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Relationships among Brazilian and worldwide isolates of *Fusarium oxysporum* f. sp. *lactucae* race 1 inferred from ribosomal intergenic spacer (IGS-rDNA) region and *EF*-1α gene sequences

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Abstract Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lactucae* (FOLac), is amongst the main diseases affecting lettuce in subtropical regions. Although nationwide surveys indicated the exclusive presence of FOLac race 1 in Brazil, no detailed studies are available providing molecular evidences if these isolates were introduced into the country via contaminated seeds or if they are endemic populations. The translation elongation factor 1α (*EF*-1α) gene and rDNA intergenic spacer (IGS-rDNA) region represent the most comprehensive databases for comparative analyses of *Fusarium* isolates. Our aim was to assess the genetic relationships of 23 Brazilian FOLac race 1 isolates with a collection of FOLac isolates of worldwide origin, using the information from these genomic regions. A consistent single-cluster pattern was observed for FOLac race 1 isolates from Brazil, California-USA, Arizona-USA, Japan, Italy, as well as the novel FOLac race 4 isolates from the Netherlands based upon the *EF*-1α (604 nucleotides) and the IGS-rDNA (1859 nucleotides) sequences. Our analysis (based upon six single nucleotide polymorphisms identified only in the IGS-rDNA sequence) allowed the identification of intra-race 1 variation with the discrimination of four haplotypes. Isolates from Brazil, Italy, and a subset from the USA were classified into a single haplotype. The low diversity levels and the presence of only a single haplotype across the entire country are strong indications that Brazilian FOLac race 1 isolates are result of recent introduction event(s). This fast and widespread distribution of FOLac race 1 in Brazil has occurred more likely via importation and planting of contaminated seeds.

Keywords *Lactuca sativa* · Lettuce · Fusarium wilt · Phylogenetic analysis

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Introduction

The Fusarium wilt [caused by four races of *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *lactucae* Matuo and Motohashi (FOLac)] is one of the main diseases of lettuce (*Lactuca sativa* L.) in tropical and subtropical regions. Plants affected by this disease are initially chlorotic and with reduced growth rate. As the disease progress, the symptoms become more severe and may include death of older leaves, wilting, and collapse of the whole plant. Susceptible plants display an intense and typical vascular discoloration (Fujinaga et al. 2001; Garibaldi et al. 2004b; Scott et al. 2010). Yield losses

due to Fusarium wilt may vary from 20 to 70%, depending upon environmental conditions, management practices, and resistance/tolerance levels of the cultivars (Pasquali et al. 2007).

FOLac is seed-transmissible (Davis et al. 1997; Garibaldi et al. 2004a; Mbofung and Pryor 2010; Gullino et al. 2014) and this characteristic can, in part, explain the current worldwide distribution of this pathogen (Gilardi et al. 2017a). In geographic terms, race 1 is the most widespread FOLac variant, being reported in Japan (Matuo and Motohashi 1967), United States (Hubbard and Gerik 1993; McCreight et al. 2005), Taiwan (Huang 1998), Iran (Millani 1999), Italy (Garibaldi et al. 2002), Portugal (Pasquali et al. 2007), Argentina (Malbrán et al. 2014) and France (Gilardi et al. 2017b). Japan is the only country so far with the presence of three distinct races (1, 2, and 3) (Fujinaga et al. 2001, 2003; Yamauchi et al. 2004). Outside Japan, race 3 was only reported in Taiwan (Lin et al. 2014). Isolates of the novel FOLac race 4 were identified so far only in the Netherlands (Gilardi et al. 2017a).

