

Geotrichum bryndzae sp. nov., a novel asexual arthroconidial yeast species related to the genus *Galactomyces*

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Ten strains of an asexual arthroconidial yeast species were isolated from Bryndza, a traditional Slovak artisanal sheep cheese, which was manufactured from raw milk during a 4-month summer production period at two Slovakian sites (the northern Ružomberok and the central-southern Tisovec areas). Sequence comparison of the D1/D2 domains of the large-subunit rRNA gene revealed that this yeast represents a novel species of the genus *Geotrichum*, which contains anamorphs of the ascogenous genus *Galactomyces*, for which the name *Geotrichum bryndzae* sp. nov. is proposed (type culture CCY 16-2-1^T = NRRL Y-48450^T = CBS 11176^T). The novel species is most closely related to *Geotrichum silvicola* NRRL Y-27641^T, although yeasts with identical or very similar sequences have been found throughout the world.

The taxonomy of the arthroconidial ascomycetous genus *Geotrichum* Link: Fr. (Hemiascomycetes) is still being developed, although these yeast-like fungi have been studied quite extensively by conventional taxonomy as well as by DNA sequence comparisons (de Hoog *et al.*, 1986; Smith *et al.*, 2000; Naumova *et al.*, 2001; Smith & Poot, 2003; de Hoog & Smith, 2004). *Geotrichum* represents the anamorphic state of species of the genera *Dipodascus* Lagerheim and *Galactomyces* Redhead & Malloch (de Hoog *et al.*, 1998a, b), as demonstrated by sequence divergence of the rRNA subunits (Kurtzman & Robnett, 1998; Ueda-Nishimura & Mikata, 2000). Until 1998, the genus *Geotrichum* contained 11 species, five of which have their teleomorph in the genus *Dipodascus* and two in the genus *Galactomyces*, while the remaining four species have no known sexual state (de Hoog *et al.*, 1998a, b). Since 1998, several revisions have been suggested and novel species have been proposed (Naumova *et al.*, 2001; de Hoog & Smith, 2004). Less closely related *Dipodascus*

species from the so-called group 2 clade were renamed *Magnusiomyces*, with the anamorph for this genus being *Saprochaete* (de Hoog & Smith, 2004).

Kurtzman & Robnett (1998) determined that domains 1 and 2 (D1/D2) (~600 nt) of the large-subunit rRNA gene are sufficiently variable to resolve individual biologically defined species. They also reported the sequences of all known ascomycetous yeasts, and Fell *et al.* (2000) published the D1/D2 sequences of known basidiomycetous yeasts. From these extensive data, Kurtzman & Robnett (1998) deduced simple rules, which generally defined strains showing 6 or more non-contiguous substitutions (1%) as likely to represent separate species (Kurtzman, 2006). Currently, the only exception to this prediction is among interfertile strains of *Clavispora lusitaniae*, which are unusually polymorphic in a 90 bp region of the D2 domain (Lachance *et al.*, 2003).

Recently, DNA sequence comparison has allowed the description of two novel species, *Geotrichum silvicola* (Pimenta *et al.*, 2005) and *Geotrichum vulgare* (Wuczkowski *et al.*, 2006).

During a survey of yeasts and fungi associated with Bryndza, a traditional Slovak artisanal sheep cheese, we isolated ten strains of asexual arthroconidial yeasts

The GenBank/EMBL/DDBJ accession number for the large-subunit rRNA gene sequence of strain CCY 16-2-1^T is EU186073, and those of small-subunit rRNA gene sequences of strain CCY 16-2-1^T are EU433455 and EU433456.

The Mycobank (<http://www.mycobank.org>) accession number for *G. bryndzae* sp. nov. is MB 512675.

identified by conventional taxonomy as *Galactomyces/Geotrichum* strains. These yeasts were found in all Bryndza samples collected from two dairies located in different geographical regions during the summer of 2003 (June–September). Sequence comparison of the D1/D2 domains of the large-subunit rRNA gene revealed that these isolates belong to the same novel *Geotrichum* species (Laurenčík *et al.*, 2008), related to *Geotrichum silvicola* (Pimenta *et al.*, 2005).

