

## Phylogenetic analysis identifies the 'megabacterium' of birds as a novel anamorphic ascomycetous yeast, *Macrorhabdus ornithogaster* gen. nov., sp. nov.

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An organism commonly referred to as 'megabacterium' colonizes the gastric isthmus of many species of birds. It is weakly Gram-positive and periodic acid–Schiff-positive and stains with silver stains. Previous studies have shown that it has a nucleus and a cell wall similar to those seen in fungi. Calcofluor white M2R staining suggests that the cell wall contains chitin, a eukaryote-specific substance, and rRNA *in situ* hybridization demonstrates that it is a eukaryote. To characterize this organism phylogenetically, DNA was extracted from purified cells. rDNA was readily amplified by PCR with pan-fungal DNA primer sets and primer sets derived from the newly determined sequence, but not with bacteria-specific primer sets. Specific primer sets amplified rDNA from isthmus scrapings from an infected bird, but not from a non-infected bird or other control DNA. The sequence was confirmed to derive from the purified organism by *in situ* rRNA hybridization using a specific probe. Phylogenetic analysis of sequences of the 18S rDNA and domain D1/D2 of 26S rDNA showed the organism to be a previously undescribed anamorphic ascomycetous yeast representing a new genus. The name *Macrorhabdus ornithogaster* gen. nov., sp. nov. is proposed for this organism. The type material is CBS 9251<sup>T</sup> (= NRRL Y-27487<sup>T</sup>).

An organism commonly referred to as 'megabacterium' infects domestic birds (Huchzermeyer *et al.*, 1993; Mutlu *et al.*, 1997; Wieliczko & Kuczkowski, 2000; Schulze & Heidrich, 2001) as well as wild (Filippich *et al.*, 1993; Pennycott *et al.*, 1998) and companion birds (Dorrestein *et al.*, 1980; Baker, 1992; Gerlach, 2001). These long, slender organisms (2–3 × 20–80 µm) stain with silver stains and periodic acid–Schiff (PAS) and are weakly Gram-positive. The 'megabacterium' is found in the isthmus between the glandular and grinding stomach, where it grows on the luminal surface and may penetrate koilin (Dorrestein *et al.*, 1980; van Herck *et al.*, 1984). It is associated with a lymphoplasmacytic gastritis in poultry (Mutlu *et al.*, 1997)

and a chronic fatal wasting disease in companion birds (Gerlach, 2001).

The organism was originally thought to be a yeast because of its staining characteristics (Dorrestein *et al.*, 1980). Subsequently, van Herck *et al.* (1984) concluded that it was a bacterium, as they were unable to demonstrate cytoplasmic organelles or a nucleus. They did, however, show nucleus-like structures in Geimsa-stained organisms, but interpreted them to be 'granules'. Scanlan & Graham (1990) reported isolating a bacterium from the stomach of a budgerigar using standard microbiological techniques. The isolated bacterium, however, was smaller than the organism observed *in vivo* and was not characterized by PAS or silver stains. Attempts by other investigators to grow this organism with standard microbiological isolation techniques have been unsuccessful. However, Gerlach (2001) reported isolation of this organism on MRS medium, but was unable to maintain it past a few passages. Huchzermeyer *et al.* (1993) also reported isolating an organism from the proventriculus of ostriches using MRS agar. This organism had the same biochemical properties as the one isolated by

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**Abbreviations:** D1/D2, domains 1 and 2; PAS, periodic acid–Schiff; PNA, peptide nucleic acid.

The GenBank/EMBL/DDBJ accession number for the partial 18S, ITS1, 5-8S, ITS2 and partial 26S rDNA sequence of *Macrorhabdus ornithogaster* is AF350243.

Scanlan & Graham (1990), but was smaller than those seen histologically, and its ability to stain with PAS and silver stains was not reported.

More recent work suggested that the 'megabacterium' is, in fact, a yeast. *In vivo* trials showed that the 'megabacterium' was susceptible to amphotericin B, but not to antibacterial antibiotics (Filippich & Perry, 1993). It stained strongly with calcofluor white M2R (Moore *et al.*, 2001) and blanchophor BA (Ravelhofer *et al.*, 1998), stains that bind chitin, a polysaccharide not found in bacteria (Monheit *et al.*, 1984). A nucleus was demonstrated by electron microscopy and *in situ* hybridization with a pan-eukaryote rRNA probe was positive (Ravelhofer-Rotheneder *et al.*, 2000).

