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Assessment of *Exserohilum turcicum* using molecular markers for sustainable maize production in Tanzania

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The *Exserohilum turcicum* fungus causes the Northern leaf blight (NLB) of maize all over the world. In Tanzania, this disease is considered as a major foliar disease with yield losses of 23.9 to 62.8%. To understand the genetic variations of *E. turcicum* from maize fields in Tanzania, a survey was conducted in four regions, including Morogoro, Iringa, Njombe, and Mbeya. Leaf samples were collected from diseased plants exhibiting NLB symptoms. Using aseptic technique, pure colonies of fungal pathogens were isolated on selective media followed with molecular analysis. The internal transcribed ribosomal DNA (ITS), six microsatellite markers of simple sequence repeat (SSR) nature, and two specific mating type primers (MAT) designed to amplify *MAT1* and *MAT2* gene sequences were used in this study. With ITS marker, 14 isolates were amplified, Sanger sequenced, and their sequences were deposited to GenBank with accession numbers MT124699-MT124712. The SSR results were scored using base pairs in each genotype and subjected to power marker software to determine genetic variations. A total of four alleles across two loci were detected, with gene diversity (0.26 - 0.58). Polymorphic bands revealed 4 and 5 genotypes using SSR 06 and SSR 024, respectively. The polymorphic information content (PIC) of SSR 06 and SSR 024 loci were 0.25 and 0.52, respectively. The specific MAT markers demonstrated dominance of *MAT 2* over *MAT 1*. The distribution of *MAT 1* and *MAT 2* in humid highlands of Mbeya, Njombe, and Iringa regions indicated possibility of sexual reproduction and good potential for sexual recombination than in the dry lowland of Morogoro region. This study is the first report on genetic diversity of *E. turcicum* in Tanzania, further studies using robust molecular and sequencing techniques is imperative in Tanzania.

Key words: *Exserohilum turcicum,* maize, microsatellite, mating type primers, ITS primers, Tanzania.

INTRODUCTION

Exserohilum turcicum (Pass.) Leonard and Suggs (1974) of maize is an important foliar disease in Tanzania (Dong et al., 2008; Nwanosike, 2016). The disease is potentially distributed all over the world (Ramathani et al., 2011). The pathogen also infects sorghum and other related grasses (Harlapur et al., 2007; PANNAR Seed Company, 2009); however, epidemic may be sporadic depending on the environment and varieties to resist infection (Degefu,

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1990).

This disease is a threat to maize production globally (Muiru et al., 2010; Levic et al., 2008; Nwanosike et al., 2017). In Tanzania, yield losses ranged from 23.9% in Morogoro to as high as 62.8% in Mbeya (Nwanosike, 2016). Population studies on the pathogen become imperative to provide understanding of the genetic diversity (Nei, 1973; Ferguson and Carson, 2007; Haasbroek et al., 2014; McDonald and McDermott, 1993).

The studies of *E. turcicum* genetic diversity in various countries concentrated on use of RAPDs, AFLP, and isozymes (Dong et al., 2008; Muiru et al., 2010), but microsatellites markers considered as choice markers for population studies (Chambers and MacAvoy, 2000; Ellegren, 2004). However, SSR has been used primarily to detect polymorphism amongst the parental cultivated varieties (Klein et al., 2000). Ferguson and Carson (2004) reported that the population genetic structure of *Setosphaeria turcica* is subjective to the result of both sexual and asexual reproduction. Although the sexual phase of *E. turcicum* has not been discovered in the field, evidence of recombination exists especially in tropical populations (Borchardt et al., 1998b).

Simple sequence repeats are sections of DNA repetitive units (2-6 bp in length) inside all eukaryotic organisms (Quellar et al., 1993). Identification of nucleotide sequences in the adjoining regions of the microsatellite facilitates development of specific primers (generally 20-25 bp) to amplify the SSR by polymerase chain reaction. Ramathani et al. (2011) reported the type of mating primers that amplified the *MAT1* and *MAT2* idiomorphs of *E. turcicum* with fragment size of 154 and 197 bp, respectively. The efficacy of such primers was used to test maize and sorghum derived *E. turcicum* in Uganda (Ramathani, 2010). However, in South Africa, Haasbroek et al. (2014) designed and developed mating types idiomorphs of the pathogen with higher fragment size for mating type 1 and mating type 2, which was used to screen fungal isolates of maize and sorghum. It has a single locus and two-allele mating system and as such exhibits two forms of mating type (Borchardt et al., 1998a; Klix et al., 2010).

