Biodiversity, pathogenicity, antifungal susceptibility and rapid identification of Fonsecaea and relatives
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Chapter I

General introduction: 
*Fonsecaea* and the black yeasts
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Black yeasts are classically defined as anamorphic fungi potentially able to produce melanized budding cell at least a part of their life cycle. This condition is known in phylogenetically highly diverse fungi, namely in some basidiomycetes and in members of the ascomycete orders Chaetothyriales, Capnodiales and Dothideales (de Hoog et al. 2000). Today, fungi are classified according to their phylogeny. Fonsecaea is a member of the order Chaetothyriales, which also contains the black yeast genus Exophiala. Cladophialophora, Cyphellophora, Fonsecaea, Phialophora and Rhinocladiella are related but do not produce yeast-like cells.

The order Chaetothyriales is remarkable in the fungal Kingdom, for two reasons. First, a large number of the infections are observed in individuals without known immune disorder. Of the about 77 species confirmed to belong to the order by sequence data (Barr 1990; Gueidan et al. 2008), about 40 of them have been encountered as etiologic agents of infections in vertebrates (Badali et al. 2008; de Hoog et al. 2000; Zeng et al. 2007). This high percentage of species with an infective potential is only matched by the order Onygenales containing the dermatophytes and classical systemic fungi. Second, the diversity in clinical pictures caused by members of the Chaetothyriales is bewildering. Species of Onygenales are very consistent in their pathology, displaying a similar clinical course by members causing cutaneous or systemic infections, whereas those of Chaetothyriales encompass a wide diversity of diseases, which are nevertheless more or less characteristic within a single species (de Hoog et al. 2000). This pathogenic potential is particularly observed in the more derived parts of the order, comprising the ascomycete family Herpotrichiellaceae (Untereiner 2000).

Several other, recurrent ecological trends in the Chaetothyriales are known; one of these is extremotolerance. Many species are found on exposed surfaces, having a competitive advantage at high temperature, dryness (Sterflinger 1998), or low nutrient availability (Satow et al. 2008). Phylogenetic trees published by Lutzoni et al. (2001) and Gueidan et al. (2008) indicate that some deep branches among the pathogenic black yeasts have a shared evolution with rock-inhabiting fungi, suggesting that this life style might be an ancestral condition. A meristematic growth form, morphologically similar to the muriform cells found in tissue of patients with chromoblastomycosis, in nature is expressed under adverse environmental conditions of nutrient depletion, high temperature and dryness. This suggests a functional change in the course of evolution, from an ancestral rock-inhabiting lifestyle to a derived strategy in which ultimately pathogenicity to vertebrate hosts enhances the fitness of species.

Human associated black yeast and relatives have been known since the end of the 19th century as a taxonomically quite heterogeneous group, sharing melanized cell walls and the formation of daughter cells by polar budding, but they still are among the most difficult fungal groups to identify and therefore the knowledge of this group is still only fragmentary (de Hoog et al. 2000). The diagnostic confusion in the past is not surprising, since the taxonomy of black yeast is now known to be much more complicated than was anticipated. With the application of molecular methods great number of undescribed species is encountered.

Knowledge of natural habitats and evolution is essential for a better understanding of
pathogenicity and opportunism. Members of different fungal orders and families tend to be differentially involved in human mycoses. Natural habitats are dead plants materials, wood, biofilters, soil polluted with toxic hydrocarbons, rock and inter surface (Badali et al. 2008). For recovery of black yeast like fungi selective isolation methods are required, e.g., the use of high temperature (Sudhadham et al. 2008), a mouse vector (Gezuele et al. 1972), extraction via mineral oil (Vicente et al. 2008) or enrichment on volatile aromatic hydrocarbons (Zhao et al. 2010). The success of the latter method, enabling isolation of black yeasts where direct plating previously had failed, has supported the hypothesis that herpotrichiellaceous black yeasts are potent degraders of aromatic hydrocarbons. Previously, the alkylbenzene enrichment technique has been applied in the biofiltration of air polluted with volatile aromatic compounds (Cox et al. 1997; Kennes and Veiga 2004; Weber et al. 1995).