In this study, budgerigars naturally infected with the 'megabacterium' were killed humanely and longitudinal strips of the stomach containing the distal proventriculus, isthmus and proximal ventriculus were fixed in buffered formalin and paraffin-embedded. Thin sections were stained with haematoxylin and eosin, PAS, methenamine silver, Brown and Brenn and calcofluor white M2R (Monheit *et al.*, 1984). Organisms obtained from scrapings of the remaining gastric isthmus were heat- or methanol-fixed and stained with PAS, methenamine silver, Gram stain and calcofluor white MR2. Additional organisms were acid-digested and Giemsa-stained.

Aliquots of the organisms were washed several times in sterile PBS and purified by layering onto a 10–40% (w/v) sucrose gradient which was centrifuged for 1 h at 14 600 g. The pellet was washed and suspended in PBS. Wet mounts of this suspension revealed no other organisms or debris. Genomic DNA was isolated from the suspension using mechanical disruption and a Puregene DNA isolation kit. Amplification and sequencing of ITS and 26S fungal ribosomal genes was done using previously described pan-fungal primers (White *et al.*, 1990; Sandhu *et al.*, 1995). New primers were designed to amplify and sequence the 18S rDNA. To this end, the sequence of *Saccharomyces cerevisiae* 18S rDNA (GenBank accession no. J01353) was compared with that of other yeasts. Primers were then selected from conserved sequences found to be present in most or all yeasts compared. The forward primers were Sm1 (ATCTGGTTGATCCTGCCAGTAGTC; positions 2–25), BIG 1 (AGTGAACTGCGAATGGCTC; 80–99), Sm3 (CTGAGAAACGGCTACCACATCC; 394–415) and Sm5 (AACTACTGCGAAAGCATTGGCC; 928–949). The reverse primers were Sm2 (CAATACGCCTGCTTTGAACACTC; 761–783), Sm4 (CTTCGATCCCCTAACTTTCGTTC; 971–993) and Sm6 (CACCTACGGAAACCTTGTTACGAC; 1756–1778). All primers are written in the 5'–3' direction. Positions refer to the *S. cerevisiae* rDNA sequence. Using these primers, DNA was routinely amplified by PCR, purified by gel electrophoresis and sequenced (Tomaszewski *et al.*, 2001). A 3004 bp sequence was obtained that contained the majority of the 18S, the entire ITS and the 5' portion of the 26S rDNA.

To verify that the amplified sequence was from a single organism, primers BIG 1 and Sm6 and ITS 5 (White *et al.*, 1990) and U2 (Sandhu *et al.*, 1995) were used to produce two large overlapping amplicons. A sequence from the 18S rDNA of the organism but not found in any reported fungal sequence was included in the overlap. The two amplicons were inserted into vectors and cloned in competent cells. Plasmid inserts from selected recombinant colonies were sequenced with universal primers T7pl, M13 and the newly developed primers.

A PCR primer (AGY1) (GGACTTATATTACTAGTCAG-ATGG; positions 620–643 in the rDNA of the organism) that did not match reported sequences of other known fungi was developed from the 18S rDNA. It was used in combination with Sm2 to show the specificity of the determined sequence. DNA extracted from scrapings of the isthmus of a naturally infected budgerigar, a budgerigar without infection, *Candida albicans* and a *Lactobacillus* sp. was subjected to PCR with these primers. Bacteria-specific PCR primers (Relman, 1993) were also used in a PCR with DNA from the purified organism to show that it was not a bacterium. DNA from *Lactobacillus* sp. was used as a positive control.

*In situ* hybridization with a commercially synthesized peptide nucleic acid (PNA) probe (AATTGAACCAGG-ACG; positions 704–718 in the rDNA of the organism) (Applied Biosystems) that targeted an rRNA sequence not found in other previously reported sequences of fungi was performed as described by Oliveira *et al.* (2001) with minor modifications. Isthmus mucosa scrapings collected from infected budgerigars were mixed with *C. albicans* and placed in wells of Teflon-coated slides and heat-fixed at 80 °C for 1 h. Wells were then covered with approximately 20 µl hybridization solution containing 10% (w/v) dextran sulfate, 10 mM NaCl, 20% formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) Ficoll, 5 nM disodium EDTA, 1% (v/v) Triton X-100, 50 mM Tris/HCl and 1 pM PNA probe, overlaid with a cover slip and incubated for 30 min at 55 °C. The cover slips were removed by immersion in PBS plus 0.1% Tween (PBST) for 1–2 min at room temperature. Slides were washed in preheated PBST (59–69 °C) for 30 min, rinsed with PBS and air-dried. Each smear was mounted with Slow Fade light antifade kit (Molecular Probes) and a cover slip. The stained cells were visualized with a fluorescence microscope and photographed.

