Presumptive synchronized nuclear divisions without cytokinesis in the \textit{Rhinosporidium seeberi} parasitic life cycle

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Despite numerous studies of the \textit{Rhinosporidium seeberi} parasitic phase, the stages of its nuclear cycle leading to the formation of endoconidia have yet to be properly described. \textit{R. seeberi} resists culture and can only be investigated on histological preparations. We have evaluated tissue sections collected from 35 host species with rhinosporidiosis searching for the presence of mitotic figures during sporangia development. This study found that soon after endoconidia release, the prominent reddish vesicles typical of this stage vanished leading to the development of juvenile sporangia (JS) \(12–70 \mu\text{m}\) in diameter. This stage possesses granular cytoplasm, a thick cell wall, and a central reddish nucleus with a conspicuous nucleolus. The first nuclear division takes place in the JS. It is a rarely encountered event characterized by the development of a distorted nucleus leading to the formation of two nuclei without cytokinesis. The finding of multiple nuclear divisions at prophase-, metaphase- and telophase-like stages without cytokinesis was detected in intermediate sporangia (IS). IS with multiple dividing nuclei seem to be at the same stage of nuclear partitioning, suggesting synchronized nuclear division. In these sporangia, the nuclei continue divisions without cytokinesis until the sporangia reach \(\geq 300 \mu\text{m}\) in diameter. The last nuclear division prior to cytokinesis appears to take place in very large sporangia with thousands of nuclei. The build-up of cytoplasm around each nucleus and the formation of a thin cell wall lead to the formation of endoconidia. This study revealed the presence of several mechanisms of pathogenesis in \textit{R. seeberi} that deserved further investigation.

\section*{INTRODUCTION}

\textit{Rhinosporidium seeberi} is the aetiological agent of rhinosporidiosis, a disease affecting the mucous membranes of mammals (including humans) (Arseculeratne, 2002; Ashworth, 1924; Easley \textit{et al.}, 1986; Kurunaratne, 1964; Thianprasit & Thagernpol, 1989) and birds (Kennedy \textit{et al.}, 1989). In these species \textit{R. seeberi} has been found to cause infection of the eyes, nose, oral cavity and genitalia. Unusual cases of rhinosporidiosis of the skin and other tissues have been sporadically reported (Arseculeratne, 2002; Thianprasit & Thagernpol, 1989). Although some investigators had claimed the isolation in culture of \textit{R. seeberi} (Arseculeratne, 2002; Vilela & Mendoza, 2012), it is now clear that this pathogen resists culture and its infection cannot be reproduced in experimental animals; thus its ecological niche still elusive.

Since the first report of the infection in 1900 (Seeber, 1900), few studies have addressed the behaviour of the nuclei of \textit{R. seeberi} during its complex parasitic life cycle (Acevedo, 1958; Ashworth, 1924; Kurunaratne, 1964). Ashworth (1924) was the first to draw attention to the stages of the \textit{R. seeberi} nuclear cycle. Similar observations taking place during prophase, metaphase, anaphase and telophase were also published by Acevedo (1958) and six years later by Kurunaratne (1964). Sadly, subsequent studies focused mostly on aspects other than the \textit{R. seeberi} nuclear cycle, so these observations were soon forgotten (Vilela & Mendoza, 2012). We have had the opportunity to study the presence of \textit{R. seeberi} nuclei at different stages of its life cycle in 35 tissue sections of different hosts with rhinosporidiosis. Our study showed that juvenile (JS), intermediate (IS) and mature sporangia (MS) tend to
display stages of the nuclear cycle consistent with morphological features of eukaryotic microbes during prophase, metaphase, anaphase and telophase, but without cytokinesis.

**METHODS**

**Hosts with rhinosporidiosis, tissue sample collection and staining.** Tissue samples from 35 hosts with rhinosporidiosis were obtained by request from several physicians (Table 1). The submitted materials were formalin-fixed paraffin-embedded tissue samples. In a few of the cases, the samples were acquired as fresh clinical material (Table 1). Before microscopic evaluation, the embedded tissue samples in paraffin blocks were sectioned into 5 μm slides and then stained with haematoxylin and eosin (HE) following standard protocols (Fischer et al., 2006). One or more slide sections were evaluated in each of the 35 hosts with the disease.