**Chromoblastomycosis**

Chromoblastomycosis is one of the most frequent diseases caused by melanized fungi. It concerns a chronic cutaneous and subcutaneous infection, characterized by slowly expanding skin lesions which eventually leads to emerging, cauliflower-like eruptions. The muriform cells are the invasive form of the fungus, provoking a granulomatous immune response at the site of a preceding transcutaneous trauma (Najafzadeh et al. 2009; Queiroz-Telles et al. 2009). Chromoblastomycosis is found worldwide, but most clinical reports are from tropical and subtropical climates (de Hoog et al. 2007; Najafzadeh et al. 2009). Infection occurs in immunocompetent individuals. To date seven species have been proven as recurrent causative agents of the disease, i.e., Fonsecaea pedrosoi (Lopez Martinez & Mendez Tovar 2007), F. monophora (de Hoog et al. 2004), F. nubica (Najafzadeh et al. 2010), Cladophialophora carrionii (Badali et al. 2008), C. samoensis (Badali et al. 2008), Phialophora verrucosa (Gugnani et al. 1978; Hofmann et al. 2005) and Rhinocladiella aquaspersa (Arango et al. 1998; Marques et al. 2004). Several Exophiala species have been reported as occasional agents of the disease (Padhye et al. 1996; Tomson et al. 2006). The etiologic agents are supposed to gain entrance through the skin by traumatic implantation of contaminated materials. The majority of lesions are observed on extremities of outdoor workers (de Hoog et al. 2007; Queiroz-Telles et al. 2009). Chromoblastomycosis is currently classified into six clinical types: nodular, tumorous, verrucose, cicatricial, plague and lymphatic (Carrion 1950; Queiroz-Telles et al. 2009). In addition, lesions can be graded according to their severity, as mild, moderate or severe (Queiroz-Telles et al. 2009). In advanced cases more than one type of lesion can be observed in the same patient. As yet it is unknown whether these types are associated with specific etiologic agents or are dependent on host responses. Treatment of chromoblastomycosis may be difficult because of the presence of the therapy-refractory muriform cells and because of differential susceptibilities between taxonomically closely related groups (Bonifaz et al. 2001). There is no drug of choice for treatment of the disorder and results may depend on the size and severity of the lesions (Queiroz-Telles et al. 2009), etiologic agent, patient status and clinical localization (Bonifaz et al. 2001). For that reason, in-depth study of the various agents and their virulence is mandatory.
**Fonsecaea**

Fonsecaea is defined by absence of budding cells, sympodial conidiogenesis and conidia arranged in short chains. Cladophialophora is different by having very long conidial chains, but some species show intermediate morphology and are difficult to attribute to either one of the genera on morphological groups. A phialidic synanamorph may be produced on nutritionally poor media. Fonsecaea pedrosoi was first isolated in 1913 as an etiological agent of chromoblastomycosis by Pedroso and later described and named by Brumpt (1922) and Negroni (1936). On the basis of ribosomal DNA internal transcribed spacer (ITS) sequence data, two species were recently recognized within the genus, i.e., *F. pedrosoi* and *F. monophora* (de Hoog et al. 2004; Xi et al. 2009). Traditionally the genus included a third species, *F. compacta* (Carrión 1950). This taxon is now known to be one of the morphological mutants occurring in Fonsecaea species, but another, molecular sibling of *F. pedrosoi*, Fonsecaea nubica, was discovered in the course of our study. We also analyzed a Fonsecaea-like strain reported previously (Shinwari et al. 1985) as Cladophialophora bantiana from the left occipital lobe of the cerebrum of an 18-month-old spayed female cat in Australia (Najafzadeh et al. 2011).

