flower	Env	Tube	*Calendula officinalis*	20.09.2013	Farm 1, El Jamal	10/5	*Candida cf. azyma, Cryptococcus flavescens*
F14-1	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	10/10	*Candida tropicalis, Candida blattariae*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
F14-2	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	10/10	*Candida quercitrusa, Hanseniaspora opuntiae, Candida carpophila, Candida blattariae*
F14-3	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	10/10	*Candida carpophila, Candida cf. quercitrusa, Hanseniaspora opuntiae*
F14-5	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	10/10	*Pichia kudriavzevii, Candida tropicalis, Candida hawaiiana, Candida boidinii*
F14-6	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	10/10	*Hanseniaspora opuntiae, Candida carpophila*
F14-7	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	12/12	*Pichia kudriavzevii, Candida quercitrusa, Metschnikowia* sp. 3
F14-8	Flower	Env	Tube	*Roystonea regia*	9.09.2014	Farm 2, El Jamal	10/10	*Candida carpophila, Kodamaea ohmeri*
F14-9	Flower	Env	Tube	Cocoa	9.09.2014	Farm 2, El Jamal	4/4	*Candida carpophila*
F14-10	Flower	Env	Tube	Cocoa	9.09.2014	Farm 2, El Jamal	10/10	*Metschnikowia* sp. 1, *Metschnikowia* sp. 2, *Candida leandrae, Candida quercitrusa*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
F14-11	Flower	Env	Tube	Cocoa	9.09.2014	Farm 2, El Jamal	0/0	
F14-12	Flower	Env	Tube	Cocoa	9.09.2014	Farm 2, El Jamal	0/0	
F14-13	Flower	Env	Tube	Cocoa	9.09.2014	Farm 2, El Jamal	2/2	*Candida conglobata*
F14-15	Flower	Env	Tube	Banana	12.09.2014	Farm 3, Saint Luis	0/0	
F14-17	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	10/10	*Candida quercitrusa, Aureobasidium melanogenum*
F14-20	Flower	Env	Tube	*Widelia triloba*	10.09.2014	Yumuri river	0/0	
F14-21	Flower	Env	Tube	*Widelia triloba*	10.09.2014	Yumuri river	0/0	
F14-23	Flower	Env	Tube	*Widelia triloba*	10.09.2014	Yumuri river	0/0	
F14-24	Flower	Env	Tube	*Widelia triloba*	10.09.20	Yumuri river	0/0	
F14-25	Flower	Env	Tube	*Widelia triloba*, with fly	10.09.2014	Yumuri river	0/0	
F14-26	Flower	Env	Tube	*Widelia triloba*, closed	10.09.2014	Yumuri river	10/10	*Pseudozyma parantarctica*
F14-27	Flower	Env	Tube	*Widelia triloba*	10.09.2014	Yumuri river	0/0	
F14-28	Flower	Env	Tube	Unidentified	10.09.2014	Yumuri river	4/4	*Candida cf. haemulonii*
F14-30	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	0/0	
F14-31	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	10/10	*Candida khmerensis, Candida conglobata*
F14-32	Flower	Env	Tube	*Roystonea*	14.09.2014	Taco Bay National	6/6	*Candida quercitrusa,*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
				regia		Park station		*Candida conglobata*
F14-33	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	10/10	*Candida carpophila, Candida conglobata*
F14-34	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	12/12	*Candida conglobata, Kodamaea ohmeri, Candida khmerensis*
F14-35	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	10/10	*Candida khmerensis, Candida conglobata*
F14-36	Flower	Env	Tube	*Roystonea regia*	14.09.2014	Taco Bay National Park station	10/10	*Kodamaea ohmeri, Candida conglobata*
F14-37	Flower	Env	Tube	Coconut palm	16.09.2014	Caguajo beach	7/7	*Candida etchellsii*
F14-39	Flower	Env	Tube	Cocoa, pollinated	19.09.2014	Farm 4, Palo Gordo	14/14	*Candida quercitrusa*
F14-40	Flower	Env	Tube	Cocoa, not pollinated	19.09.2014	Farm 4, Palo Gordo	0/0	