Isolation and characterization of yeasts

The strains described in this study were isolated from samples of Bryndza collected from two dairies, one located in Ružomberok, in the north of Slovakia (49° 04' 45" N 19° 18' 09" E; altitude 464 m), and the other in Tisovec, in central-southern Slovakia (48° 34' 31" N 19° 57' 04" E; altitude 310 m). Monthly samples were taken during June–September 2003, 14 days after clothing, and they were transported immediately to the laboratory, maintained at 4 °C and analysed in triplicate within 24 h. Detailed isolation procedures were described by Laurenčík *et al.* (2008). Yeasts were characterized by standard methods (Yarrow, 1998) and identified using the keys of Kurtzman & Fell (1998) and Barnett *et al.* (2000). Their ability to form asci was examined on sporulation media [Gorodkova agar, 5% malt extract agar, cornmeal agar, McClary's acetate agar and yeast sporulation medium (0.1% yeast extract, 1% potassium acetate, 0.05% glucose and 2% agar)].

DNA sequence analysis

Cultivation, DNA extraction and PCR were carried out as described by Laurenčík *et al.* (2008). Briefly, the divergent D1/D2 domain (nucleotides 63–642 for *Saccharomyces cerevisiae*) at the 5' end of the rRNA gene of the cytoplasmic large ribosomal subunit was amplified by PCR with primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5'-GGTCCGTGTTTCAAGACGG) as described by Kurtzman & Robnett (1998). To obtain clear sequences, PCR products were cloned in the pCR-Blunt II-TOPO vector of the Zero Blunt TOPO PCR Cloning kit (Invitrogen) according to the manufacturer's instructions.

Mitochondrial DNA (mtDNA) was prepared by a modification of the original protocol described by Defontaine *et al.* (1991). Parts of the gene for the small rRNA subunit (*rns*) were amplified by two sets of primers, SSU1 (5'-GTGCCAGCAGTCGCGGTAATAC) plus SSU2 (5'-GGA-TTATCGAATTAATAACATGCTCCACTG) and SSU3 (5'-CAGTGGAGCATGTAAATTCGATAATCC) plus SSU5 (5'-CAGGTTCCCCTACAGTAACTGTATTTCAACTT), in 36 PCR cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 2 min.

Individual PCR products and those cloned in plasmids were purified with the Novagen SpinPrep Gel DNA kit or

Qiagen Plasmid Mini kit (Qiagen). Purified DNA was sequenced directly with one of the amplification primers (or sequencing primers –21M13F or –48M13R) using an Applied Biosystems BigDye Terminator v3.1 cycle sequencing kit and ABI3100-Avant Genetic Analyzer (Applied Biosystems). Sequences were edited in CHROMAS version 1.45 and compared with the NCBI database (<http://www.ncbi.nlm.nih.gov/>) by the BLASTN program (Altschul *et al.*, 1990, 1997).

The sequences were aligned with the CLUSTAL W multiple sequence alignment program included in the CLUSTAL_X 1.8 package (Thompson *et al.*, 1997). Rooted phylogenetic trees were constructed with the neighbour-joining method of the CLUSTAL_X 1.8 package. The stability of individual branches was assessed by the bootstrap method (Felsenstein, 1988), and numbers given at nodes are scores with which a given internode appeared in 1000 bootstrap replicates.

The number of nuclei was examined by DAPI staining, according to the specific fluorescence pattern as previously described (Marinoni *et al.*, 1999), using a fluorescent microscope equipped with a DAPI optical filter.

