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ORIGINAL PAPER

Intraspecific nucleotide divergence in *Saccharomycodes ludwigii*, and proposal of *Saccharomycodes pseudoludwigii* sp. nov, a new apiculate yeast isolated from China

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Abstract The six synonyms currently accepted under *Saccharomycodes ludwigii* were investigated for by phenotypic properties, however, the sequence diversity of the rRNA and protein coding genes have not yet been determined. Nine strains including the type strains of synonyms of *S. ludwigii* deposited in the CBS yeast collection, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, were analyzed using a multi-locus sequence analysis (MLSA) approach that included sequences of 18S ribosomal RNA (rRNA), the D1/D2 domains of the 26S rRNA, the ITS region (including the 5.8S rRNA) and fragments of genes encoding the largest subunit of the RNA polymerase II (RPB1 and RPB2) and translation elongation factor 1-α (TEF1). Our results showed that the nine strains have identical D1/D2, 18S and RPB2 sequences and similar ITS, RPB1 and TEF1 sequences, which indicated that they are conspecific. In addition, a novel species of *Saccharomycodes*, *S. pseudoludwigii* sp. nov. (type CGMCC 2.4526 ^{T}) that was isolated from fruit and tree bark in China, is proposed. The MycoBank number of this new species is MB 811,650.

Keywords Phylogeny · *Saccharomycodes* · Taxonomy · Yeasts · One new taxon

Introduction

The genus *Saccharomycodes* contains previously two species, namely *S. ludwigii*, the type species of *Saccharomycodes*, and *S. sinensis* (Kurtzman and Robnett 1998; Boundy-Mills et al. 2011). As *S. sinensis* was reclassified as *Yueomyces sinensis* based on a multi-gene sequence analysis (Wang et al. 2015b, c, a), currently it is not assigned to *Saccharomycodes*, and the genus *Saccharomycodes* includes only one species, *S. ludwigii*. *Saccharomycodes vini* (Kroemer and Heinrich) ex Kudryavtsev (1954) [MB#338772] (= *Saccharomycodes ludwigii var. vini* Kroemer and Heinrich (1922) [MB#456537]), *Saccharomycodes bisporus* Castelli (1941) [MB#290745] (= *Saënkia bispora* Castelli ex Kudryavtsev (1960)

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[MB#456541] = *Saccharomycodes ludwigii* var. *bisporus* Castelli ex Hjort (1954) [MB#352485]) and *Saccharomycodes lipophora* Bachinskaya (1941) [MB#456536] were assigned as synonyms of *S. ludwigii* as determined from phenotypic characters (Miller and Phaff 1998; Boundy-Mills et al. 2011). However, the genetic diversity within these synonymized species is unclear. Nine strains of *S. ludwigii* deposited in the CBS yeast collection, Westerdijk Fungal Biodiversity Institute, CBS 820 (type of *S. vini*), CBS 821 (type of *S. ludwigii*), CBS 2624 (type of *S. bisporus*), CBS 1168, CBS 1169, CBS 2625, CBS 5929, CBS 7780 and CBS 8338, were phylogenetically analyzed based on five loci, including the 18S rRNA and 26/28S rRNA (D1/D2 domains) genes, and fragments of genes encoding the first and second large subunits of RNA polymerase II (RPB1 and RPB2) and translation elongation factor 1-α (TEF1) in this study. Additionally, a new species of *Saccharomycodes*, *S. pseudoludwigii*, represented by three strains isolated from fruit and tree bark in China, is proposed.