**The study of biodiversity**

The first step in any biodiversity study is the establishment of species borderlines. Morphology in the Chaetothyriales is essential for the ecology and survival for the organism, but is often less significant to classify species. Therefore the development of a molecular species concept is necessary. This concept is primarily based on genealogical concordance of several gene trees made for the set of species under investigation. The result is then compared for consistency with other data sets, such as AFLP and morphology. Subsequently the geographic distribution of species is investigated, for which particularly AFLP is suitable. We then wish to develop rapid diagnostic techniques; to this aim we tested LAMP and RCA as novel approaches in species recognition.

**Sequencing**

In fungi, species previously diagnosed by morphological species recognition (MSR) frequently appear to be composed of more than one species when applying phylogenetic species recognition (PSR) (Taylor et al. 2000). In PSR, individuals are grouped objectively, but the decision on the exact delimitation of species remains arbitrary. To avoid the subjectivity of determining the limits of a species, Taylor and his coworkers applied multilocus sequence typing to recognize fungal species to establish the principle of GCPSR, genealogical concordance phylogenetic species recognition. The strength of GCPSR lies in a comparison of the congruency between several gene genealogies enabling the detection of recombination events (Taylor & Fisher 2003). Using GCPSR, multilocus sequence typing (MLST) schemes have been developed to investigate species delimitation in human or animal pathogenic fungi. In this thesis distinction of species is determined by morphological and multilocus sequences of the ribosomal internal transcribed spacers (ITS) and partial sequences of the
β-tubulin (BT2), actin (ACT1), and cell division cycle (cdc42) genes.

A subsequent step is the identification of strains and their attribution to the species defined above. We used novel methods for detection and identification of isolates such as AFLP, LAMP and RCA.

**AFLP**

The distribution of species defined by sequencing and phenetic characters can be studied by AFLP (Amplified Fragment Length Polymorphism), a relatively new technique which has a high discriminatory power and high reproducibility that makes it suitable for species identification as well as for strain typing (Savelkoul et al. 1999; Vos et al. 1995). The technique has emerged as a major epidemiological tool with broad application in ecology, population genetics, pathotyping, DNA fingerprinting and quantitative trait loci (QTL) mapping (Mueller & Wolfenbarger 1999). AFLP fingerprinting has been shown to be useful for the molecular characterization of microorganisms with relatively large genomes including various fungal species (Ball et al. 2004; Boekhout et al. 2001; Gupta et al. 2004; Theelen et al. 2001; Warris et al. 2003).

The AFLP protocol includes four steps: 1) the digestion of genomic DNA with two restriction endonucleases, 2) the ligation of digested DNA to double-stranded nucleotide adaptors, 3) pre-selective amplification of genomic fragments containing an adaptor at each end, and 4) selective amplification using primers with selective base extensions (Fig. 1). Only those fragments with complementary nucleotides extending beyond the restriction site will be amplified by the selective primers under stringent annealing conditions. This reduces the complexity of the mixture (Groenewald 2009). The number of fragments that will be generated can be modulated by extending the amplification primer(s) at the 3’ site with one or more selective nucleotides.

**LAMP**

Loop-mediated Isothermal Amplification (LAMP) is a powerful innovative gene amplification technique for early detection and identification of microbial diseases (Abliz et al. 2008). It was firstly described and initially evaluated for detection of hepatitis B virus DNA (Notomi et al. 2000) and was further developed by Eiken Chemical Co. (http://loopamp.eiken.co.jp). Nucleic acid amplification takes place with high specificity, efficiency and rapidity under isothermal conditions. It is characterized by the use of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and anti sense strands of the target DNA initiates LAMP. Strand displacement DNA synthesis is primed by an outer primer releasing single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA and a new
stem–loop DNA with a stem twice as long (Notomi et al. 2000; Tomita et al. 2008) (Fig. 2). Amplification and detection of genes can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). It provides high amplification efficiency, with DNA being amplified $10^9$–$10^{10}$ times in less than an hour. The amplification products can be easily detected by visual assessment of turbidity, electrophoresis or by the naked eye.