F14-41	Flower	Env	Tube	Palm tree	27.09.2015	Zoologico de Piedras, Alto de Boqueron	0/1	*Wickerhamomyces ciferrii, Candida quercitrusa, Kodamaea ohmeri*
F14-42	Flower	Env	Tube	Palm tree	27.09.2015	Zoologico de Piedras, Alto de Boqueron	0/1	*Candida quercitrusa*
F14-43	Flower	Env	Tube	Palm tree	27.09.2016	Zoologico de	0/1	*Candida hawaiiana*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
						Piedras, Alto de Boqueron		
I-1B	Insect	Env	Tube	*Drosophila*-like fly	20.09.2013	Farm 1, El Jamal	20/15	*Candida sorboxylosa, Hanseniaspora opuntiae, Pichia terricola*
I-2B	Insect	Env	Tube	*Drosophila*-like fly	20.09.2013	Farm 1, El Jamal	10/9	*Pichia terricola, Hanseniaspora* sp.1
I14-1	Insect	Env	Plate	*Drosophila*-like	10.09.2014	Yumuri river	10/10	*Pichia kluyveri, Saccharomycopsis crataegensis*
I14-2	Insect	Env	Plate	*Drosophila*-like	10.09.2014	Yumuri river	10/10	*Saccharomycopsis crataegensis, Pichia occidentalis, Pichia kluyveri, H. opuntiae*
I14-3	Insect	Env	Plate	*Drosophila*-like	10.09.2014	Yumuri river	0/0	
I14-4	Insect	Env	Plate	*Drosophila*-like	10.09.2014	Yumuri river	10/10	*Pichia kluyveri, Pichia manshurica, Saccharomycopsis crataegensis, Candida orthopsilosis*
I14-6	Insect	Env	Plate	*Drosophila*-	12.09.2014	Farm 3, Saint Luis	10/10	*Wickerhamomyces*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
				like				*anomalus, Hyphopichia burtonii*
I14-7	Insect	Env	Plate	*Drosophila*-like	15.09.2014	Farm 3, Saint Luis	0/0	
I14-8	Insect	Env	Plate	*Drosophila*-like	15.09.2014	Farm 3, Saint Luis	0/0	
I14-9	Insect	Env	Plate	*Drosophila*-like	16.09.2014	Farm 3, Saint Luis	10/10	*Hyphopichia burtonii, Cryptococcus cf. laurentii*
I14-10	Insect	Env	Plate	*Drosophila*-like	16.09.2014	Farm 3, Saint Luis	10/10	*Cryptococcus cf. laurentii, Candida orthopsilosis*
I14-11	Insect	Env	Plate	Termite	16.09.2014	Caguajo beach	0/0	
I14-12	Insect	Env	Tube	Particle from termite tunnel	16.09.2014	Caguajo beach	0/0	
L-B1	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B2	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B3	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B4	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B5	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B7	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B8	Leaf	Env	Swab	Cocoa	13.09.2013	Farm 1, El Jamal	0/0	
L-B9	Leaf	Env	Swab	Banana	13.09.2013	Farm 1, El Jamal	2/0	
L-B10	Leaf	Env	Swab	Unknown tree	13.09.2013	Farm 1, El Jamal	0/0	

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
L-B11	Leaf	Env	Swab	Banana steam	13.09.2013	Farm 1, El Jamal	0/0	
SP-1A	Leaf	Env	Swab	Dead palm leaf, site A	20.09.2013	Farm 1, El Jamal	15/7	*Candida carpophila*
SP-2A	Leaf	Env	Swab	Dead palm leaf, site A	20.09.2013	Farm 1, El Jamal	10/7	*Candida hawaiiana*
L14-1	Leaf	Env	Swab	Banana	9.09.2014	Farm 2, El Jamal	0/0	
L14-2	Leaf	Env	Swab	Banana	9.09.2014	Farm 2, El Jamal	0/0	
L14-3	Leaf	Env	Swab	Dead palm leaf	9.09.2014	Farm 2, El Jamal	0/0	
L14-4	Leaf	Env	Swab	Dead palm leaf	9.09.2014	Farm 2, El Jamal	10/10	*Ustilago/Sporisorium/Macalpinomyces complex*
L14-5	Leaf	Env	Swab	Dead palm leaf	9.09.2014	Farm 2, El Jamal	2/2	*Candida conglobata*
L14-6	Leaf	Env	Swab	Green palm leaf	9.09.2014	Farm 2, El Jamal	0/0	
L14-7	Leaf	Env	Swab	Green palm leaf	9.09.2014	Farm 2, El Jamal	0/0	
L14-9	Leaf	Env	Swab	Dead palm leaf	10.09.2014	Yumuri river	7/7	*Clavispora lusitaniae*
L14-10	Leaf	Env	Swab	Dead palm leaf	10.09.2014	Yumuri river	0/0	

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
L14-11	Leaf	Env	Swab	Dead palm leaf	14.09.2014	Humboldt NP, Bahia de Taco	10/10	*Pseudozyma hubeiensis*