Materials and methods

Strains and phenotypic characteristics

The *S. ludwigii* strains, CBS 820 (type of *S. vini*), CBS 821 (type of *S. ludwigii*), CBS 1168, CBS1169, CBS 2624 (type of *S. bisporus*), CBS 2625, CBS 5929, CBS 7780 and CBS 8338, obtained from the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, were used in this study (Table 1). Strain CGMCC 2.4525 (= SD 169 = TAli83-3B = CBS 12,643) was isolated from a fruit of *Pyrus sorotina*, collected from Tai'an, Shandong province in China (Coordinate: 117°08′40″N 36°18′32″E). CGMCC 2.4526 ^{T} (= SD 139 = AQSZP6L-3 = CBS 12,644) and CGMCC 2.4527 (= SD 200 = AQSZP6L-1 = CBS 12,645) were isolated from the bark of *Diospyros kaki*, collected from Anqiu, Shandong province in China (Coordinate: 119°02′13″N 36°43′20″E). The bark samples were obtained from around the bases of the trees using a sterile scalpel and deposited in sterile 15-ml conical tubes. The fruit samples were collected using sterile sampling bags in the field, cut into small pieces and filled with enrichment medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 8% v/v ethanol, 200 μg chloramphenicol ml^{-1} and 1 ml 1 M HCl per liter) described by Wang et al. (2012) and incubated at 25 °C for one or two weeks. The cultured liquid was gradiently diluted and spread on YM plates (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast, 2.0% agar, pH 6.0 with chloramphenicol 100 μg/ml) and incubated at 25 °C for 2–5 days. Single yeast colonies were picked and purified on YM agar plates. The phenotypic and biochemical characters were examined according to standard methods for yeast classification (Kurtzman et al. 2011). The assimilation of carbon and nitrogen compounds were examined in liquid medium at 25 °C. The sexual reproduction of these strains were examined using YPD and McClary acetate agars. A loopful of cells of individual strains were spotted in the agar plate and incubated at 25 °C for four weeks and examined weekly under a microscope for ascosporulation.

Molecular phylogenetic analysis

Genomic DNA was extracted using the method as described by Wang and Bai (2008). Sequences of the ribosomal cistron, including ITS (ITS1 + 5.8S + ITS2), the D1/D2 domains of 26S rRNA, 18S rRNA, and RPB1, RPB2 and TEF1 gene sequences were determined as described by Wang et al. (2014, 2015a, b). Purified PCR products of the ITS region from strain CGMCC 2.4525 were cloned using the pUCm-T Vector system (BBI) and transformed into *E. coli* DH5α competent cells. White clones were randomly picked and sequenced using the procedure described by Wang et al. (2014). Sequences were aligned with the MUSCLE program in MEGA 7 (Kumar et al. 2016). A general time-reversible model of DNA substitution that assumes a percentage of invariable sites and Γ-distributed substitution rates at the remaining sites (GTR + I + G) was selected for Maximum likelihood (ML) analyses conducted in MEGA 7 using 1000 bootstrap replicates analysis. All sites including the gaps in the alignments were used to construct the ML trees. A phylogenetic network was constructed in SplitsTree4 (Huson and Bryant 2006) using the Consensus Network algorithm with default parameter settings based on the five genes studied i.e. 18S rRNA, 26S rRNA D1/D2, ITS, RPB2 and TEF1, to infer the phylogenetic relationships between the

Table 1 Yeast strains studied

Species	Strain	Original name	Source	18S RNA	ITS + D1/D2	RPB1	RPB2	TEF1
S. pseudoludwigii sp. nov	CGMCC 2.4526 ^{T}	–	Bark of *Diospyros kaki* in Anqiu, Shandong province, China, in August 2008	KP866237	KP866237	–	KP866230	KP866226
	CGMCC 2.4525	–	Fruit of *Pyrus sorotina,* in Tai'an, Shandong province, China, in August 2008	KP866232	KP866238 KP866246 KP866234	–	KP866229	KP866227
	CGMCC 2.4527	–	Bark of *Diospyros kaki* in Anqiu, Shandong province, China, in August 2008	KP866236	KP866236	–	KP866231	KP866225
S.ludwigii	CBS 820	type of *S. vini*	Juice, Germany	MT937297	MT937305	MT940416	MT940425	–
	CBS 821 ^{T}	type of *S. ludwigii*	Wine, Germany	AY046261	MT937309	MT940424	MT940426	AF402074
	CBS 1168	–	Unknown	MT937298	MT937313	MT940417	MT940427	MT940415
	CBS 1169	–	Fermenting grape must, Italy	MT937299	MT937306	MT940418	MT940428	MT940409
	CBS 2624	type of *S. bisporus*	Grape must, Italy	MT937300	MT937311	MT940423	MT940429	MT940412
	CBS 2625	–	liquid medium, from culture of Mycobacter aceti, unknown	MT937301	MT937312	MT940419	MT940430	MT940410
	CBS 5929	–	Garden soil, South Africa	MT937302	MT937310	MT940420	MT940431	MT940411
	CBS 7780	–	bottled cider, Belgium	MT937303	MT937307	MT940421	MT940432	MT940414
	CBS 8338	–	white wine, the Netherlands	MT937304	MT937308	MT940422	MT940433	MT940413
Saccharomyces cerevisiae	NRRL Y-12632	–	–	Z75578	NR_111007 JQ6890177	JQ713023	JQ698955	JQ699041