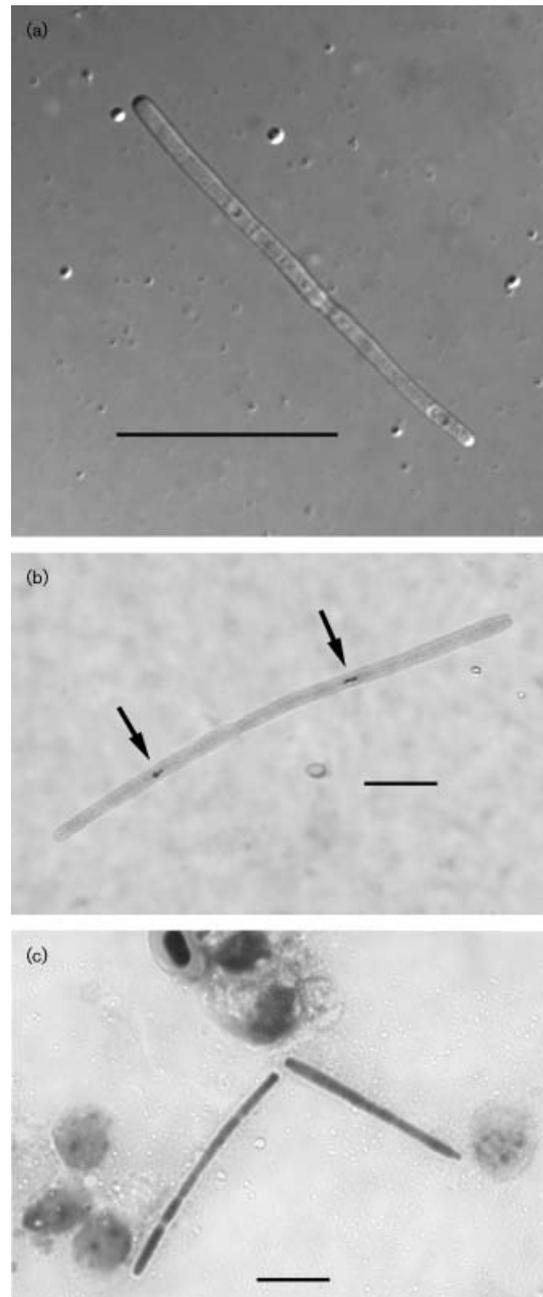
Initial placement of the bird pathogen among the ascomycetous yeasts resulted from a BLAST search of 18S rDNA sequences maintained in GenBank. Following this, phylogenetic relationships were evaluated further from comparison of domains 1 and 2 (D1/D2) of 26S rDNA with sequences from all currently known ascomycetous yeasts (Kurtzman & Robnett, 1998; and subsequent GenBank entries). The dataset was analysed by maximum parsimony as well as by neighbour joining with the Kimura two-parameter correction using PAUP 4.03a (Swofford, 1998).

Sequence data were aligned visually with Qedit 2.15 (SemWare) and regions of uncertain alignment, including indels, were removed from the dataset before analysis. The dataset comprised 487 characters, of which 219 characters were parsimony-informative.

Haematoxylin and eosin-stained sections of the isthmus demonstrated numerous densely packed, long, filamentous organisms that were identical in size (2–3  $\mu\text{m}$  wide and 20–80  $\mu\text{m}$  long), shape and staining characteristics to the so-called megabacterium. The organisms were PAS- and methenamine silver-positive but were only weakly Gram-positive. Organisms from scrapings were of the same dimensions (Fig. 1a) and staining characteristics. Gram-stained preparations showed that organisms longer than 20  $\mu\text{m}$  were, in fact, chains of organisms each separated by a transverse septum (Fig. 1c). The number of cells ranged from one to four, with two and three being most common. Small, probably new, growing cells were often seen at either end of a chain of cells. Acid-digested and Giemsa-stained organisms contained one to four evenly spaced, dense, elongate and oval basophilic structures (Fig. 1b).