In Brazil, the lettuce crop occupies an area of approximately 87,000 ha with the main producing States being São Paulo, Rio de Janeiro, Minas Gerais, Paraná, Rio Grande do Sul, and the Federal District (IBGE 2017). The main varietal groups of lettuce cultivated in the country are: loose-leaf (62.1%), iceberg-type (25%), butter-head (10.2%), and romaine (2.7%). The fusarium wilt of lettuce was initially reported in the State of Espírito Santo (Ventura and Costa 2008) and, subsequently, in other States of the Southeast region (Rio de Janeiro, São Paulo, and Minas Gerais) as well as in States of the Southern subtropical region (Rio Grande do Sul, Santa Catarina, and Paraná). Recent nationwide surveys and characterization of Fusarium wilt agents via pathogenicity tests and molecular markers revealed the exclusive presence of FOLac race 1 isolates in all major lettuce-producing regions in Brazil (Cabral et al. 2014). However, no detailed studies are available providing evidences if their nationwide spread was done via importation and planting of contaminated seeds, and, if so, from which geographic location(s) these isolates were originated. Alternatively, these isolates may represent potentially endemic fungal populations.

Distinct genomic regions have been employed to estimate the genetic relationships among populations and *formae speciales* within members of the *F. oxysporum* species complex (FOSC). For the genus *Fusarium*, the partial *EF*-1α gene sequence plus the entire IGS-rDNA region are currently the more comprehensive public sequence databases available for comparative analyses of isolates (Kawabe et al. 2005; Llorens et al. 2006; Mbofung et al. 2007; Enya et al. 2008; Dissanayake et al. 2009; O'Donnell et al. 2009; Amatulli et al. 2010; Srinivasan et al. 2011; Gilardi et al. 2017a). In this context, the aim of the present study was to examine the genetic relationships of these FOLac race 1 isolates obtained from infected lettuce plants across major producing regions of Brazil with a worldwide set of FOLac isolates based upon sequence analyses of the partial *EF*-1α gene and the entire IGS-rDNA region.

Material and methods

Brazilian Fusarium oxysporum isolates associated with lettuce and genomic DNA purification Twenty-three monoconidial isolates were collected infecting lettuce plants in different producing regions, encompassing seven Brazilian States (see Table 1 for the information about geographical origin of the isolates). All these 23 monosporic isolates were classified as FOLac race 1 since they were unable to induce disease in the cultivar 'Costa Rica No. 4', but were highly virulent to the cultivar 'Banchu Red Fire' (Cabral et al. 2014). Race 1 classification of these 23 FOLac isolates was also confirmed in PCR assays using primer pair Hani (5′ GAA-CCC-TCC-AAC-ATT-CAA-CA 3′) and Hanilatt3rev (5' CCT-CCA-ACA-TTC-AAC-AAC-AAT-G 3′), essentially as described by Pasquali et al. (2007) but with a modification (30 cycles with an extension step of 72° C for 30 s). Four non-pathogenic *F. oxysporum* isolates associated with lettuce plants (Cabral et al. 2014) were collected in the Federal District ($n = 1$) and Espírito Santo State ($n = 3$) and also included in the present study (Table 1). All fungal isolates were maintained either in Petri dishes filled with Potato Dextrose Agar medium supplemented with penicillin (PDA-t), or in glass tubes filled with sterile distilled water (Castellani 1939) or in a deep freezer at -80° C (Dingra and Sinclair 1995). The total DNA of the fungal isolates was extracted using a CTAB 2X (pH = 8.0) protocol, employing chloroform/isoamyl alcohol as solvents (Boiteux et al. 1999). Purified DNA was stored in microcentrifuge tubes containing Tris:EDTA buffer (10: 0.5 mM) in a freezer (at -20 °C) until use. DNA was stored at -20 °C before its use in PCR assays.

Table 1 Collection of *Fusarium oxysporum* f. sp. *lactucae* isolates associated with lettuce (*Lactuca sativa* L.) from Brazil and a distinct set of *Fusarium* species and *formae speciales* (species, accession number, original host plant, and geographic origin of the isolate), which translation elongation factor 1α (*EF*-1α) gene and the rDNA intergenic spacer (IGS-rDNA) region sequences were employed in diversity analyses