TF14-1	Tree flux	Env	Tube	Coconut palm	9.09.2014	Farm 2, El Jamal	10/10	*Candida suratensis*
TF14-2	Tree flux	Env	Tube	Coconut palm	9.09.2014	Farm 2, El Jamal	10/10	*Candida suratensis*
TF14-3	Tree flux	Env	Tube	Coconut palm	9.09.2014	Farm 2, El Jamal	0/0	
TF14-4	Tree flux	Env	Swab	Coconut palm	9.09.2014	Farm 2, El Jamal	0/0	
TF14-5	Tree flux	Env	Tube	Thick liquid from cut banana trunk on the ground	19.09.2014	Farm 4, Palo Gordo	10/10	*Candida intermedia*
TF14-6	Tree flux	Env	Tube	Thick liquid from cut banana trunk on the ground	19.09.2014	Farm 4, Palo Gordo	10/10	*Candida intermedia, Candida tropicalis*
SN	Knife	Eq	Swab	Machete	20.09.2013	Farm 1, El Jamal	15/10	*Candida carpophila, Hanseniaspora opuntiae*
K-C2	Knife	Eq	Swab	Machete	12.09.2014	Farm 3, Saint Luis	10/10	*Pichia kudriavzevii, Candida tropicalis, Wickerhamomyces anomalus, Hanseniaspora thailandica, Hypopichia burtonii*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
K-C1	Knife	Eq	Swab	Machete used for cutting palm leaf	12.09.2014	Farm 3, Saint Luis	12/10	*Candida conglobata, Cryptococcus cf. laurentii, Pichia kluyveri, Wickerhamomyces anomalus*
K-E	Knife	Eq	Swab	Machete	19.09.2014	Farm 4, Palo Gordo	0/0	
G-E	Glove	Eq	Swab	Glove, after use	19.09.2014	Farm 4, Palo Gordo	10/10	*Hanseniaspora opuntiae, Pichia occidentalis, Pichia kluyveri, Hyphopichia burtonii*
C-B	Container	Eq	Swab	Yagua	13.09.2013	Farm 1, El Jamal	20/20	*Hanseniaspora opuntiae, Candida orthopsilosis, Candida intermedia, Torulaspora delbrueckii*
C-C	Container	Eq	Swab	Yagua	12.09.2014	Farm 3, Saint Luis	10/10	*Yamadazyma* sp.*, Clavispora lusitaniae, Pichia terricola*
C-G	Container	Eq	Swab	Yagua	15.09.2014	Farm 3, Saint Luis	10/10	*Hanseniaspora opuntiae, Pichia manshurica*
C-E	Container	Eq	Swab	Yagua	18.09.2014	Farm 4, Palo Gordo	10/10	*Candida carpophila, Hanseniaspora opuntiae, Hyphopichia burtonii,*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
								Candida cf. sorbosivorans, Meyerozyma caribbica, Hanseniaspora thailandica
C-E1	Container	Eq	Swab	Yagua	19.09.2014	Farm 4, Palo Gordo	0/0	
BS-A	Bag inner surface	Eq	Swab	Bag A	13.09.2013	Farm 1, El Jamal	8/17	*Trichosporon asahii, Pichia kudriavzevii, Candida cf. sorbosivorans, Candida orthopsilosis*
BS-C	Bag inner surface	Eq	Swab	Bag C	13.09.2014	Cooperative, El Jamal	10/10	*Pichia kluyveri, Saccharomycopsis cf. crataegensis, Hanseniaspora thailandica*
BS-E	Bag inner surface	Eq	Swab	Bag E	19.09.2014	Farm 4, Palo Gordo	10/10	*Hanseniaspora opuntiae, Zygoascus hellenicus*
B1-1	Fermentation surface	Eq	Swab	Concrete surface at site A	14.09.2013	Cooperative, El Jamal	20/19	*Trichosporon asahii, Torulaspora delbrueckii, Hanseniaspora opuntiae, Hyphopichia burtonii, Candida tropicalis*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
B2-1	Fermentation surface	Eq	Swab	Concrete surface site B	14.09.2013	Cooperative, El Jamal	6/6	*Trichosporon asahii*
B3	Fermentation surface	Eq	Swab	Concrete surface	13.09.2014	Cooperative, El Jamal	10/10	*Candida tropicalis, Pichia kluyveri*
B5	Fermentation surface	Eq	Tube	Particles from old, unused wooden fermentation box	18.09.2014	Farm 4, Palo Gordo	0/0	
BC-1	Heap cover	Eq	Swab	Plastic cover before touching the beans	14.09.2013	Cooperative, El Jamal	0/0	
BC-2	Heap cover	Eq	Swab	Plastic cover before touching the beans	14.09.2013	Cooperative, El Jamal	20/20	*Pichia manshurica, Pichia kudriavzevii*
BC-3	Heap cover	Eq	Swab	Plastic cover before touching the beans	13.09.2014	Cooperative, El Jamal	1/1	*Yamadazyma* sp.