Classification and ecology

Typical characteristics for the members of *Galactomyces/Dipodascus/Geotrichum* clade and also for the novel strains are the assimilation of D-glucose, D-galactose, L-sorbose, glycerol, DL-lactate, succinate and ethanol as carbon sources. Some differences were observed when the novel species was compared with *Galactomyces geotrichum*. The novel species does not use ribitol or D-mannitol. In order to separate the novel species from *Geotrichum silvicola*, its closest relative, assimilation of D-glucitol and D-glucosamine can be used; *Geotrichum silvicola* assimilates D-glucitol and does not assimilate D-glucosamine.

The anamorphic species of the genus *Geotrichum* can be distinguished from their teleomorphs in the genera *Dipodascus* and *Galactomyces* by their inability to form asci. However, typical asci were not found after cultivation on the most common yeast sporulation media. In contrast, *Galactomyces geotrichum* NRRL Y-17569^T produced a number of spherical asci, each containing a single spore which can be seen on DAPI staining (not shown).

The large-subunit rRNA gene D1/D2 domain sequence was analysed for five of the ten novel isolates. Three strains provided identical sequences and the other two isolates had single and double nucleotide polymorphisms in different positions. The DNA polymorphism was confirmed by sequencing of PCR products cloned in pCR-Blunt II-TOPO. Sequence comparison with the GenBank database revealed 12 nucleotide differences in a 545 bp sequence from *Galactomyces geotrichum* NRRL Y-17569^T and 16 substitutions in comparison with *Galactomyces* sp. NRRL Y-6418 (Kurtzman & Robnett, 1998). Additionally, the DNA sequence exhibited variation in 18 nucleotides from

the sequence of the type strain of the recently described species *Geotrichum vulgare* (Wuczkowski *et al.*, 2006) and 6 substitutions in 542 nucleotides from the sequence of the type strain of *Geotrichum silvicola* (Pimenta *et al.*, 2005).

When the rate of divergence and the inability to form asci are considered together, the isolate found in Bryndza is most likely an anamorphic form of a novel *Geotrichum* species.

Phylogenetic analysis of the *Dipodascus* clade (Kurtzman & Robnett, 1998) involving the novel species supported this conclusion, especially when the high bootstrap values show clear separation on the phylogenetic tree for all the lineages (Fig. 1). The novel species belonged to a clade of several *Galactomyces* species. Kurtzman & Robnett (1998) suggested that there is no basis for maintaining the separate genera *Galactomyces* and *Dipodascus*, as they are phylogenetically intertwined. However, Naumova *et al.* (2001) have disagreed because of the common mating type system and the high DNA–DNA reassociation.

A high degree of divergence was also shown by two sequences obtained from mitochondrial DNA (*rns* gene), where 12 substitutions in the 504 bp amplified segment and 12 substitutions in the second 350 bp PCR product were found when the sequences of strain CCY 16-2-1^T (GenBank accession numbers EU429455 and EU429456) were aligned with those of *Galactomyces geotrichum* NRRL Y-17569^T (EU429453, EU429454).

Galactomyces/Geotrichum species are quite ubiquitous since they are involved in biodegradation and depollution. They are recognized as an integral part of the microbiota of some food products. In particular, *Geotrichum candidum* is naturally present in raw milk (Desmasures *et al.*, 1997) and it has long been accepted to be an important component of the microbiota of soft cheeses such as Camembert and semi-fresh goat's and ewe's milk cheeses (reviewed by

Boutrou & Guéguen, 2005). Yeasts with the same D1/D2 sequence as the novel strains identified in Bryndza cheese have been found in Asia among cow rumen microbiota (uncultured fungus clone FE24) and also in Chinese koji (daqu) (GenBank accession no. DQ912852; not shown in Fig. 1). Conspecific strains (with minor divergences in the sequence) are preserved in the Chinese Industry Culture Collection (GenBank accession no. DQ912846) and among microbes degrading fats and oils in Vietnam (DQ377646).