Fusarium/ *formae speciales*/ species	GenBank accession number		Isolate designation	Host	Geographic origin
	EF -1α	IGS-rDNA			
F. oxysporum f. sp. *lactucae* race 1	KY561356	KY352887	Fus–171	Lettuce	Espírito Santo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561357	KY352888	Fus–172	Lettuce	Espírito Santo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561358	KY352889	Fus–173	Lettuce	Santa Catarina – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561359	KY352890	Fus–174	Lettuce	Santa Catarina – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561360	KY352891	Fus–187	Lettuce	Minas Gerais – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561361	KY352892	Fus–202	Lettuce	Minas Gerais – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561362	KY352893	Fus–203	Lettuce	Espírito Santo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561363	KY352894	Fus–205	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561364	KY352895	Fus–206	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561365	KY352896	Fus–207	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561366	KY352897	Fus–208	Lettuce	Rio de Janeiro – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561367	KY352898	Fus–209	Lettuce	Rio de Janeiro – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561368	KY352899	Fus–210	Lettuce	Rio de Janeiro – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561369	KY352900	Fus–219	Lettuce	Paraná – Brazil
F.oxysporum	KY561352	KY352901	Fus–221	Non-pathogenic	Espírito Santo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561370	KY352902	Fus–222	Lettuce	Santa Catarina-Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561371	KY352903	Fus–223	Lettuce	Paraná – Brazil
F. oxysporum	KY561354	KY352904	Fus–224	Non-pathogenic	Espírito Santo – Brazil
F. oxysporum	KY561353	KY352905	Fus–225	Non-pathogenic	Espírito Santo – Brazil
F. oxysporum f. sp. *lactucae* race1	KY561372	KY352906	Fus–227	Lettuce	Santa Catarina – Brazil
F. oxyporum	KY561355	KY352907	Fus–237	Non-pathogenic	Distrito Federal – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561373	KY352908	Fus–240	Lettuce	Rio Grande do Sul – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561374	KY352909	Fus–241	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561375	KY352910	Fus–242	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561376	KY352911	Fus–243	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561377	KY352912	Fus–252	Lettuce	São Paulo – Brazil
F. oxysporum f. sp. *lactucae* race 1	KY561378	KY352913	Fus–253	Lettuce	Paraná – Brazil
F. oxysporum f. sp. *lactucae* race 1	DQ837658	DQ831864	BMP 1300	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837659	DQ831865	BMP 1301	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837660	DQ831866	BMP 1306	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837661	DQ831867	BMP 1307	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ 837662	DQ831868	BMP 1308	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ8363	DQ831869	BMP 1323	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ 837664	DQ831870	BMP 1324	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ 837665	DQ831871	BMP1326	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837666	DQ831872	BMP1331	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ 837667	DQ831873	BMP1333	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837668	DQ831874	HL-1	Lettuce	California – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837669	DQ831875	HL-2	Lettuce	California – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837670	DQ831879	BMP1880	Lettuce	Arizona – USA

Table 1 (continued)