NLB is a destructive foliar disease in maize growing areas of Tanzania and is reflected as a limiting biotic factor hampering successful cultivation of maize. Despite efforts of the poor resource for farmers to improve yield, it remains low 1.3 to 1.5 tons/ha (Nkonya et al., 1998). Therefore, this study will apply SSR loci and species-specific primers designed based on internal transcribed ribosomal DNA (ITS) and 5.8s rDNA sequences to determine genetic diversity and mating types of Tanzania isolates, respectively.

MATERIALS AND METHODS

Leaf samples showing lesions were collected in 38 fields during a survey in 2012 to 2013. Twenty-eight (28) samples were collected from Southern highland regions including Iringa (10), Njombe (8), and Mbeya (10), and ten samples from Eastern region (Morogoro), using the Global Positioning System. Samples were surface sterilised and incubated for 2 days at 25±3°C, in laboratory. The fungus were isolated on V8 agar medium (Harlapur, 2005). *E. turcicum* cultured in a liquid media, the mycelia were collected and preserved at 4°C (Muiru et al., 2010).

DNA was extracted using Cetyltrimethylammonium bromide (CTAB) protocols by Moller et al. (1993) and adapted by Muiru et al. (2010). The quality and quantity of extracted DNA was measured on NanoDrop Spectrophotometer (Thermofisher, Waltham, MA) at ratio of 260/280 wavelength. The extracted DNA was stored at -20°C for use in PCR experiments.

PCR reactions and cycles

The identity of the isolates was confirmed through PCR sequence information from product targeting of the internal transcribed spacer 1 and 2 (ITS) of the 5.8S ribosomal RNA region using specific primers that were previously used by Ramathani (2010) (Table 1). The PCR amplicons were Sanger sequenced and blasted to the NCBI GenBank to identify isolate with more hits to our sequences.

Six microsatellite primers (Table 2) obtained from several related loci of ascomycetes and two specific MAT markers; *MAT1* and *MAT2* (Table 1) were used to test for polymorphism of 20 *E. turcicum* maize isolates using the protocol described by Ramathani (2010) and Ramathani et al. (2011), with amendments.

Amplifications were performed in a thermo cycler (Gene Amp PCR system 9700 Applied Bio Systems, 850 Lincoln Centre Drive Foster City, CA 94404 USA) using the following program: 1 cycle of 1 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 45 to 50°C and 45 s at 72°C, with a final extension of 7 min at 72°C, for SSR while the cycling condition for MAT primers was 1 cycle of 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final extension of 9 min at 72°C.

Amplified products were separated by electrophoresis (Bio-Rad, model 96, Bio-Rad Laboratories, Inc. Life Science Research Group 2000 Alfred Nobel Drive Hercules, CA 94547 USA) at 110 V for 2.0 h on 1.5% agarose gels and stained with intercalating ethidium bromide (0.5 µg ml^{-1}) using 1.0 × TBE buffer (89 mMTris, 89 mM borate, 2 mM EDTA pH 8.3). Gels were documented under UV-Trans-illuminator 2000 (Sagrate, Milan Italy). Isolates that were amplified with ITS primers, were submitted to Molecular and Cellular Imaging Center (MCIC), Wooster Ohio for Sanger sequencing.

Amplicons separated on a gel were scored using base pair (bp) size, in reference to the molecular marker for each genotype. Estimates of similarity among strains were calculated from the data matrices in the form of dissimilarity units and expressed as Euclidean genetic distance (Hintze, 1998). Polymorphic bands of SSR06 and SSR024 were subjected to power marker software for genetic variation calculation amongst the isolates.

Sanger sequenced products (forward and reverse) per isolate were assembled using MacVector software version 17.5.2 (Apex, NC, USA) to create a consensus sequence that was later blasted to the NCBI database for identification.