**RCA**

Rolling circle amplification (RCA) is a sensitive, specific and reproducible isothermal DNA amplification technique for rapid molecular identification of microorganisms, the process discovered in the mid 1990s (Fire & Xu 1995; Liu 1996). RCA-based diagnostics are characterized by good reproducibility, with less amplification errors compared to PCR.
Fig. 2. Primer design of the LAMP reaction. For ease of explanation, six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c and B3 from the 5’ end. As c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence. (b) Starting structure producing step. DNA synthesis initiated from FIP proceeds as follows. The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 3’ end (structure 3). DNA synthesis proceeds with the single-strand DNA as the template, and BIP and B3 primer, in the same manner as described earlier, to generate structure 5, which possesses the loop structure at both ends (dumbbell-like structure). (c) Cycling amplification step. Using self-structure as the template, self-primed DNA synthesis is initiated from the 3’ end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5, and structure 5 is produced from structure 8 in a reaction similar to that which led from structures 5–7. Structures 9 and 10 are produced from structures 6 and 8, respectively, and more elongated structures (11, 12) are also produced. (Nature Protocols 3: 877–882, 2008).
Chapter 1

Fig. 3. (A) Typical design of a circularizable padlock probe as exemplified by the Fonsecaea pedrosoi specific (FOP) probe. The probe comprises (i) a 5’-phosphorylated end, (ii) a “backbone” containing binding sites for the RCA primers (RCA primers 1 and 2, respectively; designated by bold uppercase letters) as well as the nonspecific linker regions (designated by bold lowercase letters), and (iii) a 3’ end. The 5’ and 3’ ends of the probe are complementary to the 5’ and 3’ termini of the target sequence in reverse, in this example to the F. pedrosoi sequence. Abbreviations: 5’-P, 5’-phosphorylated binding arm; 3’, 3’ binding arm. (B) Pictorial representation of the RCA method. Step 1, hybridization. Hybridization of padlock probe, containing target-complementary segments, to a target DNA sequence. Step 2, ligation. The probe is circularized by DNA ligase. Step 3, RCA and primer extension I. Ligated probe and binding of RCA primer 1 for RCA. Tandem repeat sequences complementary to the circular probe are generated by RCA. The reverse primer (RCA primer 2) binds to each tandem repeat generated by the rolling circle. Step 4, RCA and primer extension II. As the original RCA strand elongates, further priming events are initiated by primer 2, generating displaced DNA strands. As a result, new priming sites for the first primer (primer 1) are generated. The two primers thus function to generate a self-propagating pattern of DNA fragment release events. Step 5, detection of amplified product. RCA may be monitored using real-time PCR or agarose gel electrophoresis. ssDNA, single-stranded DNA (Zhou et al. 2008; Najafzadeh et al. 2011).
(Demidov 2002). The method uses a padlock probe, a circularizable oligonucleotide consisting of two segments complementary to the 3’ and 5’ ends of the target and a linker sequence (Nilsson et al. 1994). When the 3’ and 5’ terminal regions of the oligonucleotide probes are juxtaposed to the sequence of interest, the probe ends can be joined by a DNA ligase to form a circular DNA molecule that can be amplified by RCA (Fig. 3).

### Antifungal susceptibility testing

Much has been written on the emerging incidence of fungal infections during the last decades related to the growing number of patients at risk, such as persons with AIDS, recipients of solid organ or hematopoietic stem cell transplants, persons with hematological malignancies, and other individuals receiving immunosuppressive treatment. Chromoblastomycosis, however, is a disease that occurs in otherwise healthy individuals, and its incidence is related to social conditions of the humans populating endemic area, for example profession, hygiene, and access to medical care. Therefore its incidence is not likely to increase. However, the disease is regionally very common, and difficult to treat due to the therapy-refractory nature of the invasive form, the muriform cell, and the frequent relapses. With growing economic standards a mutilating disease as chromoblastomycosis will no longer be accepted, and we thus have to develop timely, effective and low-cost therapy. With orphan diseases like chromoblastomycosis there is still a long way to go. The problem may be aggravated by the advent of resistance to antifungal agents. Determination of an efficient strategy for treatment of the disease is an important issue in clinical mycology (Clark & Hajjeh 2002; Najafzadeh et al. 2009).