Fusarium/ *formae speciales*/ species	GenBank accession number		Isolate designation	Host	Geographic origin
	EF -1α	IGS-rDNA			
F. oxysporum f. sp. *lactucae* race 1	DQ837671	DQ831876	BMP1363	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837672	DQ831877	BMP1370	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837673	DQ831878	BMP1375	Lettuce	Arizona – USA
F. oxysporum f. sp. *lactucae* race 1	DQ837657	DQ831863	S-1	Lettuce	Japan
F. oxysporum f. sp. *lactucae* race 1	KU840919	KU840912	ATCCMya-3040	Lettuce	Italy
F. oxysporum f. sp. *lactucae* race 1	KU840921	KU840914	Fuslat 1.14	Lettuce	Italy
F. oxysporum f. sp. *lactucae* race 1	KU840920	KU840913	Fuslat 3.14	Lettuce	Italy
F. oxysporum f. sp. *lactucae* race 2	DQ837693	DQ831893	F9501	Lettuce	Japan
F. oxysporum f. sp. *lactucae* race 2	DQ837694	DQ831892	FK09701	Lettuce	Japan
F. oxysporum f. sp. *lactucae* race 3	KU840924	KU840917	MAFF744085	Lettuce	Japan
F. oxysporum f. sp. *lactucae* race 3	KU840925	KU840918	MAFF744086	Lettuce	Japan
F. oxysporum f. sp. *lactucae* race 4	KU840923	KU840916	PD015/04750896	Lettuce	Netherlands
F. oxysporum f. sp. *lactucae* race 4	KU840922	KU840915	PD015/04750888	Lettuce	Netherlands
F. oxysporum	DQ837676	DQ831881	BMP1389	Non-pathogenic	Arizona – USA
F. oxysporum	DQ837674	DQ831880	BMP 1385	Non-pathogenic	Arizona – USA
F. oxysporum	DQ837675	DQ831883	BMP 1388	Non-pathogenic	Arizona – USA
F. oxysporum	DQ837677	DQ831882	BMP1397	Non-pathogenic	Arizona – USA
F. oxysporum f. sp. *cepae*	DQ837681	DQ831891	NRRL22538	*Allium*	Germany
F. oxysporum f. sp. *vasinfectum*	DQ837680	DQ831896	NRRL25231	*Gossypium*	*N. Carolina* – USA
F. oxysporum f. sp. *perniciosum*	AF008506	FJ985461	NRRL22550	*Albizia*	–
F. oxysporum f. sp. *canariensis*	AF008485	FJ985485	NRRL26035	*Cactus*	–
F. oxysporum f. sp. *rhois*	DQ837683	DQ831898	NRRL26227	*Rhus*	Israel
F. oxysporum f. sp. *mathiolae*	DQ837682	DQ831899	NRRL22545	*Matthiola*	Germany
F. oxysporum f. sp. *fabae*	DQ837684	DQ831902	NRRL26411	*Vicia*	USA
F. oxysporum f. sp. *heliotropa*	DQ837685	DQ831903	NRRL26411	*Heliotropium*	USA
F. oxysporum f. sp. *phaseoli*	DQ837686	DQ831900	NRRL26445	*Phaseolus*	*S. Carolina* – USA
F. oxysporum f. sp. *opuntiarum*	DQ837689	DQ831884	NRRL28934	*Opuntia*	Netherlands
F. oxysporum f. sp. *medicaginis*	DQ837690	DQ831901	NRRL22546	*Medicago*	Asia
F. oxysporum f. sp. *vasinfectum*	DQ837695	DQ831885	FOV14	*Gossypium*	California – USA
F. oxysporum f. sp. *asparagi*	DQ837691	DQ831886	FOA50	*Asparagus*	Australia
F. oxysporum f. sp. *lycopersici*	DQ837692	DQ831894	FOLR2	Tomato	California – USA
F. oxysporum f. sp. *callistephi*	DQ837679	DQ831897	NRRL22536	*Rhus*	Germany
F. oxysporum f. sp. *batatas*	DQ837678	DQ831895	NRRL22535	*Potato*	Germany
F. oxysporum f. sp. *spinaciae*	DQ837687	DQ831888	NRRL26871	*Spinacia*	Japan
F. oxysporum f. sp. *melonis*	DQ837696	DQ831887	TX388	*Cucumis melo*	Texas – USA
F. oxysporum f. sp. *cubense*	AF008493	FJ985483	NRRL26029	*Musa*	–
F. commune	AF246832	HM057283	NRRL28387	*Pisum sativum*	Netherlands
F. subglutinans	DQ837698	DQ831904	BMP1462	*Ananas*	Arizona – USA

PCR assays of the EF-1α gene and IGS-rDNA region of the 27 Brazilian F. oxysporum isolates associated with lettuce PCR assays were carried out using as template total genomic DNA of all 27 *F. oxysporum* Brazilian isolates with the primer pair 'EF-1H' and 'EF-2 T' (Table 2), which were designed to target conserved

Table 2 PCR primers targeting the translation elongation factor 1α (*EF*-1α) gene and the rDNA intergenic spacer region (IGS-rDNA) that were used in the present study to amplify the corresponding genomic regions of 23 isolates of *Fusarium oxysporum* f. sp. *lactucae* race 1 and four non-pathogenic *F. oxysporum* isolates found in association with lettuce plants in Brazil