isolates and *Saccharomycodes* species. The GenBank accession numbers obtained during this study are listed in Table 1.

Results and discussion

Saccharomycodes vini (= *S. ludwigii* var. *vini*), *S. bisporus* (= *Saënkia bispora* = *S. ludwigii* var.

bisporus) and *S. lipophora* are current synonyms of *S. ludwigii*. *S. lipophora* was described without a Latin description by Bachinskaya (1941), which is invalid according to Art. 39.1 of the International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code, Turland et al. 2018). The type strain of *S. lipophora* is not available in the Russian Collection of Microorganisms (VKM) nor the CBS Collections. It is unclear whether the type of *S. lipophora* was lost in other collections, therefore it was not included in this study. Nine strains of *S. ludwigii*, CBS 820 (type of *S. vini*), CBS 821 (type of *S. ludwigii*), CBS 2624 (type of *S. bisporus*), CBS 1168, CBS 1169, CBS 2625, CBS 5929, CBS 7780 and CBS 8338, have identical D1/D2, 18S and RPB2 sequences, and differed from each other by 5 (including 3 substitutions and 2 inserts/deletions), 4 synonymous substitutions and 5 nucleotides (nt) (1 non-synonymous and 4 synonymous substitutions) in the ITS region, RPB1 and TEF1 genes, respectively. The above results indicated that the nine CBS strains are conspecific as indicated before by phenotypic data (Boundy-Mills et al. 2011).

Three strains isolated from bark and fruit, namely CGMCC 2.4525, CGMCC 2.4526 ^{T} and CGMCC 2.4527, are characterized by lemon-shape cells with bipolar budding. These three strains have identical 18S rRNA and D1/D2 sequences, but CGMCC 2.4526 ^{T} and CGMCC 2.4527 with identical ITS sequences were found to differ from the nine clone sequences of CGMCC 2.4525 by 0–4 nt mismatches (3 substitutions and 1 insert/deletion) in the ITS region. The ITS sequences of CGMCC 2.4525 is polymorphic and have 0–4 nt differences (3 substitutions including 2 transitions and 1 transversion, and 1 insert/deletion) in the ITS1 region among 9 clones (GenBank numbers KP866238-KP866246). A BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using these sequences revealed that these strains were close relatives to *S. ludwigii*. CGMCC 2.4526 ^{T} differs from the type strain of *S. ludwigii* by 4 substitutions and 1 inserts/deletions in the D1/D2 domains, and by 21 nt (including 12 substitutions and 9 inserts/deletions, 3%) in the ITS regions. Forty three nt (6%, 39 synonymous and 4 non-synonymous substitutions) and 21 nt (2%, 18 synonymous and 3 non-synonymous substitutions) differences were observed between these strains and the type strain of *S. ludwigii* in the RPB2 and TEF1 genes, respectively.