RESULTS

Selection of *E. turcicum* DNA by species specific primers

The rDNA ITS specific primers were detected in 20 out of

Table 1. Sequences of internal transcribed spacer ribosomal DNA (ITS) and mating type primers used for classification of *Exserohilum turcicum.*

Primer name	Sequence (5'… 3')	Gene type	Expected size (bp)	References
ITS1	TGTGTGTGTGTGTGTGTGTGT ATAAGACGGCCAACACCAAG	Specie specific	344	
MAT2-specific	ACCGATTGCTTCG CAAACATCTCAAGGCGGAA	Mat 1-2 & Mat2-2	195	Ramathan (2010)
MAT1 α-clone	GTGAACCGACCCTCAAC GTCCATGGGATACGCTACG	Mat 1-1 gene	190	

Source: Ramathan (2010).

Table 2. Simple sequence repeat primers used to characterize *Exserohilum turcicum* populations.

Primer name	Sequence	Accession No.	Reference
SSR01	5'-TAGTTGCAACCGAACAGG-3' 5'-CTCCGTAGGTATGATGGTGT-3'	AJ303015	Molina et al. (2001)
SSR06	5'-CGAACAGGACGAAAGAATAG-3' 5'-GTTTGTTCCAGTTCGTCAAG-3'	AJ303023	
SSR24	5'-TCAAGAGGAGAAGTTGA-3' 5'-GGTTCTGATCAAGAGGAGGA-3'	AJ303034	
SSR36	5'-ATTCCAGGTACGGCTACAC-3' 5'-ATTCAGATCTGGTCTGGTTG-3'	AJ303040	
SSR10	5'-GAGAGCATGAAAAGTGGAAA-3' 5'-CGTGACACTCGTCAGTTACA-3'	AJ303026	
SSR14	5'-ATTTGGTGAATGGGGTAAG-3' 5'-ACAGAGGGAAGCAAGTTTTT-3'	AJ303027	

Source: Molina et al. (2001).

38 isolates from maize samples, yielding the expected 344 base pairs (Figure 1). Fourteen isolates were Sanger sequenced and had 98 to 100% nucleotide identity with MN918438.1, MN918291.1, and query coverage of 94 to 100 with reference sequences in the NCBI Genbank. The sequenced isolates were annotated and deposited in the NCBI GenBank. The isolates, locations, altitude, and NCBI accession numbers are indicated in Table 3.

Genetic variation of *E. turcicum* based on SSR analysis

Molecular variations were observed among isolates based on microsatellite repeat makers used in this study (Figure 2). Two microsatellite regions, SSR 06 (Figure 2a) and SSR 024 (Figure 2b) were polymorphic, while SSR01, SSR10, SSR14 and SSR36 loci were monomorphic on the 14 *E. turcicum* isolates (Figure 2). A total of four alleles were identified in each locus (SSRs), and the genetic diversity ranged from 0.26 (SSR06) to 0.58 (SSR24). The analysis of the polymorphic bands using power marker software detected 4 and 5 genotypes for SSR 06 and SSR 024, respectively (Table 4).

The genotypes were distinguished by the type of polymorphic bands (Figure 2). The amplicons of isolates 2, 5 and 10 (Figure 2a) and 2, 5 and 6 (Figure 2b) were distinctly different genotypes, 4 and 8 are different from that of 9 and 13 genotypes while the other isolates genotypes were of the same strain, indicating five