**Evaluation of microscopic nuclear features in tissue sections of *R. seeberi*.** To identify the *in situ* *R. seeberi* sporangia displaying the presence of putative mitotic figures, we have adopted the ontologic nomenclature proposed by Kennedy et al. (1995) and recently updated by Vilela & Mendoza (2012). In brief, spherical structures between 4 and 10 μm in diameter containing multiple reddish vesicles inside or outside MS were named endoconidia; 10–70 μm in diameter spherical structures without a nucleus or with a single central nucleus and a thick cell wall were referred to as juvenile sporangia (JS); 70–290 μm structures were termed intermediate sporangia (IS); and the ≥300 μm spherical structures containing well-developed endoconidia, MS. The identification of each of these structures in the infected hosts was based on the proposed sizes.

**Strategy to screen the tissue sections.** The main strategy of this study was to first microscopically screen the whole HE-stained tissue section under investigation to locate the presence of endoconidia, JS, IS and MS using low magnification (×10–×20). Once the structures of interest were properly located, higher magnification (×40–×100) was used. High magnification was essential to monitor the presence of putative mitotic figures similar to that reported by Acevedo (1958), Ashworth (1924) and Kurunaratne (1964).

**Transmission electron microscopy (EM) studies.** Within the 35 host tissue samples, two specimens, also studied by Herr et al. (1999a, b) and Silva et al. (2005), containing *R. seeberi* mitotic structures were also processed for EM. In brief, the samples were fixed in 2.5 % glutaraldehyde with 0.05 M sodium cacodylate-buffered saline (pH 7.4) at room temperature for 2 h, followed by three washes with the same reagent for 20 min each. The samples were then treated with 1 % OsO4 at room temperature for 4 h, followed by three 20 min distilled water washes and dehydration in acetone. The samples were transferred to 33 % and 66 % Spurr resin in acetone solution for 30 min at each concentration. The samples were then treated with 100 % Spurr resin and incubated overnight. An ultramicrotome was used to obtain ultrathin sections of the samples (~100 nm slices). The sections were placed onto grids and then post-stained with uranyl acetate for 30 min. A thin layer of carbon was evaporated onto the surface of the post-stained grids and the samples were examined with a Philips CM-10 electron microscope.

**RESULTS**

**Frequency of mitotic figures in the studied samples with rhinosporidiosis**

Of the 35 evaluated specimens with rhinosporidiosis, only 11 consistently displayed mitotic figures (four human, five swan, one dog and one horse) in their tissue sections. Although the typical spherical phenotypes at different stages of the *R. seeberi* life cycle were observed in the remaining 24 samples, the presence of a nucleus was only detected in 10 of them, mainly in the JS stage. The remaining samples (*n*=14) displayed spherical phenotypes at different stages of development, but no nuclear structures could be found within their granular cytoplasm (data not shown). Although little information was available on the processing protocols followed after collection for some of the samples devoid of nuclei or mitotic figures, the data available showed that some of them were formalin-fixed several hours after sample collection. In contrast, tissue samples consistently displaying nuclei and mitotic figures (*n*=11) were all formalin-fixed immediately or within the first hour after collection.

**Microscopic evidence of the first nuclear divisions in *R. seeberi***

In HE, the mature endoconidia (4–10 μm), inside or outside MS, were characterized by the presence of a thin cell wall and numerous 0.5–2.0 μm, prominent, reddish vesicles within each endoconidium (Fig. 1a, b). The reddish vesicles within the endoconidia were devoid of internal structures (Fig. 1). In EM, these vesicles appeared as electron-dense dark vesicles (Fig. 2a). The presence of a nucleus inside endoconidia was very difficult to locate in

**Table 1. Studied tissue samples infected with *R. seeberi*: their origin, hosts and other features**

<table>
<thead>
<tr>
<th>Host</th>
<th>Tissue sample</th>
<th>Fixed</th>
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<th>Number</th>
<th>Location</th>
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<tr>
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<tr>
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<td>Yes (1)</td>
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<td>No</td>
<td>2</td>
<td>USA</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td>Yes</td>
<td>No</td>
<td>3</td>
<td>India, Sri Lanka, Venezuela</td>
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<tr>
<td></td>
<td>Mouth</td>
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<td>Yes (1)</td>
<td>5</td>
<td>India (4), Sri Lanka (1)</td>
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<td>Yes (1)</td>
<td>6</td>
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<tr>
<td></td>
<td>Eye</td>
<td>Yes</td>
<td>Yes (5)</td>
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HE preparations. However, in EM, a single nucleus 2–4 μm in diameter was found in some of the evaluated endoconidia (Fig. 2a, arrow). The integrity of the endoconidia cells outside the sporangia was maintained at all times and did not have physical disruption in any of the studied tissue samples (Figs 1 and 2). In some sections, the presence of spherical endoconidia-size structures (8–12 μm) lacking the reddish vesicles, all containing granular cytoplasm and a thin cell wall, was a common finding (Fig. 1c, arrows). The actual size of these structures was verified by serial sectioning (data not shown).