The 18S, D1/D2, ITS, RPB2 and TEF1 were aligned using the MUSCLE program, resulting in alignments of 1 583, 538, 801, 712 and 866 nucleotide lengths, respectively. 2, 5, 22, 47 and 24 variable sites were found between all strains of *Saccharomycodes* in the 18S, D1/D2, ITS, RPB2 and TEF1 alignments, respectively. Our multi-locus sequence analysis showed that the strains CGMCC 2.4525, CGMCC 2.4526 ^{T} and CGMCC 2.4527 form a clade distinct from *S. ludwigii* (Fig. 1). The ITS tree including all polymorphic sequences of CGMCC 2.4525 showed that those three strains formed a separate clade from the *S. ludwigii* clade (Fig. 2). The five-gene phylogenetic network supported that those three strains and *S. ludwigii* strains formed separate lineage (Fig. 3). The single gene trees of 18S, D1/D2, RPB2 and TEF1 showed a topology in agreement with the five-gene tree (Fig. S1). Thus, a new species of *Saccharomycodes*, namely *Saccharomycodes pseudoludwigii* sp. nov., is proposed to accommodate these three strains.

Description of Saccharomycodes pseudoludwigii Q.M. Wang and T. Boekhout sp. nov. MycoBank MB811650

Etymology: The specific epithet *pseudoludwigii* refers to the morphology of this species being similar to *Saccharomycodes ludwigii*.

In YM broth, after 3 days at 25 °C, the cells are lemon-shaped (apiculate), 4.0–6.5 × 8.0–24 µm, and occur singly or in pairs. Budding is bipolar. After 1 month at 25 °C, a sediment and ring are present. After 1 month at 25 °C, the streak culture is cream-colored to light yellow, butyrous, smooth, and the margins are entire or somewhat curved. Pseudohyphae are not observed on corn-meal agar. Sporulation is observed on YPD and McClary acetate agars after 5 days at 25 °C. Sausage or lemon-shaped asci are unconjugated and contain two or four globose ascospores (Fig. 4). Ascus walls do not lyse when the spores are mature.

Glucose, sucrose and raffinose are fermented, but galactose, maltose and lactose are not. Glucose, sucrose, raffinose, cellobiose, ethanol, glycerol and salicin are assimilated; whereas galactose, *L*-sorbose, maltose, lactose, trehalose, *D*-ribose, inulin, melibiose, melezitose, soluble starch, *D*-xylose, *L*-arabinose, *D*-arabinose, *L*-rhamnose, *D*-glucosamine,

Fig. 1 Multi-locus ML tree depicting relationships of *S. pseudoludwigii* sp. nov. and *S. ludwigii*. Phylogenetic tree constructed from ML analysis of the combined sequences of the18S rRNA, D1/D2 domains of the 26S rRNA, the ITS region (including the 5.8S rRNA), the RPB2 and TEF1, depicting *S. pseudoludwigii* sp. nov. and *S. ludwigii* relationships. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown. The tree is rooted with *Saccharomyces cerevisiaae*. Bar = 0.02 indicates 2% sequence divergence