S1	Shovel	Eq	Swab	Shovel before	16.09.2013	Cooperative, El	5/3	*Hanseniaspora opuntiae*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
				turning		Jamal		
S3	Shovel	Eq	Swab	Shovel before turning	13.09.2014	Cooperative, El Jamal	10/10	*Hanseniaspora thailandica, Yamadazyma* sp.*, Pichia kluyveri*
BC-A0	Bag content	F 2h	Bag	Bag A content 2h after harvest	13.09.2013	Farm 1, El Jamal	16/16	*Pichia kudriavzevii, Hanseniaspora thailandica, Hanseniaspora opuntiae, Rhodosporidium paludigenum*
BC-A1	Bag content	F4h	Bag	Bag A content on arrival	13.09.2013	Cooperative, El Jamal	20/20	*Pichia kudriavzevii, Hanseniaspora opuntiae, Hanseniaspora thailandica*
BC-A2	Bag	F 8h	Bag	Bag A content 6h	13.09.2013	Cooperative, El Jamal	20/22	*Hanseniaspora opuntiae*
BC-A3	Bag	F 20h	Bag	bag A content	14.09.2013	Cooperative, El Jamal	20/11	*Hanseniaspora opuntiae, Pichia kudriavzevii*
FE1-1	Fermentation	F 24h	Bag	Heap 1, site A	14.09.2013	Cooperative, El Jamal	20/9	*Hanseniaspora opuntiae, Hanseniaspora thailandica, Pichia terricola*
FE1-2	Fermentation	F 28h	Bag	Heap 1, site A	14.09.2013	Cooperative, El Jamal	15/13	*Hanseniaspora opuntiae, Pichia kudriavzevii, Saccharomyces cerevisiae,*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
FE1-3	Fermentation	F 48h	Bag	Heap 1, site A	15.09.2013	Cooperative, El Jamal	17/17	*Candida tropicalis, Torulaspora delbrueckii Pichia kudriavzevii, Torulaspora delbrueckii, Pichia manshurica, Saccharomyces cerevisiae*
FE1-4	Fermentation	F 56h	Bag	Heap 1, site A	15.09.2013	Cooperative, El Jamal	0/0	
FE1-5	Fermentation	F 72h	Bag	Heap 1, site A	16.09.2013	Cooperative, El Jamal	5/3	*Pichia terricola, Pichia kudriavzevii*
FE1-7	Fermentation	F 96h	Bag	Heap 1, site A	17.09.2013	Cooperative, El Jamal	2/2	*Schwanniomyces etchellsii*
FE1-9	Fermentation	F 144h	Bag	Heap 1, site A	19.09.2013	Cooperative, El Jamal	1/1	*Candida catenulata*
FE1-10	Fermentation	F 168h	Bag	Heap 1, site A	20.09.2013	Cooperative, El Jamal	0/0	
BC-B0	Bag content	F 2h	Bag	Bag B content 2h after harvest	13.09.2013	Farm 1, Jamal	14/13	*Hanseniaspora opuntiae, Bensingtonia* sp.
BC-B1	Bag content	F 4h	Bag	Bag B content on arrival	13.09.2013	Cooperative, El Jamal	16/15	*Pichia terricola, Hanseniaspora thailandica*
BC-B2	Bag	F 8h	Bag	Bag B content	13.09.2013	Cooperative, El	20/19	*Pichia kudriavzevii, Pichia*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
				6h		Jamal		*terricola, Torulaspora delbrueckii, Hansenispora opuntiae, Hanseniaspora thailandica*
BC-B3	Bag	F 20h	Bag	Bag B content	14.09.2013	Cooperative, El Jamal	20/8	*Pichia terricola, Hansenispora opuntiae*
FE2-1	Fermentation	F 24h	Bag	Heap 1, site B	14.09.2013	Cooperative, El Jamal	20/13	*Hanseniaspora opuntiae, Hanseniaspora* sp.2
FE2-2	Fermentation	F 28h	Bag	Heap 1, site B	14.09.2013	Cooperative, El Jamal	20/18	*Torulaspora delbrueckii, Pichia kudriavzevii, Saccharomyces cerevisiae*
FE2-3	Fermentation	F 48h	Bag	Heap 1, site B	15.09.2013	Cooperative, El Jamal	10/12	*Pichia kudriavzevii, Pichia manshurica, Candida ethanolica, Saccharomyces cerevisiae*
FE2-4	Fermentation	F 56h	Bag	Heap1, site B	15.09.2013	Cooperative, El Jamal	15/15	*Pichia kudriavzevii, Saccharomyces cerevisiae*
FE2-5	Fermentation	F 72h	Bag	Heap 1, site B	16.09.2013	Cooperative, El Jamal	3/2	*Schwanniomyces etchellsii*
BC-C1	Bag content	F 10min	Bag	Bag C content after harvest	12.09.2014	Farm 3, Saint Luis	10/10	*Yamadazyma* sp.*, Candida tropicalis, Zygoascus hellenicus*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
BC-C2	Bag content	F 4h	Bag	Bag C content	12.09.2014	Farm 3, Saint Luis	10/10	*Zygoascus hellenicus, Pichia kluyveri*
BC-C3	Bag content	F 8h	Bag	Bag C content	12.09.2014	Farm 3, Saint Luis	10/10	*Candida hawaiiana*
FE3-1	Fermentation	F 24h	Bag	Heap 2	13.09.2014	Cooperative, El Jamal	10/10	*Pichia kluyveri, Yamadazyma* sp.*, Saccharomycopsis cf. crataegensis*
FE3-2	Fermentation	F 28h	Bag	Heap 2	13.09.2014	Cooperative, El Jamal	10/10	*Pichia kluyveri, Yamadazyma* sp.*, Candida tropicalis*
FE3-3	Fermentation	F 32h	Bag	Heap 2	13.09.2014	Cooperative, El Jamal	10/10	*Yamadazyma* sp.
FE3-5	Fermentation	F 48h	Bag	Heap 2	14.09.2014	Cooperative, El Jamal	10/10	*Pichia manshurica, Yamadazyma* sp.
FE3-6	Fermentation	F 56h	Bag	Heap 2	14.09.2014	Cooperative, El Jamal	10/10	*Pichia manshurica, Yamadazyma* sp.