After the endoconidia, which appeared in great numbers in some sections (Fig. 1a–c), the most common phenotype was the 12–70 μm in diameter JS. These structures were characterized by their size; the presence of granulated cytoplasm; a thick cell wall; and a single, 2–4 μm in diameter, reddish nucleus with a prominent nucleolus, both surrounded by a delicate nuclear membrane (Fig. 3a). In EM, the JS displayed the same characteristics as those in histological preparations. The presence of a single nucleus containing a nucleolus, both surrounded by a well-defined nuclear membrane, was typical of this stage (Fig. 2b). Tissue samples with nuclear structures always showed the presence of JS, with or without the presence of a single nucleus, and a prominent nucleolus (Figs 2b and 3a). A distorted nucleus with what appeared to be four nuclear protuberances prior to nuclear division was found in two of the 11 specimens with nuclear arrangements (Fig. 3b). This was a sporadically encountered phenotype. Equally uncommon was the finding of JS with two nuclei in late
Fig. 3. (a) A typical *R. seeberi* JS with a thick cell wall containing granular cytoplasm and a distinctive reddish nucleus with a noticeable nucleolus, a feature that contrasts with the nucleus of its infected host. Bar, 10 μm. (b–d) The first *R. seeberi* nuclear division. It starts with the distortion of the nucleus and the formation of at least four protuberances (b; bar, 10 μm). (c) JS in late telophase (bar, 12 μm). (d) The development of two nuclei in the absence of cytokinesis (bar, 10 μm). (e) IS at prophase (top left; bar, 15 μm) comparable to that in (b) and in Fig. 4(b), metaphase (top right; bar, 15 μm), telophase (bottom left; bar, 15 μm) and several nuclei at resting stage similar to that in Fig. 4(a) (bottom right; bar, 15 μm). (f) Prior to cell-wall development, the evidence of cytoplasm accumulation as a reddish pattern around each nuclei. The lower right section depicts MS with numerous cell-walled endoconidia. Bar, X μm.

telophase (Fig. 3c). Also, some small JS structures (15–30 μm) showed evidence of the first nuclear division, whereas larger (~50 μm) JS displayed a single nucleus but without evidence of nuclear partitioning. After the first nuclear division without cytokinesis, the formation of two well-defined nuclei, both having a distinct nucleolus, was found in a few of the investigated JS displaying stages of the *R. seeberi* nuclear cycle (Fig. 3d).

Evidence of synchronized nuclear divisions without cytokinesis in IS

According to the arrangements of the *R. seeberi* phenotypic cell cycle, after the formation of two nuclei the JS seems to increase in size and gain an increased number of nuclei with prominent nucleoli, becoming an IS. The presence of IS with numerous nuclei showing mitotic figures at different stages of development was detected in the 11 tissue sections displaying different stages of the *R. seeberi* nuclear cycle (Fig. 3e). One particular difference between JS and IS at this transitional stage was the presence in the IS of a prominent translucent inner layer in closer association to the sporangium cell wall (Figs 3e, f and 4a–d). In this phenotype, the presence of multiple distorted nuclei with what appeared to be four protuberances was also found (Fig. 4b). Based on the morphological features of this phenotype, the lower sketch in Fig. 4b describes the interpretation of the nuclear arrangement of *R. seeberi* at prophase stage. The number of chromosomes in the sketch is not accurate and was used only to illustrate the event. The distorted nuclei was a rarely encountered phenotype. The morphological feature of the distorted nucleus with four protuberances in JS at prophase (Fig. 3b) was identical to those found in the IS phenotype with multiple nuclei at prophase stage with distorted nuclei (Fig. 3e, top left; Fig. 4b, circle). As the prophase IS nuclei evolved, a short linear condensation of nuclear material in metaphase was found in some IS (Fig. 3e, top right; Fig. 4c, circle). The sketch in the lower section of Fig. 4c depicts the interpretation of the *R. seeberi* nuclei at this stage. The separation of nuclear material in anaphase is shown in Fig. 4d (oval). The nuclear material at this stage showed numerous filaments irradiating from two well-condensed dark spots (Fig. 4d, oval). The nuclear cycle stage depicted below Fig. 4d illustrates the *R. seeberi* chromosome condensation. Despite the low resolution of the HE staining it can be noted that the other condensate nuclei in Fig. 4d seem to be at a similar partitioning stage.