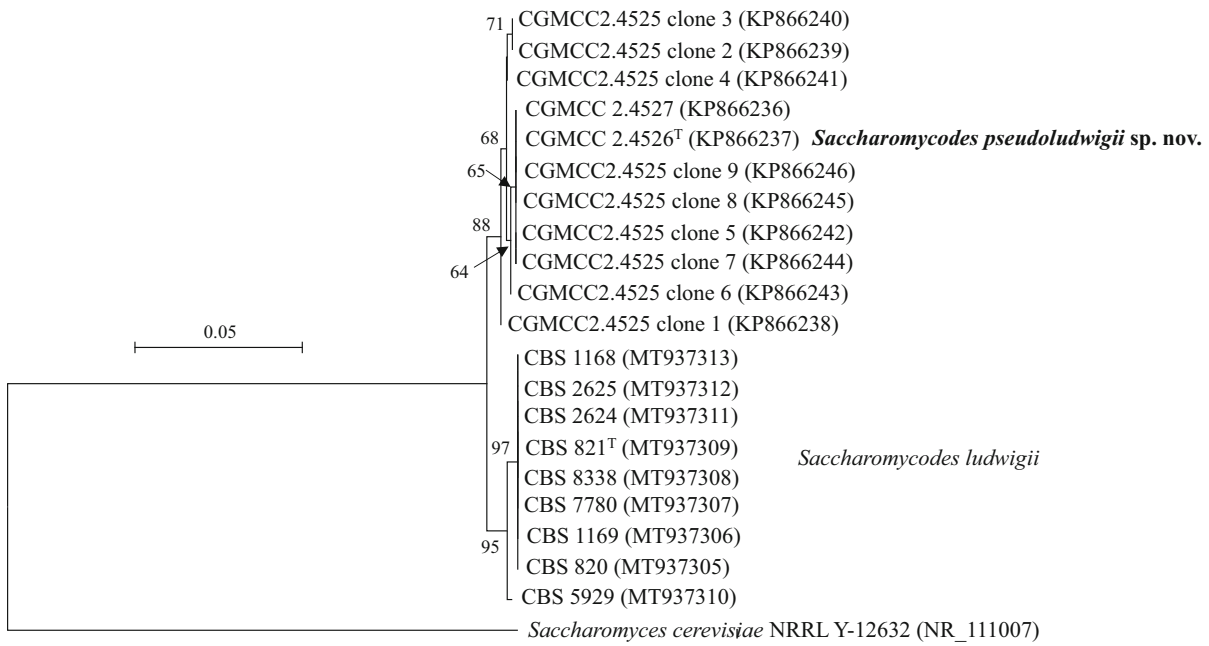

Fig. 2 The ITS ML tree depicting relationships of *S. pseudoludwigii* sp. nov. and *S. ludwigii*. Phylogenetic tree constructed from ML analysis of the combined sequences of the ITS region (including the 5.8S rRNA), depicting *S. pseudoludwigii* sp. nov. and *S. ludwigii* relationships. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown. The tree is rooted with *Saccharomyces cerevisiaae*. Bar = 0.05 indicates 5% sequence divergence

methanol, erythritol, ribitol, galactitol, *D*-mannitol, α-methyl-*D*-glucoside, *DL*-lactic acid, succinic acid, citric acid, inositol and hexadecane are not. Ammonium sulfate, ethylamine hydrochloride, cadaverine hydrochloride and *L*-lysine are assimilated, whereas potassium nitrate and sodium nitrite are not. Starch-like compounds are not produced. Growth in vitamin-free medium is absent. Maximum growth temperature is 32 °C. Growth occurred in 0.0001% cycloheximide, but not in media containing 0.001% cycloheximide or 50% glucose. Urease activity and diazonium blue B reaction are negative.

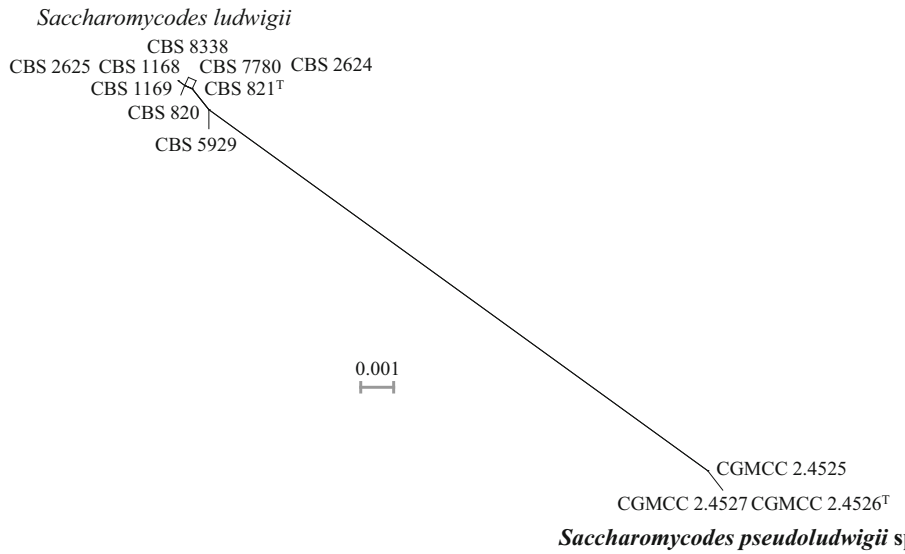

Fig. 3 The phylogenetic network tree depicting relationships of *S. pseudoludwigii* sp. nov. and *S. ludwigii*. Phylogenetic network of the strains of *Saccharomycodes*. Single gene ML trees inferred with MEGA 7 were investigated in Splitstree 4.13.1 using the ConsensusNetwork algorithm under default settings. Bar = 0.001 substitutions per nucleotide position