FE3-7	Fermentation	F 80h	Bag	Heap 2	15.09.2014	Cooperative, El Jamal	0/0	
FE3-8	Fermentation	F 104h	Bag	Heap 2	16.09.2014	Cooperative, El Jamal	0/0	
FE3-9	Fermentation	F 128h	Bag	Heap 2	17.09.2014	Cooperative, El Jamal	0/0	

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
FE4-1	Fermentation	F 24h	Bag	Heap 3	13.09.2014	Cooperative, El Jamal	10/10	*Hanseniaspora opuntiae, Pseudozyma hubeiensis*
FE4-2	Fermentation	F 28h	Bag	Heap 3	13.09.2014	Cooperative, El Jamal	10/10	*Hanseniaspora guilliermondii, Candida orthopsilosis*
FE4-3	Fermentation	F 32h	Bag	Heap 3	13.09.2014	Cooperative, El Jamal	10/10	*Pichia manshurica, Yamadazyma* sp.
FE4-7	Fermentation	F 80h	Bag	Heap 3	15.09.2014	Cooperative, El Jamal	0/0	
FE4-8	Fermentation	F 104h	Bag	Heap 3	16.09.2014	Cooperative, El Jamal	0/0	
FE4-9	Fermentation	F 128h	Bag	Heap 3	17.09.2014	Cooperative, El Jamal	0/0	
BC-E1	Bag content	F 10min	Bag	Bag E content after harvest	19.09.2014	Farm 4, Palo Gordo	10/10	*Pichia terricola*
BC-E2	Bag content	F 2h	Bag	Bag E content	19.09.2014	Farm 4, Palo Gordo	12/10	*Pichia occidentalis, Zygoascus hellenicus, Kodamaea ohmeri, Hanseniaspora opunitae*
BC-E3	Bag content	F 4h	Bag	Bag E content	19.09.2014	cocoa collection site Palo Gordo	10/10	*Hanseniaspora opunitae, Wickerhamomyces cf. pijperi, Zygoascus*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
								hellenicus, Candida natalensis
FE5-1	Fermentation	F 8h	Bag	Heap 4	19.09.2014	Cooperative, El Jamal	10/10	*Hanseniaspora opuntiae, Pichia manshurica*
FE5-2	Fermentation	F 24h	Bag	Heap 4	20.09.2014	Cooperative, El Jamal	10/10	*Hanseniaspora opuntiae, Pichia kudriavzevii*
FE5-3	Fermentation	F 28h	Bag	Heap 4	20.09.2014	Cooperative, El Jamal	10/10	*Pichia kudriavzevii, Pichia occidentalis, Pichia manshurica*
FE5-4	Fermentation	F 32h	Bag	Heap 4	20.09.2014	Cooperative, El Jamal	10/10	*Pichia kudriavzevii, Pichia occidentalis, Pichia manshurica*
FE5-5	Fermentation	F 48h	Bag	Heap 4	21.09.2014	Cooperative, El Jamal	10/10	*Pichia kudriavzevii*
FE5-6	Fermentation	F 56h	Bag	Heap 4	21.09.2014	Cooperative, El Jamal	10/10	*Pichia kudriavzevii, Pichia occidentalis, Pichia manshurica*
FE5-7	Fermentation	F 80h	Bag	Heap 4	22.09.2014	Cooperative, El Jamal	10/10	*Pichia kudriavzevii*
FE6-1	Fermentation	F 30min	Bag	Heap D1	20.09.2014	Farm 5; road to Mata	9/9	*Pichia occidentalis, Starmerella bacillaris*
FE6-2	Fermentation	F 4h	Bag	Heap D1	20.09.2014	Farm 5; road to	13/10	*Hanseniaspora opuntiae,*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
						Mata		*Pichia occidentalis, Saccharomycopsis cf. cratengensis*
BC-G1	Bag content	F 10min	Bag	Yagua content after harvest	15.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica, Hanseniaspora opuntiae*
FE7-1	Fermentation	F 4h	Bag	Heap D2	15.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica, Meyerozyma caribbica*
FE7-1A	Fermentation	F 4h	Tube	Liquid from heap D2	15.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica*
FE7-2	Fermentation	F 8h	Bag	Heap D2	15.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica, Candida intermedia, Hanseniaspora opuntiae*
FE7-3	Fermentation	F 12h	Bag	Heap D2	15.09.2014	Farm 3, Saint Luis	10/10	*Hanseniaspora thailandica, Saccharomycopsis cf. crataegensis, Pichia kluyveri, Pichia manshurica*
FE7-4	Fermentation	F 24h	Bag	Heap D2	16.09.2014	Farm 3, Saint Luis	10/10	*Saccharomycopsis cf. crataegensis, Pichia manshurica, Hanseniaspora opuntiae*
FE7-5	Fermentation	F 28h	Bag	Heap D2	16.09.2014	Farm 3, Saint Luis	10/10	*Pichia occidentalis,*