Few IS displaying what appears to be telophase patterns were found (Fig. 3e, bottom left). Nuclear division in telophase was characterized by the presence of an early nuclear membrane around each nuclear matter and the presence of nucleoli (Fig. 3e, bottom left). This event increased the number of nuclei within the IS (Fig. 3e, right bottom; Fig. 4a, circle), and the cycle of nuclear divisions without cytokinesis continued. Fig. 4(a) (circle) and the sketch below both depict the typical *R. seeberi* nucleus at a resting stage. Even though the HE staining has low resolution, the inspected nuclei appeared to be at the same resting stage without cytokinesis. The EM data (Fig. 2c) showed the presence of multiple nuclei in an IS structure, reinforcing the histological findings of Fig. 4(a).

Last synchronized nuclear division

As the number of nuclei continued rising, the IS also increased in size. At this stage, some sporangia can reach ≥300 μm in diameter (Fig. 3f). Prior to the last nuclear division the IS appeared spherical, containing multiple nuclei with nucleoli. The build-up of cytoplasmic-like material around each nucleus appears as a reddish
coloration (Fig. 3f). Large IS with thousands of nuclei were observed and the presence of a cell wall around each nuclei was found in several MS (Fig. 3f, lower right). The arrangement of small immature endoconidia at the opposite side of the pore and large immature endoconidia at the centre and near the pore was the main feature of the inspected MS.

**DISCUSSION**

Although the early photographic documentation published in the first part of the 20th century suggested that *R. seeberi* was a typical eukaryotic microbe with complex stages of its nuclear cycle (Acevedo, 1958; Ashworth, 1924; Kurunaratne, 1964), these observations were conveniently overlooked by the studies of the last 40 years (Apple, 1983; Arseculeratne, 2002; Bader & Grueber, 1970; Easley et al., 1986; Herr et al., 1999b; Kannan–Kutty & Teh, 1974, 1975; Kennedy et al., 1995; Savino & Margo, 1983; Silva et al., 2005; Teh, 1974; Teh & Kannan-Kutty, 1975; Thianprasit & Thagarngpol, 1989; Vanbreuseghem, 1973). Ashworth (1924) described for the first time in *R. seeberi* the stages of the nuclear cycle during prophase, metaphase, anaphase and telophase. In his studies, he showed on page 343, plate I, figures 3–8, drawings of JS with a single nucleus, the condensation of the nuclear material, interpreted by him as the presence of four ‘chromosomes’ (figures 9 and 10 of Ashworth, 1924), and the first nuclear division (figures 14 and 15 of Ashworth, 1924). He showed also in figures 16 and 17, drawings of IS containing numerous nuclei in different stages of nuclear partitioning without cell walls. Later, Acevedo (1958) and Kurunaratne (1964) presented photographic documentation of four nuclear protuberances in prophase and other mitotic nuclear stages confirming the observations of Ashworth (1924). In our study, we were able to corroborate the presence of identical phenotypic nuclear arrangements as those described by these investigators. Ashworth (1924) and Kurunaratne (1964) believed that the four nuclear protuberances represented the condensation of four chromosomes and we tend to agree with this notion. In our study, the formation of four nuclear swellings was found in only three histological sections suggesting that this is a transient event. The JS consistently showed a single nucleus with a prominent nucleolus and was one of the most common phenotypes (Figs 2b and 3a), a finding also reported by many (Apple, 1983; Arseculeratne, 2002; Kennedy et al., 1995; Kurunaratne, 1964; Savino & Margo, 1983; Teh, 1975; Thianprasit & Thagarngpol, 1989). A prominent nucleolus could indicate that during the development of endoconidia, the production of large quantities of rRNA is required to cope with the production of key nuclear proteins required to maintain multiple nuclear divisions without cytokinesis.

Our data also suggest that *R. seeberi* sporangia possess sophisticated cell cycle machinery that simultaneously and efficiently coordinates the timing of nuclear division in each of the newly formed nuclei. They also indicate that during evolution *R. seeberi* may have acquired genes that allow the formation of multiple nuclei without the hassle of cytokinesis, which could facilitate the formation of thousands of cells in a short period of time. We believe that the strategy is used by this unique pathogen to overwhelm the cellular and humoral immunity of the infected hosts ensuring the survival of at least some of its progeny. This finding also agrees with the hypothesis of...
Mendoza et al. (1999) that the MS acts as a single mother cell that actively participates in an organized manner in the process of nuclei formation and endoconidia release. Despite the low resolution of the HE staining, the evidence collected suggests multiple synchronized nuclear divisions in the studied sporangia (Figs 3 and 4). Recently, Suga & Ruiz-Trillo (2013) reported the occurrence of synchronized nuclear division without cytokinesis in the mesomyzclozoan (ichthyosporean) 19. This finding suggests that the development of metazoan multicellularity may have some evolutionary links with the mesomyzclozoans, including R. seeberi, a discovery that further validates our study.