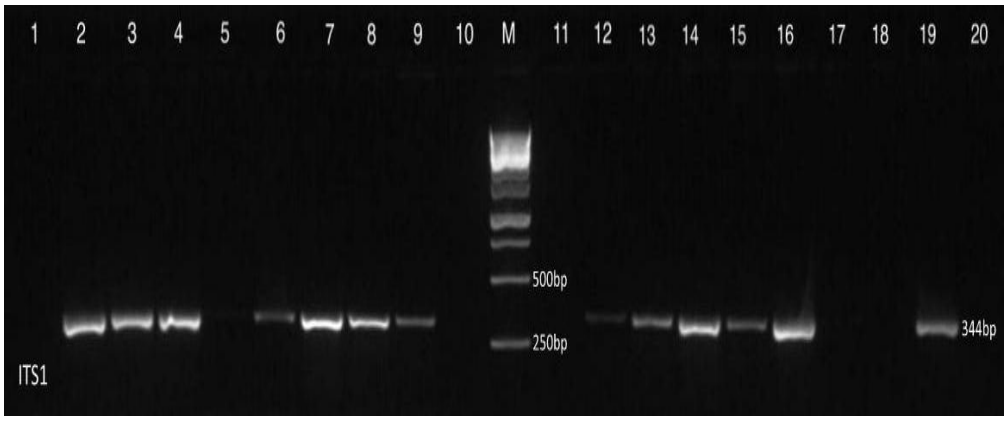

Figure 1. Gel image of rDNA ITS1 species specific primer showing 344 base pair PCR products specific to *E. turcicum* isolated from maize. Lanes 1-14 represents the sequenced isolates; MOR2, MOR3, IR4, MBY5, IR7, IR8, MOR9, MBY12, MBY13, IR14, MOR15, MOR16, IR19, IR20 in ascending order. Locations are described in Table 2, M = Marker
Source: Nwanosike, 2016.

Table 3. Tanzania strains of *Exserohilum turcicum* used in the study.

S/N	Region	Village	Co-ordinates	Altitude (masl)	*E. t* code	Sequenced DNA	MAT type
1	Mbeya	Mbaliu	8° 54' 11.53"S 33°26' 23.12"E	1777.1	MBY1	MT124702	NA
2	Morogoro	Msumbe	6° 53' 31.39"S 37°33' 41.98"E	566.1	MOR2	MT124699	2
3	Morogoro	Nanenane	6° 48' 05.24"S 37°40' 22.35"E	515.8	MOR3	MT124700	2
4	Iringa	Ifunda	8° 02' 51.08"S 35°28' 11.91"E	1869	IR4	MT124701	2
5	Mbeya	Uyole	8° 53' 46.55"S 33°32' 41.31"E	1913.2	MBY5	×	2
6	Mbeya	Igurusi	8° 49' 19.79"S 33°50' 33.09"E	1270.6	MBY6	NA	NA
7	Iringa	Mgama	8° 05' 56.56"S 36°30'51.61"E	365.2	IR7	MT124703	1
8	Iringa	Kalenga	7° 48' 13.40"S 35° 36' 04.14"E	1548.1	IR8	MT124704	2
9	Morogoro	Mazimbu	6° 46' 30.52"S 37°39' 31.27"E	506.1	MOR9	MT124705	2
10	Morogoro	Mikese	6° 43' 59.38"S 37°55' 12.63"E	393.9	MOR10	NA	NA
11	Njombe	Halal	3°25' 40.92"S 34°46' 00.00"E	1240.3	MBY11	NA	NA
12	Njombe	Makambako	8°50' 51.15"S 34°50' 50.36"E	1778.1	MBY12	MT124706	1
13	Mbeya	Iyunga	8°56' 13.90"S 33°25' 17.52"E	1733.9	MBY13	MT124707	1
14	Iringa	Ikengeza	7° 07' 36.40"S 35° 41' 00.00"E	1155.5	IR14	MT124708	2
15	Morogoro	Matombo	7° 07' 26.98"S 37° 48' 31.43"E	449.7	MOR15	MT124709	2
16	Morogoro	Bwakila	7° 57' 19.66"S 38° 12' 46.06"E	223.2	MOR16	MT124710	2
17	Iringa	Mfyome	7° 53' 56.38"S 35° 14' 04.05"E	1790.3	IR17	NA	NA
18	Iringa	Kibebe	7° 48' 10.00"S 35° 45' 23.08"E	1647.1	IR18	NA	NA
19	Iringa	Luganga	7° 14' 58.22"S 36° 06' 00.00"E	1249	IR19	MT124711	1
20	Iringa	Kitayawa	8° 01' 52.10"S 36° 11'58.39"E	987.1	IR20	MT124712	2

E. t = *Exserohilum turcicum*, x = PCR positive DNA isolates, masl = meters above sea level, MBY=Mbeya, IR=Iringa, MOR=Morogoro, NA = not applicable representing the strains with low quality DNA, 1 represents mating type 1, 2 represents mating type 2.
Source: Nwanosike, 2016.

genotypes of the pathogen. Polymorphic information content of SSR06 and SSR 024 loci were 0.25 and 0.52, respectively, with mean of 0.39 (Table 4). Although the two loci were genetically informative, SSR 024 indicated high heterozygosity (0.23) and gene diversity (0.58) compared to SSR 06.