On the basis of the results presented here, a novel species of the genus *Geotrichum* is described to accommodate these strains from Bryndza cheese, *Geotrichum bryndzae* sp. nov.

Latin diagnosis of *Geotrichum bryndzae* Sulo, Laurenčík, Poláková, Minárik et Sláviková sp. nov.

Cultura in agar multi post dies 7 (25 °C) plana, sicca, capillata et candida. Post dies 7 mycelium verum et arthroconidia formantur. In agar Solanum tuberosum et glucosum post dies 7 mycelium verum et arthroconidia formantur. Asci nec ascospores non formantur. Glucosum non fermentatur. D-Glucosum, D-galactosum, D-xylosum, L-sorbosum (lente), glucosaminum (lente), glycerolum, 2-ketogluconatum (lente), acidum lacticum, acidum succinicum, acidum citricum (lente), ethanolum propan-1,2-diolum, butan-2,3-diolum et assimilantur, at non D-ribosum, L-arabinosum, D-arabinosum, L-rhamnosum, maltosum, α , α -trehalosum, cellobiosum, salicinum, arbutinum, melibiosum, sucrosum, lactosum, raffinose, melezitose, inulinum, amyllum solubile, erythritolum, ribitololum, xylitololum, L-arabinitolum, D-mannitololum, galactitololum, myo-inositololum, D-glucono-1,5-lactonum, 5-keto-D-gluconatum, D-gluconatum, D-glucuronatum, et methanololum. Ethylaminum et cadaverinum assimilantur et non natrium nitricum, natrium nitrosolum et lysinum. Ad crescentiam vitaminarum externarum non necessariae sunt. Augmentum in 28 °C, at non 42 °C. Typus

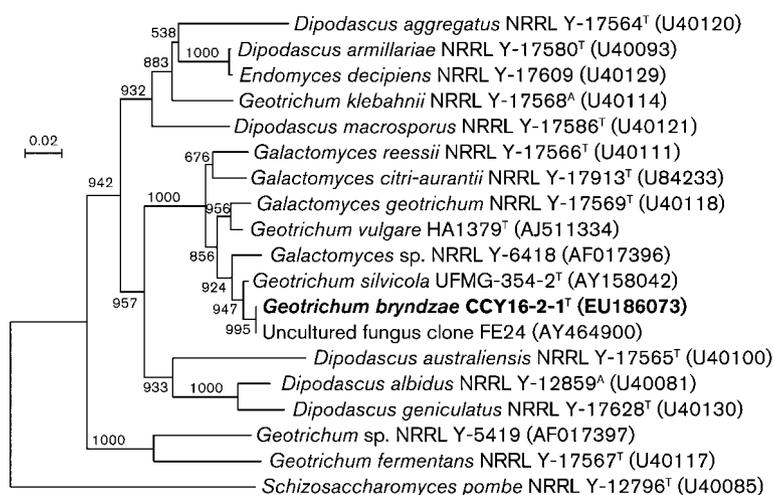


Fig. 1. Phylogenetic tree of the *Dipodascus* clade containing the novel species *Geotrichum bryndzae*. Neighbour-joining tree based on nucleotide sequences of the D1/D2 region in the 26S rRNA gene of the novel *Geotrichum* species and its relatives from the *Dipodascus* clade (Kurtzman & Robnett, 1998). The tree also includes the recently described species *Geotrichum vulgare* (Wuczkowski *et al.*, 2006) and *Geotrichum silvicola* (Pimenta *et al.*, 2005). Branch lengths are proportional to nucleotide differences and numbers at nodes are frequencies of a given branch appearing in 1000 bootstrap replications (frequencies under 50% not given). The outgroup in this analysis was *Schizosaccharomyces pombe* NRRL Y-12796^T. Bar, 2% sequence divergence.

CCY 16-2-1^T (=NRRL Y-48450^T =CBS 11176^T), *collectione zymotica* Culture Collection of Yeasts in the Institute of Chemistry, Slovak Academy of Sciences.