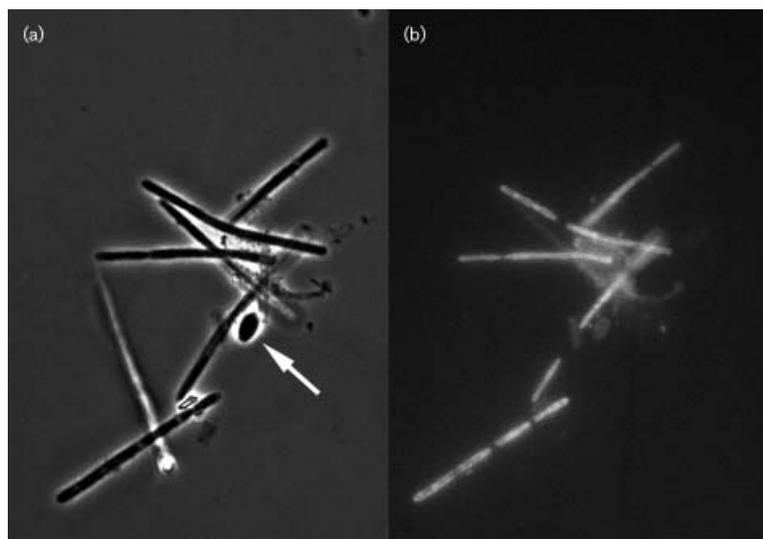
The sequence determined by sequencing the overlapping clones was identical to that obtained by sequencing the original smaller overlapping PCR products. Comparison of this sequence with those in GenBank showed that, as expected, it contained the majority of the SSU rDNA, the entire 5·8S, ITS regions and 697 bp of the 5' end of the LSU rDNA. The sequence obtained was verified to be from the organism seen histologically by PCR with specific primers and *in situ* hybridization with a specific PNA probe. The specific PCR primer set (AGY1/SM2) only amplified DNA from the isthmus scraping from the infected budgerigar and not from a non-infected budgerigar or from *C. albicans* or *Lactobacillus* DNA. The sequence of the amplified product was identical to the expected sequence. DNA from *Lactobacillus* was amplified with the bacteria-specific primer pairs, but these primers did not produce an amplicon from DNA of the organism purified from isthmus scrapings nor from *C. albicans* DNA. The organisms from isthmus scrapings hybridized with the rRNA PNA probe, but *C. albicans* did not (Fig. 2a, b).

A comparison of the 18S rDNA sequence with those in GenBank placed the budgerigar fungus well within the ascomycetous yeast clade but, because relatively few 18S sequences are available for yeasts, only broad relationships were discerned. Further analysis was made from the D1/D2 domain of 26S rDNA, because these sequences are available for all currently recognized ascomycetous yeasts (Kurtzman & Robnett, 1998; and subsequent GenBank entries). Phylogenetic trees from both analyses were concordant and strongly supported placement of the budgerigar fungus within the ascomycetous yeast clade (bootstrap value 97%), as well as showing it to be a unique species. However, neither gene sequence provides strong internal branch support and the location of the budgerigar



**Fig. 1.** Micrographs of *Macrorhabdus ornithogaster* gen. nov., sp. nov. in scrapings of the isthmus from a budgerigar: wet mount (a), acid-digested and Giemsa-stained (b) and Gram-stained (c). Structures believed to be nuclei are seen in the Giemsa-stained organism (b, arrows). Bars, 30  $\mu\text{m}$  (a) and 10  $\mu\text{m}$  (b, c).

yeast within the ascomycetous yeast clade is only approximate from these datasets. The 26S D1/D2 analysis suggests the budgerigar yeast to be weakly associated with and basal to the *Dipodascus* and *Metschnikowia* clades, as shown from its placement among representative species in Fig. 3. Further phylogenetic resolution will require analysis of multiple gene sequences.



**Fig. 2.** Bright-field (a) and PNA fluorescence *in situ* hybridization (b) images of cells of *M. ornithogaster* gen. nov., sp. nov. and *C. albicans* (arrow). The *M. ornithogaster*-specific probe for rRNA is selectively localized in the cytoplasm of *M. ornithogaster* cells.

Our rDNA sequence findings are consistent with those of previous investigators, who showed by *in situ* hybridization that the organism contained eukaryote rRNA and a nucleus (Ravelhofer-Rotheneder *et al.*, 2000). These findings are also consistent with the observations that this organism stains with both calcofluor MR2 and blanchophor BA, stains that detect chitin, a molecule that is only found in eukaryotes. Given that this organism is a yeast, we conclude that the regularly spaced 'granules', first observed by van Herck *et al.* (1984), are, in fact, nuclei.