Genetic variability of *E. turcicum* based on mating type genes

Gel electrophoresis identified both *MAT 1* and *MAT 2* (Figure 3). Amplification of isolates that were *MAT 1* (α-clone locus) yielded a fragment size of 190 bp (Figure 3a)

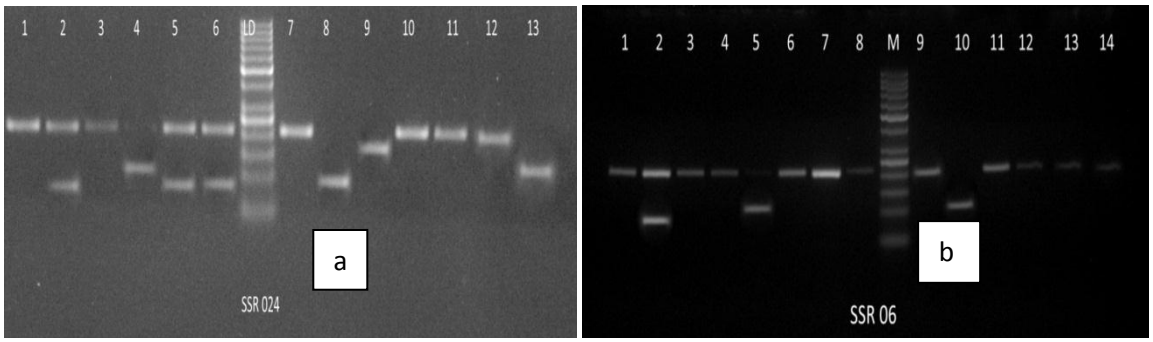

Figure 2. The polymerase chain reaction amplicons of maize derived *Exserohilum turcicum* strains generated by SSRs primer in a 1.5% agarose gel. Samples 1 - 14 and M/L DNA 100 base pair ladder, a = SSR 06 primer, b = SSR 024 primer.
Source: Nwanosike, 2016.

Table 4. Primer description, amplification, alleles, diversity, heterozygosity and polymorphic content of two SSR pairs, amplified polymorphic regions on 14 maize strains of *E. turcicum*.

Locus	Allele frequency	Genotype No.	Sample Size	No. of obs.	N_A	Availability	H	Heterozygosity	PIC
SSR 24	0.58	5	14	13	4	0.93	0.58	0.23	0.52
SSR 06	0.86	4	14	14	4	1.00	0.26	0.14	0.25
Mean	0.72	4.5	14	13.5	4	0.96	0.42	0.18	0.39

SSR = Simple sequence repeats, N_A = number of alleles, H = gene diversity, PIC = polymorphic information contents.
Source: Nwanosike, 2016.

while isolates for *MAT 2* (*MAT 2*- specific locus) produced fragment size of 195 bp (Figure 3b). Of the 14 isolates, four *MAT 1* (MBY 5, IR 8, IR 19 and MBY 13) and 10 *MAT 2* idiomorphs were observed. All Morogoro samples were *MAT 2* gene, while *MAT 1* and *2* existed in Mbeya, Njombe, and Iringa regions, however in an unequal proportion.

DISCUSSION

Information from the sequenced DNA and mating types indicated evidence of gene flow between populations of *E. turcicum* in the different maize fields of Tanzania. The microsatellites SSR06 and SSR24 loci and MAT type markers (*MAT1* and *MAT2*) polymorphism revealed strains of *E. turcicum*. The microsatellite regions of SSR06 and SSR24 loci fragment analysis of the 14 isolates revealed 4 allele. Genetic diversity of 0.42 and polymorphic information content of 0.39 (Table 4) demonstrated that sexual reproduction may have been responsible for the genotypes. Such results showed that five different strains of *E. turcicum* existed in the population. In South Africa, Haasbrock et al. (2014) reported 90 alleles across 13 SSR loci and gene diversity of 0.074 to 0.929 per locus, with average diversity of 0.602 in maize and sorghum *E. turcicum*. The study also showed minimum detection of 2 and maximum of 19 allele with amplification length of 191 to 493 bp, respectively.