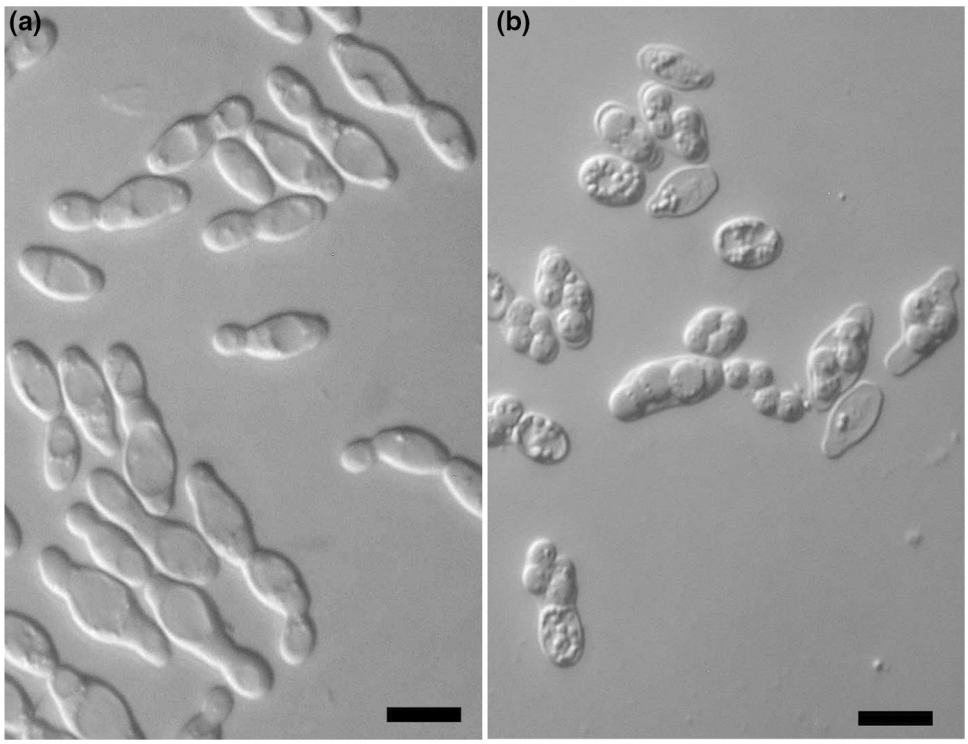

Fig. 4 Picture of the cells and asci. *Saccharomycodes pseudoludwigii* sp. nov. CGMCC 2.4526 asexual cells grown in YM broth for 3 days at 25 °C **a** and asci and ascospores formed on McClary acetate agar after 5 days at 25 °C **b**. Bars, 10 μm

Physiologically, *S. pseudoludwigii* differs from *S. pseudoludwigii* in the inability to assimilate *DL*-lactic acid.

The holotype CGMCC 2.4526 ^{T} was isolated from the bark of *Diospyros kaki* in Anqiu, Shandong province, China, in August 2008, and is deposited in a metabolically inactive state in the China General Microbiological Culture Collection Center (CGMCC), Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. The ex-type culture has been deposited in the CBS yeast collection of the Westerdijk Institute, Utrecht, The Netherlands, as CBS 12,644 (= SD 139 = AQSZP6L-3). The paratypes SD 169 = (TAli83-3B) isolated from the fruit of *Pyrus sorotina* and SD 200 = (AQSZP6L-1) isolated from the bark of *Diospyros kaki*, have been deposited in the above two collections, as CGMCC 2.4525 (= CBS 12,643), CGMCC 2.4527 (= CBS 12,645), respectively.

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Author contributions Q-M.W. conceived and designed the project. Q-M.W. performed sampling and new yeast isolation. M-M. W, F.W. and Y–T.G. performed phenotypic characterization and analyzed the molecular data. M-M. W, Q-M.W., M.G. and T.B. wrote the paper. M.G. and T.B. supplied strains conserved in CBS collection.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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