Description of *Geotrichum bryndzae* Sulo, Laurenčík, Poláková, Minárik and Sláviková sp. nov.

Geotrichum bryndzae (brynd'zæ. N.L. gen. n. *bryndzae* of Bryndza, a traditional Slovak artisanal raw sheep milk cheese, the source of isolation of the first strains).

After 7 days on malt extract-yeast extract agar at 25 °C, colonies are white, flat, dry and powdery to finely hairy. Arthroconidia are abundantly present (Fig. 2). The hyphae are 3–7 µm wide with early disarticulation into cubic arthroconidia containing mostly three or four nuclei. Arthroconidia are 5.8–6.6 µm wide and 6.6–13.2 µm long. Abundant true mycelium and arthroconidia are formed on slide cultures with potato dextrose agar after 1 week. Glucose is not fermented. The following carbon compounds are assimilated: D-glucose, D-galactose, D-xylose, L-sorbose (weak), D-glucosamine (weak), glycerol, 2-keto-D-gluconate (weak), DL-lactate, succinate, citrate (weak), ethanol, propane-1,2-diol and butane-2,3-diol. No growth on D-ribose, L-arabinose, D-arabinose, L-rhamnose, maltose, α,α-trehalose, cellobiose, salicin, arbutin, melibiose, sucrose, lactose, raffinose, melezitose, inulin, starch, erythritol, ribitol, xylitol, L-arabinitol, D-mannitol, glucitol, galactitol, myo-inositol, D-glucono-1,5-lactone, 5-keto-D-gluconate, D-gluconate, D-glucuronate or methanol. Ethylamine and cadaverine are assimilated, but not nitrate, nitrite or lysine. Growth in a vitamin-free medium is positive. Exhibits good growth at 28 °C, weak and variable growth at 37 °C and no growth at 42 °C. There is also no

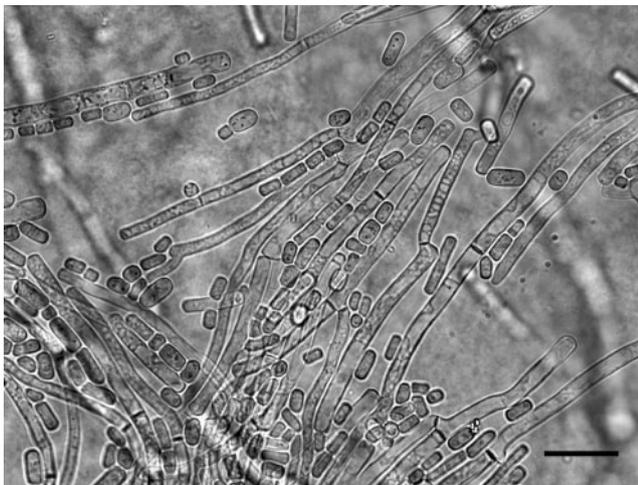


Fig. 2. Bright-field micrograph of growth of *Geotrichum bryndzae* sp. nov. CCY 16-2-1^T. True hyphae and arthroconidia are shown after 3 days of growth on yeast extract/malt extract agar at 25 °C. Bar, 20 µm.

growth in 50 % glucose. Growth occurs at 0.01 % cycloheximide, while growth at 0.1 % is variable. Urease activity is negative; does not produce starch or extracellular amyloid compounds.

The type strain is CCY 16-2-1^T (=NRRL Y-48450^T =CBS 11176^T), which is deposited in the Culture Collection of Yeasts in the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia. The Mycobank accession number is MB 512675.