First, Minchin & Fantham (1905) and then Beattie (1906) introduced the concept of spore-morulae. These authors postulated that the reddish vesicles of R. seeberi (also designated EDB) each possessed a nucleus. They suggested that the endoconidium cell wall is then disrupted and the EDB are released, becoming new infecting cells. Both investigators showed drawings of R. seeberi endoconidia containing several vesicles with nuclei, but failed to provide photographic documentation to support their claims. Based on numerous microscopic and EM studies (Easley et al., 1986; Teh, 1975; Vanbreuseghem, 1973), however, these vesicles are electron-dense formations devoid of internal structures, a concept also supported by the findings in this study (Figs 1 a, b and 2a). Although some investigators using staining protocols recorded the presence of nucleic acids in the EDB (Arseculeratne, 2002) the finding of a true nucleus inside these structures has not yet been documented (Kannan-Kutty & Teh, 1974, 1975, Teh, 1974; Vanbreuseghem, 1973; Vilela & Mendoza, 2012). Conversely, some histochemical analyses found that these vesicles contain lipidic and proteinaceous elements (Arseculeratne, 2002; Bader & Gruber, 1970; Vanbreuseghem, 1973), a finding that agrees with the position of Ashworth (1924) and Easley et al. (1986). Furthermore, our data and other studies showed that the nuclei of R. seeberi are ~2–4 μm in diameter (Easley et al., 1986; Thianprasis & Thagernpol, 1989; Kennedy et al., 1995; Pereira et al., 2005; Savino & Margo, 1983; Vanbreuseghem, 1973; Vilela & Mendoza, 2012), thus the presence of a nucleus inside a 0.5–3.0 μm-diameter vesicle is highly questionable. Besides, there is not a single example of a eukaryotic pathogen releasing their nucleated progeny without a protective cell wall.

According to our observations and those recorded by others (Ashworth, 1924; Kannan-Kutty & Teh, 1974, 1975; Kennedy et al., 1995; Mendoza et al., 2002; Teh & Kannan-Kutty, 1975; Thianprais & Thagernpol, 1989; Vanbreuseghem, 1973), each endoconidium possesses a single nucleus and maintains its cell wall integrity at all times (Pereira et al., 2005; Vilela & Mendoza, 2012), a concept that is in sharp contrast with the spore-morulae hypothesis. Conversely, Kannan-Kutty & Teh (1975) believe that the concentric 0.5–1.5 μm diameter ring structures, also termed laminated bodies (LB) and frequently encountered in EM preparations, are involved in the development of endoconidia. However, the presence of LB within nucleated endoconidia has also been recorded (Easley et al., 1986). Thus, the connection of the LB with the formation of endoconidia is doubtful. Furthermore, our study did not find such structures in any of the examined histological preparations at high magnification in HE. These structures had been found only in EM within the cytoplasmic content of several IS. Our data support the concept proposed by some that the reddish vesicles are nutritional structures used to nourish the endoconidium leading to the formation of JS (Ashworth, 1924; Easley et al., 1986). The fact that cases of rhinosporidiosis have been found in extremely dry areas of the Middle East after sand storms also suggests that the R. seeberi endoconidia can survive long periods of time in these types of environments (Arseculeratne, 2002; Thianprais & Thagernpol, 1989). Thus, it is possible that the reddish vesicles could provide the JS with the nutritional elements not only to continue its parasitic life cycle in infected hosts, but perhaps also to provide the elements needed for the transition from endoconidia to resistant spores in nature.

Recently Vilela & Mendoza (2012) mentioned the work done by the early investigators on the nuclear activities in R. seeberi. We now provide photographic evidence that R. seeberi endoconidia are formed in JS, IS and MS through repeated mitotic nuclear divisions, but without cytokinesis. We also confirmed that the last nuclear division more likely takes place in large MS after the formation of a cell wall around each nucleus. The data in this study imply that the life cycle of R. seeberi is unique and thus previous hypotheses on the subject are no longer suitable to explain the life cycle of this mesomyzclozoa. The occurrence of a putative synchronized nuclear division without cytokinesis in R. seeberi opens the possibility for the investigation of novel drugs targeting pathways involved in this type of nuclear partitioning. This could be of extreme importance since proteins involved in multiple fission pathways could well be absent in the genome of the infected hosts with rhinosporidiosis, making them ideal targets.

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