Therapy for chromoblastomycosis is challenging because there is no consensus regarding the treatment of choice. Several treatment options have been applied, but these tend to result in protracted disease, low cure rates, and frequent relapses (Bonifaz et al. 2004; Esterre & Queiroz-Telles 2006; Garnica et al. 2009; Queiroz-Telles et al. 2009). The therapeutic outcomes are variable and are allegedly dependent on the site of infection, lesion size, and the patient’s general condition (Bonifaz et al. 2001), and perhaps also on the etiological agent. In order to improve antifungal therapy, we first need to acquire more information on the in vitro antifungal susceptibility of *Fonsecaea* species against various antifungal agents. These are determined according to the Clinical and Laboratory Standards Institute (CLSI) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi (CLSI 2008). In this method, isolates are cultured on potato dextrose agar (35°C) for up to 7 days, and inocula are prepared by gently scraping the surface of the fungal colonies with sterile cotton swab moistened with sterile physiological saline containing 0.05% Tween 40. Large particles in the cell suspensions were allowed to settle for 3 to 5 min at room temperature, and then the concentration of spores in the supernatant is adjusted spectrophotometrically at 530 nm to a percent transmission in the range 68 to 71, corresponding to $1.5 \times 10^4$ to $4 \times 10^4$ CFU/ml, as controlled by quantitative colony counts on SGA. Antifungal drugs are obtained as reagent-grade powders and dissolved as prescribed by CLSI; stock solutions are prepared in DMSO.
or water. Antimycotics are diluted in RPMI 1640 medium, buffered to pH 7.0 with 0.165 morpholinepropanesulfonic acid and dispensed into 96-well microdilution trays at different concentration ranges (dependent on the drugs used) and stored at –70°C prior to use. After inoculation of conidial suspensions to microdilution trays, they are incubated at 35°C for 72 h. Minimum inhibitory concentrations (MICs) are determined visually by comparison of the growth in the wells containing the drug with the drug-free control. Minimum effective concentrations (MECs) are determined microscopically as the lowest concentration of drug promoting the growth of small, round, compact hyphae relative to the appearance of the filamentous forms seen in the control wells.

**Aim of the thesis**

The research presented in this thesis provides taxonomic, morphological and ecological aspects of the genus *Fonsecaea*. A multilocus DNA sequence data set is established to study the biodiversity and phylogenetic relationship among the *Fonsecaea* species. A first aim is to redefine taxonomic entities on the basis of molecular data and compare these with classical taxonomy.

With newly defined entities, the molecular epidemiology of *Fonsecaea* species is to be analyzed by AFLP. In addition we aim to develop a sensitive, specific and rapid method for the detection of members of the genus *Fonsecaea*, in the laboratory as well as on non-sterile adhesive tape widely used for environmental screening. We also describe a rapid and sensitive assay for identification of *Fonsecaea* species without sequencing using rolling circle amplification (RCA) by designing three specific padlock probes using informative nucleotide polymorphisms in the *Fonsecaea* ITS database. Additionally we provide the data for appropriate antifungal therapy of chromoblastomycosis that is caused by *Fonsecaea*. These data will help defining treatment recommendations and establishing guidelines for antifungal therapy. Because of the presence of therapy-refractory muriform cells and differential susceptibilities between taxonomically closely related groups, cure rate is low and there is frequent relapse.

In Chapter 9 we report an example with a case of chromoblastomycosis that relapsed after treatment with itraconazole for 4 months.

**References**


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