Number	Sample	Cat.	Type	Description	Date	Location	Isolates Cuba/ Belgium	Species
								Saccharomycopsis cf. crataegensis, Hanseniaspora opuntiae
FE7-6	Fermentation	F 32h	Bag	Heap D2	16.09.2014	Farm 3, Saint Luis	13/13	*Pichia manshurica, Saccharomycopsis cf. crataegensis, H. opuntiae*
FE7-7	Fermentation	F 48h	Bag	Heap D2	17.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica, Saccharomycopsis cf. crataegensis*
FE7-8	Fermentation	F 56h	Bag	Heap D2	17.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica, Candida ethanolica*
FE7-9	Fermentation	F 80h	Bag	Heap D2	18.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica*
FE7-10	Fermentation	F 104h	Bag	Heap D2	19.09.2014	Farm 3, Saint Luis	10/10	*Pichia manshurica*
FE7-11	Fermentation	F 128h	Bag	Heap D2	20.09.2014	Farm 3, Saint Luis	11/11	*Pichia manshurica, Pichia* sp.*, Candida ethanolica, Candida stellimalicola*
FE8-1	Fermentation	F 24h	Bag	Heap 5	21.09.2014	Cooperative, El Jamal	2/2	*Pichia kudriavzevii, Saccharomyces cerevisiae*

Supplementary Table S2: Distribution of yeasts during Cuban cocoa bean fermentations in transport bags, heaps and a yagua (Cuban royal palm leaf container). The assembly of heaps and exact sampling times are shown in Fig. 1. As different fermentations were assembled and sampled at different times, time points were categorized as shown in Table S1. Presence or absence of a species in independent samples is indicated by numbers. Isolates of the same species from the same sample were not considered as independent and are not shown. The second column provides the sum of occurrences of the species (N). Industrial-scale heap 5 and domestic-scale heap D2 were not considered in this table because of their partial sampling.

	N	10 min	2 h	4 h	8 h	12 h	20 h	24 h	28 h	32 h	48 h	56 h	72 h	80 h	96 h	104 h	128 h	144 h
Pichia manshurica	23	1		2	2	1		1	1	3	5	4		1		1	1	
Hanseniaspora opuntiae	20	1	3	2	4		2	5	2	1								
Pichia kudriavzevii	16		1	1	1		1	1	3	1	3	2	1	1				
Yamadazyma sp.	8	1						1	1	2	2	1						
Pichia terricola	7	1	1	1	1		1	1					1					
Hanseniaspora thailandica	6		1	2	1	1		1										
Saccharomycopsis cf. crataegensis	6					1		2	1	1	1							
Pichia occidentalis	5		1						2	1		1						
Saccharomyces cerevisiae	5								2		2	1						
Pichia kluyveri	4			1		1		1	1									
Torulaspora delbrueckii	4				1				2		1							
Zygoascus hellenicus	4	1	1	2														
Candida ethanolica	3										1	1					1	
Candida tropicalis	3	1							2									
Schwanniomyces etchellsii	3												1		1			1

	N	10 min	2 h	4 h	8 h	12 h	20 h	24 h	28 h	32 h	48 h	56 h	72 h	80 h	96 h	104 h	128 h	144 h
Bensingtonia sp.	1		1															
Candida catenulata	1																	1
Candida hawaiiana	1				1													
Candida intermedia	1				1													
Candida natalensis	1			1														
Candida orthopsilosis	1								1									
Candida stellimalicola	1																1	
Hanseniaspora guilliermondii	1								1									
Hanseniaspora sp. 2	1							1										
Kodamae ohmeri	1		1															
Meyerozyma caribbica	1			1														
Pichia sp.	1																1	
Pseudozyma hubeiensis	1							1										
Rhodosporidium paludigenum	1		1															
Wickerhamomyces cf. pijperi	1			1														

Supplementary Table S3: Yeast species isolated from the environment and from equipment used for cocoa harvest and fermentation during the Cuban cocoa bean fermentations. The second column provides the sum of occurrences of the species (N). The number of the samples analyzed is provided in brackets.