**Fig. 3.** Phylogenetic tree with selected representative taxa showing the placement of *M. ornithogaster* gen. nov., sp. nov. near the *Dipodascus* and *Metschnikowia* clades, as represented by one of two most-parsimonious trees derived from maximum-parsimony analysis of LSU rDNA domain D1/D2. Branch lengths, given below each branch, are proportional to nucleotide differences. Numbers above nodes are percentage frequencies with which a given branch appeared in 1000 bootstrap replicates. Frequencies under 50% are not given. Tree length, 394; consistency index, 0.807; retention index, 0.424; rescaled consistency index, 0.342; homoplasy index, 0.193. Each of the species shown is represented by the type strain or an authentic strain when no living type material was available. GenBank accession numbers for reference taxa are those given by Kurtzman & Robnett (1998). *Schizosaccharomyces pombe* was the outgroup species in the analysis. T, Type strain; NT, neotype strain; A, authentic strain.

Previously, some investigators have concluded that the so-called megabacterium could be cultured from the ventriculus by using standard microbiological techniques and is, in fact, a bacterium (Scanlan & Graham, 1990). The potential pitfall associated with culturing a lesion is that an organism that grows readily under the culture conditions, but is present in relatively small numbers, can appear to be the only organism present if the common organism in the lesion does not grow under these same conditions. It is therefore incumbent on investigators to prove that the organism that they have cultured is the same one that was seen *in situ*. The descriptions of bacteria, believed to be the 'megabacterium', that have been isolated from the gastric mucosa of birds have not been rigorous. Silver, PAS and calcofluor white M2R staining were not reported. Additionally, in two instances, the isolated organism was considerably smaller than the organisms seen *in situ* (Scanlan & Graham, 1990; Huchzermeyer *et al.*, 1993). Based on our findings, we conclude that the bacteria isolated by these investigators represent either permanent or transient flora of the stomach of the birds they cultured, but are not the PAS-positive, silver-staining and calcofluor-positive organism that we have shown to be an ascomycetous yeast.

#### Latin diagnosis of *Macrorhabdus* Tomaszewski, Logan, Snowden, Kurtzman & Phalen gen. nov.

*Cellulae vegetativae elongatae, fissione divisae, singulae aut brevi catena compositae. Ascosporae non fiunt. Parasitus avium. Species typicam, Macrorhabdus ornithogaster.*

#### Description of *Macrorhabdus* Tomaszewski, Logan, Snowden, Kurtzman & Phalen gen. nov.

*Macrorhabdus* (Mac.ro.rhab'dus. Gr. masc. adj. *macro* from *macro* long; N.L. masc. n. *rhabdus* from Gr. masc. n. *rabdos* rod; N.L. n. *Macrorhabdus* long rod).

Vegetative cells are elongate, divide by fission and are single

or in short chains. Ascospores are not formed. The organism is parasitic to birds. The type species is *Macrorhabdus ornithogaster*.

**Latin diagnosis of *Macrorhabdus ornithogaster* Tomaszewski, Logan, Snowden, Kurtzman & Phalen sp. nov.**

*Cellulae vegetativae elongatae* (2–3 × 8–20 µm), *fissione divisae*. *Cellulae singulae aut brevi seri usque et cellulas quattuor catenatae*. *Ascosporae non fiunt*. *Parasitus avium*. *Typus*: NRRL Y-27487<sup>T</sup>, *designat stirpem typicam*. *Isolata a Melopsittacus undulatus*, College Station, TX, USA, *deposi-tata in collectione culturarum* ARS (NRRL), Peoria, IL, USA.

**Description of *Macrorhabdus ornithogaster* Tomaszewski, Logan, Snowden, Kurtzman & Phalen sp. nov.**

*Macrorhabdus ornithogaster* (or.ni.tho.gas'ter. Gr. gen. fem. or masc. n. *ornitho* from *ornis* bird; Gr. fem. n. *gaster* stomach; N.L. gen. n. *ornithogaster* of the stomach of a bird).

Vegetative cells are elongate (2–3 × 8–20 µm) and divide by fission. Cells are single or in short chains of two to four cells. Ascospores are not formed. The species is parasitic to birds and is found in gastric tissue. The type material was excised from an infected budgerigar in College Station, TX, USA, and consists of infected preserved tissue that is deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as CBS 9251<sup>T</sup> and the Agriculture Research Service Culture Collection (NRRL), Peoria, IL, USA as NRRL Y-27487<sup>T</sup>. This is the type specimen for the genus *Macrorhabdus*.

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**References**

Baker, J. R. (1992). Megabacteriosis in exhibition budgerigars. *Vet Rec* **131**, 12–14.