The existence *MAT 2* dominated *MAT 1* in *E. turcicum* strains in an un-equal proportion, may be due to low frequency of genetic recombination, affected by location and genetic variation. The observed clones and mutants of *E. turcicum* isolated from Mbeya region could be due to frequent contact of sexually compatible strains, rapid and abundant inoculum and production of multiple generations and cycles of asexual reproduction. This may lead to high prevalence of disease in the Southern regions of Tanzania (Nwanosike et al., 2017). Maize is widely cultivated in Tanzania, particularly in the humid Southern Highlands, thus, the different climatic zones and cropping patterns as well as systems employed in different maize growing regions of the country may have also subjected the pathogen to different agro-ecological environments resulting in different mating types and genetic diversity.

The frequency and distribution of *MAT 1* and *MAT 2* in humid southern highlands of Mbeya, Njombe and Iringa regions indicated a possibility of sexual reproduction and a good potential for sexual recombination than in the dry lowland of Morogoro Region (Table 3). Random

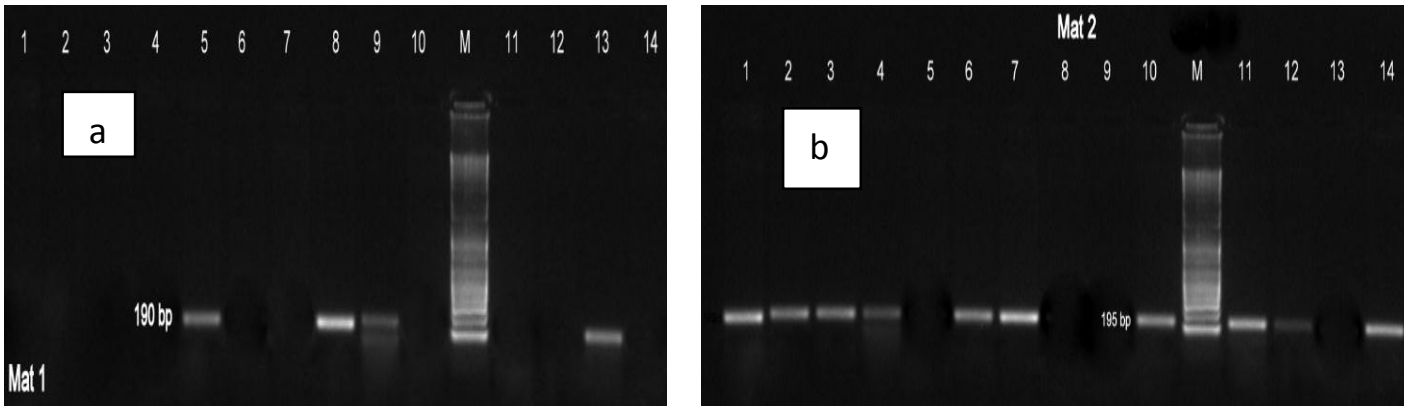

Figure 3a and b. PCR amplicons of *E. turcicum* strains a = α-clone gene (190 bp-*MAT 1*) and b = *MAT 2* specific gene (195 bp).
Source: Nwanosike, 2016.

distribution of mating types in the field may have enhanced chances of sexual reproduction (Welz et al., 1996). Borchardt et al. (1998b) also reported that severe epidemics of NLB in tropical environment resulted in higher population densities and more sexually compatible strains of *E. turcicum*. Similar condition was reported in Mbeya and Iringa regions, possibly due to consistent cultivation of susceptible maize cultivars, high *E. turcicum* population density and favourable climatic factors (Nwanosike et al., 2015).

Conclusion

The SSR and specific MAT markers used in this study explained variation in the genotypes of the 14 fungal isolates in Tanzania. The occurrence and distribution of *MAT 1* and *MAT2*, revealed the *E. turcicum* collected from Southern regions of Tanzania (Mbeya, Njombe, and Iringa) to be more diverse than isolates from Eastern regions (Morogoro). Following the current findings, there is a need for further diversity study on *E. turcicum* isolates collected from different zones of Tanzania by using robust molecular and sequencing techniques.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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