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References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Barnett, J. A., Payne, R. W. & Yarrow, D. (2000). In *Yeasts: Characteristics and Identification*, 3rd edn, pp. 388–394. Cambridge: Cambridge University Press.
- Boutrou, R. & Guéguen, M. (2005). Interests in *Geotrichum candidum* for cheese technology. *Int J Food Microbiol* **102**, 1–20.
- Defontaine, A., Lecocq, F. M. & Hallet, J. N. (1991). A rapid miniprep method for the preparation of yeast mitochondrial DNA. *Nucleic Acids Res* **19**, 185.
- de Hoog, G. S. & Smith, M. T. (2004). Ribosomal gene phylogeny and species delimitation in *Geotrichum* and its teleomorphs. *Stud Mycol* **50**, 489–515.
- de Hoog, G. S., Smith, M. T. & Guého, E. (1986). A revision of the genus *Geotrichum* and its teleomorphs. *Stud Mycol* **29**, 1–131.
- de Hoog, G. S., Smith, M. T. & Guého, E. (1998a). *Dipodascus* de Lagerheim. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 181–193. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- de Hoog, G. S., Smith, M. T. & Guého, E. (1998b). *Galactomyces* Readhead & Malloch. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 209–213. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Desmaures, N., Bazin, F. & Guéguen, M. (1997). Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. *J Appl Microbiol* **83**, 53–58.
- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Felsenstein, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Annu Rev Genet* **22**, 521–565.
- Kurtzman, C. P. (2006). Yeast species recognition from gene sequence analyses and other molecular methods. *Mycoscience* **47**, 65–71.
- Kurtzman, C. P. & Fell, J. W. (editors) (1998). *The Yeasts: a Taxonomic Study*, 4th edn. Amsterdam: Elsevier.
- 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.

- Lachance, M. A., Daniel, H. M., Meyer, W., Prasad, G. S., Gautam, S. P. & Boundy-Mills, K. (2003).** The D1/D2 domain of the large subunit rDNA of the yeast species *Clavispora lusitaniae* is unusually polymorphic. *FEMS Yeast Res* **4**, 253–258.
- Laurenčík, M., Sulo, P., Sláviková, E., Piecková, E., Seman, M. & Ebringer, L. (2008).** The diversity of eukaryotic microbiota in the traditional Slovak sheep cheese – Bryndza. *Int J Food Microbiol* **127**, 176–179.
- Marinoni, G., Manuel, M., Petersen, R. F., Hvidtfeldt, J., Sulo, P. & Piškur, J. (1999).** Horizontal transfer of genetic material among *Saccharomyces* yeasts. *J Bacteriol* **181**, 6488–6496.
- Naumova, E. S., Smith, M. Th., Boekhout, T., de Hoog, G. S. & Naumov, G. I. (2001).** Molecular differentiation of sibling species in the *Galactomyces geotrichum* complex. *Antonie van Leeuwenhoek* **80**, 263–273.
- Pimenta, R. S., Alves, P. D. D., Corrêa, A., Jr, Lachance, M. A., Prasad, G. S., Rajaram, Sinha, B. R. & Rosa, C. A. (2005).** *Geotrichum silvicola* sp. nov., a novel asexual arthroconidial yeast species related to the genus *Galactomyces*. *Int J Syst Evol Microbiol* **55**, 497–501.
- Smith, M. Th. & Poot, G. A. (2003).** Genome comparisons in the genus *Dipodascus* de Lagerheim. *FEMS Yeast Res* **3**, 301–311.
- Smith, M. Th., Poot, G. A. & de Cock, A. W. A. M. (2000).** Re-examination of some species of the genus *Geotrichum* Link: Fr. *Antonie van Leeuwenhoek* **77**, 71–81.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Ueda-Nishimura, K. & Mikata, K. (2000).** Two distinct 18S rRNA secondary structures in *Dipodascus* (*Hemiascomycetes*). *Microbiology* **146**, 1045–1051.
- Wuczkowski, M., Bond, C. & Prillinger, H. (2006).** *Geotrichum vulgare* sp. nov., a novel asexual arthroconidial yeast. *Int J Syst Evol Microbiol* **56**, 301–303.
- Yarrow, D. (1998).** Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.