	N	Flowers (54)	Tree fluxes (6)	Insects (14)	Leafs (25)	Cocoa pod surfaces (7)	Knifes (5)	Glove (1)	Palm leaf containers (5)	Transport bags (4)	Fermentation surfaces (4)	Heap covers (3)	Shovels (3)
Hanseniaspora opuntiae	15	3		2			1	1	3	2	1		2
Candida carpophila	11	6			1	2	1		1				
Candida conglobata	10	8			1		1						
Candida quercitrusa	9	9											
Candida tropicalis	9	4	1				1				2		1
Pichia kluyveri	9			3			1	1		1	1		2
Candida orthopsilosis	8			3		2			1	2			
Komadae ohmeri	7	6											1
Hyphopichia burtonii	7			3			1	1	1		1		
Hanseniaspora thailandica	5						1		1	2			1
Pichia kudriavzevii	5	1					1			1		1	1
Candida hawaiiana	4	3			1								
Trichosporon asahii	4									2	2		
Candida blattariae	3	3											
Candida intermedia	3		2						1				
Candida khmerensis	3	3											
Candida sorboxylosa	3	2		1									

	N	Flowers (54)	Tree fluxes (6)	Insects (14)	Leafs (25)	Cocoa pod surfaces (7)	Knifes (5)	Glove (1)	Palm leaf containers (5)	Transport bags (4)	Fermentation surfaces (4)	Heap covers (3)	Shovels (3)
Cryptococcus cf. laurentii	3			2			1						
Pichia manshurica	3			1					1			1	
Pichia occidentalis	3	1		1				1					
Pichia terricola	3			2					1				
Saccharomycopsis crataegensis	3			3									
Torulaspora delbrueckii	3								1	1	1		
Wickerhamomyces anomalus	3			1			2						
Yamadazyma sp.	3								1			1	1
Candida cf. sorbosivorans	2							1		1			
Candida suratensis	2		2										
Clavispora lusitaniae	2				1				1				
Meyerozyma caribbica	2					1		1					
Ustilago, Sporisorium, Macalpinomyces complex	2	1			1								
Aureobasidium melanogenum	1	1											
Candida aaseri	1	1											
Candida boidinii	1	1											
Candida cf. azyma	1	1											
Candida cf. haemulonii	1	1											
Candida cf. quercitrusa	1	1											
Candida etchellsii	1	1											
Candida leandrea	1	1											
Candida metapsilosis	1									1			

	N	Flowers (54)	Tree fluxes (6)	Insects (14)	Leafs (25)	Cocoa pod surfaces (7)	Knifes (5)	Glove (1)	Palm leaf containers (5)	Transport bags (4)	Heap covers (3)	Fermentation surfaces (4)	Shovels (3)
Cryptococcus flavescens	1	1											
Hanseniaspora sp. 1	1			1									
Metschnikowia sp. 1	1	1											
Metschnikowia sp. 2	1	1											
Metschnikowia sp. 3	1	1											
Pseudozyma aphidis	1	1											
Pseudozyma hubeiensis	1				1								
Pseudozyma parantarctica	1	1											
Rhodosporidium cf. paludigenum	1	1											
Rhodosporidium paludigenum	1	1											
Rhodosporidium toruloides	1				1								
Saccharomyces cerevisiae	1						1						
Saccharomycopsis cf. crataegensis	1									1			
Wickerhamomyces ciferrii	1	1											
Zygoascus hellenicus	1									1			

Supplementary Table S4: Physiological characterization of yeasts isolated from Cuban cocoa bean fermentation heaps. Strains of *Hanseniaspora* (*H.*), *Torulaspora* (*T.*), *Saccharomyces* (*S.*), *Pichia* (*P.*), and *Candida* (*C.*) species were selected to represent environmental (Env), equipment (Eq), and fermentation (F) isolates. +, growth; -, no growth; d, delayed; w, weak

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	45750	FE2-2-3	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	Env	F	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Eq	F	Env
Fermentation																																							
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	wd	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+
D-Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	wd	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
Methyl α-D-Glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	wd	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
α,α-Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	+	-	-	+	-	-	-	-	wd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	Env	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Eq	F	Env
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	wd-		wd+		-	-	-	-	-	-	-	-	-	-	-	-	wd-	-	-	-	-	-	-	-	-	-
Carbon assimilation																																					
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	w	-	+	-	-	-	-	-	-	-	+	+	+	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-	-	-	+	w	-	w	-	-	-	-	-	-	-	+	+	+	-
D-Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	w	-	-	-	-	-	-	-	-	-	+	-
D-Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	w	-	+	-	-	-	-	-	-	-	-	+	+	-
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	w	-	-	-	w	-	-	-	-	-	+	+	+	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-	-	-	-	-	-	-	-	+	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	w	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	w	-	w	+	w	-	-	w	-	+	-	+	-	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	+	w	-	-	+	-	-	-	-	-	-	-	w	+	+	+	-
α,α-Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	+	+	w	w	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
Methyl α-D-Glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Eq	F	Env
Cellobiose	+	+	+	+	+	+	w	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	w	+	-
Salicin	+	+	+	w	w	+	w	+	+	+	w	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutin	w	w	w	w	w	w	w	w	+	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	+	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
Inulin	-	-	-	-	w	-	-	-	-	-	-	-	+	+	w	+	-	w	-	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	w	w	w	w	-	-	-	-	-	w	-	+	w	+	w	w	-	-	w	w	+	+	-	-	+	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	w	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucitol	-	-	-	-	-	-	-	-	-	-	-	-	+	+	w	+	-	-	-	-	-	w	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Eq	F	Env
Galactitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myo-Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucono-1,5-lactone	w	w	w	w	w	w	-	w	w	w	w	w	w	-	-	w	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
2-Keto-d-Gluconate	w	w	w	w	-	w	-	w	w	+	w	w	w	w	w	+	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
5-Keto-d-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	w	+	+	+	+	-
D-Gluconate	-	w	-	w	-	+	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
D-Glucuronate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Galacturonate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DL-Lactate	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	w	-	-	-	-	-	-	w	w	-	w	w	-	-	-	-	-	-	-	-	-	-	w