Dorrestein, G. M., Zwart, P. & Buitelaar, M. N. (1980). Problems arising from disease during the periods of breeding and rearing canaries and other aviary birds. *Tijdschr Diegeneesk* **105**, 535–543.

Filippich, L. J. & Perry, R. A. (1993). Drug trials against Megabacteria in budgerigars (*Melopsittacus undulatus*). *Aust Vet Pract* **23**, 184–189.

Filippich, L. J., Boyle, D. A., Webb, R. & Fuerst, J. A. (1993). Megabacteria in birds in Australia. *Aust Vet Pract* **23**, 71–76.

Gerlach, H. (2001). Megabacteriosis. *Semin Avian Exotic Pet Med* **10**, 12–19.

Huchzermeyer, F. W., Henton, M. M. & Keffen, R. H. (1993). High mortality associated with megabacteriosis of proventriculus and gizzard in ostrich chicks. *Vet Rec* **133**, 143–144.

Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**, 331–371.

Monheit, J. E., Cowan, D. F. & Moore, D. G. (1984). Rapid detection of fungi in tissues using calcofluor white and fluorescence microscopy. *Arch Pathol Lab Med* **108**, 616–618.

Moore, R. P., Snowden, K. F. & Phalen, D. N. (2001). A method of preventing transmission of so-called 'megabacteria' in budgerigars (*Melopsittacus undulatus*). *J Avian Med Surg* **15**, 283–287.

Mutlu, O. F., Seckin, S., Ravelhofer, K., Hildebrand, R.-A. & Grimm, F. (1997). Proventriculitis in domestic fowl (*Gallus gallus* var. dom. L., 1758) caused by megabacteria. *Tierärztl Prax* **25**, 460–462.

Oliveira, K., Haase, G., Kurtzman, C., Hyldig-Nielsen, J. J. & Stender, H. (2001). Differentiation of *Candida albicans* and *Candida dubliniensis* by fluorescent *in situ* hybridization with peptide nucleic acid probes. *J Clin Microbiol* **39**, 4138–4141.

Pennycott, T. W., Ross, H. M., McLaren, I. M., Park, A., Hopkins, G. F. & Foster, G. (1998). Causes of death of wild birds of the family Fringillidae in Britain. *Vet Rec* **143**, 155–158.

Ravelhofer, K., Rotheneder, R., Gareis, M., Suttner, R., Wolf, O., Matiello, R. & Kösters, J. (1998). Megabakteriosen bei verschiedenen Vogelspezies. In *DVG Tagung über Vogelkrankheiten*, vol. 11, pp. 95–104. Gießen: Deutsche Veterinärmedizinische Gesellschaft.

Ravelhofer-Rotheneder, K., Engelhardt, H., Wolf, O., Amann, R., Breuer, W. & Kösters, J. (2000). Taxonomic classification of "megabacteria" isolates originating from budgerigars (*Melopsittacus undulatus* Shaw, 1805). *Tierärztl Prax* **28**, 415–420.

Relman, D. A. (1993). The identification of uncultured microbial pathogens. *J Infect Dis* **168**, 1–8.

Sandhu, G. S., Kline, B. C., Stockman, L. & Roberts, G. D. (1995). Molecular probes for diagnosis of fungal infections. *J Clin Microbiol* **33**, 2913–2919.

Scanlan, C. M. & Graham, D. L. (1990). Characterization of a Gram-positive bacterium from the proventriculus of budgerigars (*Melopsittacus undulatus*). *Avian Dis* **34**, 779–786.

Schulze, C. & Heidrich, R. (2001). Megabakterien-assoziierte proventridulitis beim nutzgefügel in Brandenburg. *Dtsch Tierärztl Wochenschr* **108**, 264–266.

Swofford, D. L. (1998). PAUP\* 4.0: phylogenetic analysis using parsimony. Sunderland, MA: Sinauer Associates.

Tomaszewski, E., Wilson, V. G., Wigle, W. L. & Phalen, D. N. (2001). Detection and heterogeneity of herpesviruses causing Pacheco's disease in parrots. *J Clin Microbiol* **39**, 533–538.

van Herck, H., Duijser, T., Zwart, P., Dorrestein, G. M., Buitelaar, M. & van der Hage, M. H. (1984). A bacterial proventriculitis of canaries. *Avian Pathol* **13**, 561–572.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Philadelphia: Academic Press.

Wieliczko, A. & Kuczkowski, M. (2000). Selected issues of infectious diseases in ostrich (*Struthio camelus*). *Med Weter* **56**, 23–28.