Succinate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	+	-	w	+	+	w	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propane 1,2 diol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Butane 2,3 diol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Quinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucarate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	+	-	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. kudriavzevii*	*P. kudriavzevii*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Env	Eq	F	Env
Palatinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	+	-	-	+	w	w	w	-	-	w	-	-	-	-	-	w	+	+	+	+	-
Levulinate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	w	-	-	-	-	-	w	-	-	-	-	-
L-Malic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	w	+	+	+	+	+	+	+	+	+
L-Tartaric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	+	-	-	w	+	-	-	-	-	-	-	-
D-Tartaric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	+	-	-	-	-	-	-	-	-	-	+	w
Meso-Tartaric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactaric acid	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	w	-	-	-	-	+	-	+	-	-
Uric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentobiose	w	+	+	+	+	+	w	w	+	+	w	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
Ethylene glycol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tween 40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Tween 60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tween 80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrogen assimilation																																							
Nitrate	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Env	Eq	F	Env
Nitrite	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethylamine	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Lysine	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cadaverine	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Creatine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Creatinine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	+	-	-	-	-	-	-	-	-
Imidazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tryptophan	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Proline	-	-	-	-	-	-	-	-	-	-	-	-	w	-	+	-	-	-	-	-	-	w	-	-	-	-	-	-	w	+	+	w	-	+	+	+	+	-
Putrescine	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vitamin requirement																																						
w/o vitamins	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	-	+	+	-	-	-	+
w/o myo-Inositol	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
w/o Pantothenate	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	w	-	+	+	w	+	w	+	+	+	+	+	w	w	+	+	+	+	+	+	+	+

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Eq	F	Env
w/o Biotin	w	-	w	w	-	w	-	-	w	w	w	w	+	+	-	+	-	-	+	-	-	-	+	-	+	+	+	+	-	w	+	+	+	w	-	-	-	+	+
w/o Thiamin	w	-	w	w	-	+	-	-	w	w	-	w	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	w	-	-	-	w	+	-	+	+	-
w/o Biotin & Thiamin	-	-	w	-	-	w	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	-	+	+	+	+	-	w	-	-	+	w	-	-	-	+	+
w/o Pyridoxine	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	w	+	+	+	+	+	+	+	+	+
w/o Pyridoxine & Thiamin	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	w	w	-	-	-	+	+	+	-	+	+
w/o Niacin	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	w	w	+	+	-	+	+	+	+	+	+
w/o PABA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	w	+	+	+	+	+	+	+	+	+
Miscellaneous tests																																							
Cycloheximide 0.01%	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	w	-	-	-	+	+	+	+	-
Cycloheximide 0.1%	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	w	-	-	-	+	-	+	+	-
Acetic acid 1%	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	-	+	-	-	-	+	-	+	+	-	+	+	+	-	-	+	-	+
10% NaCl	-	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+	-	+	-	+	-	+	-	+	-	-	+	-	w	w	-	-	-	-	+	-	-	-	+
16% NaCl	-	-	-	-	-	+	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH=3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+	w	+	+	w	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+
pH=9.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	F	Env	Eq	Eq	F	F	F	Eq	Env	F	F	F	Env	Env	Eq	F	Env
Hexadecane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	w	-	+	w	-	-	-	-	+
Benzoic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+
Ethanol	-	-	-	+	-	-	-	+	-	-	-	-	wd	wd	wd	wd	-	-	-	-	-	wd	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
Additional characteristics																																							
Starch formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid acetic production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	+	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diazonium Blue B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50% d-Glucose	-	-	+	+	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
Arbutin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Temperature growth tests																																							
25ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37ºC	w	-	-	+	-	-	-	w	w	-	-	+	-	-	-	-	+	w	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Species	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. opuntiae*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*H. thailandica*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*T. delbrueckii*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. kudriavzevii*	*P. manshurica*	*P. terricola*	*P. terricola*	*P. terricola*	*C. ethanolica*	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*	*C. hawaiiana*
Strain	I-1B-4	F14-2-2	I14-2-5	C-B-8	BS-B-21	FE1-2-15	BC-B0-15	FE2-1-11	BS-B-1	BC-A0-1	FE1-1-13	FE1-1-19	B1-1-11	BS-B-8	B1-1-4	FE2-2-10	FE2-2-3	45750	45754	FE2-2-7	FE1-2-9	FE1-3-14	FE2-4-4	F14-5-1	BC-2-10	BS-B-10	BC-A0-16	FE2-4-1	FE1-3-16	BC2-19	I-1B-1	BC-B1-2	FE1-5-1	FE2-3-2	F14-1-1	TF14-6-8	B1-1-14	FE1-2-10	F14-5-4
Source	Env	Env	Env	Eq	Eq	F	F	F	Eq	F	F	F	Eq	Eq	Eq	F	F	Env	Env	F	F	F	F	Env	Eq	F	F	F	F	Eq	Env	F	F	F	Env	Env	F	Env
Tests in tubes																																					
Ethanol tolerance 5%	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol tolerance 10%	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	-	+	-	-	-	-	-
Ethanol tolerance 15%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol assimilation 0.5%	-	-	-	+	-	-	-	+	-	-	-	-	w	w	w	w	-	-	-	-	-	w	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
pH=2,5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH=3,5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH=5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50% d-Glucose	-	-	+	+	-	+	+	-	w	-	w	w	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Citrate assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-