Primer code	Genomic region	Primer sequence	Reference
EF-1H[a]	*EF*-1α	5'-ATGGGTAAGGAAGACAAGAC-3′	O'Donnell et al. (1998)
EF-2T[b]	*EF*-1α	5'-GGAAGTACCAGTGATCATGTT-3′	O'Donnell et al. (1998)
CNL12[a]	IGS-rDNA	5'-CTGAACGCCTCTAAGTCAG-3′	Anderson and Stasovski (1992)
CNS1[b]	IGS-rDNA	5'-GAGACAAGCATATGACTACTG-3′	White et al. (1990)
RCN61[b]	IGS-rDNA	5'-AGCCGACATCAAATTGACC-3′	Mbofung et al. (2007)
U46.67[b]	IGS-rDNA	5'-AATACAAGCACGCCGACAC-3′	Mbofung et al. (2007)
CN34[b]	IGS-rDNA	5'-CCAACACATGGGTGGTACCG-3′	Mbofung et al. (2007)
CNS12[b]	IGS-rDNA	5'-GCACGCCAGGACTGCCTCGT-3′	Mbofung et al. (2007)

[a] forward primers

[b] reverse primers

segments of the *EF*-1α gene. The complete IGS-rDNA region of all 27 isolates was amplified using 'CNL12' as the forward primer and 'CNS1' as the reverse primer (Table 2). PCR reactions were composed by 3 μL of fungal genomic DNA (20 ng/μL), 2 μL 10X buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1 μL $MgCl_2$ (50 mM), 1.6 μL dNTPs (2.5 M each), 0.4 μL *Taq* DNA polymerase (5 units/μL), 1.6 μL of each primer and 8.8 μL Milli-Q (Millipore Co., Bedford, MA, USA) water, with a final reaction volume of 20 μL. The PCR conditions were carried out for both *EF*-1α gene and IGS-rDNA region essentially as described by Mbofung et al. (2007) with an initial denaturation step (94 °C for 5 min) followed by 35 cycles of 94 °C for 1 min, 62 °C for 1.5 min., 72 °C for 2 min., and a final extension step (72 °C for 5 min). Amplicons were analyzed in agarose gel (1%) electrophoresis, stained with ethidium bromide and visualized under UV light.

Phylogenetic analysis The *EF*-1α gene-derived amplicons (≈ 700 bp) were purified from the gel using a PureLink® Quick Gel Extraction Kit (Invitrogen, Waltham–MA) and, subsequently, they were directly sequenced using the same primer pair. Sequencing was done at an ABI 3100 (Applied Biosystems) sequencer from the Genomic Analysis Laboratory (at CNPH, Brasília–DF, Brazil), using the kit ABI Prism BigDye version 3.1 (Applied Biosystems, Foster City–CA). Due to their large size, the sequencing reactions of the IGS-rDNA fragments (≈ 2800 bp) were carried out employing a subset of internal primers (viz. 'RCN61', 'U46.67', 'CN34', 'CNS12', and 'CNS1') as described by Mbofung et al. (2007) (for primer information see Table 2). Sequencing was performed in both forward and reverse directions in order to increase the accuracy of the final nucleotide sequences. The *EF*-1α and IGS-rDNA sequences were assembled and edited according to base pair quality as evaluated using the Lasergene Molecular Biology Package (DNAstar, Madison, WI, USA). Ambiguities and other errors were verified in the corresponding electropherograms and then removed and/or corrected manually. All *EF*-1α and IGS-rDNA sequences of the FOLac race 1 isolates from Brazil were submitted to GenBank (Table 1). Sequences of all available FOLac isolates in addition to a subset of reference *F. oxysporum* species complex (FOSC) isolates as well as the selected outgroups (*F. commune* and *F. subglutinans*) were retrieved from the GenBank (Table 1). A dataset of 604 bp was obtained for *EF*-1α and 1859 bp for IGS-rDNA. The multiple sequence alignments of the *EF*-1α and IGS-rDNA sequences were performed with the help of the MAFFT software (Katoh and Standley 2013) as a plugin of Geneious R8. The phylogenetic analyses were carried out for each gene individually and also by concatenating the sequences of both regions (*EF*-1α and IGS-rDNA). The substitution model of MEGA 6.0 software (Tamura et al. 2013) was chosen with the command "Find best DNA/Protein Models". The phylogenetic analysis was performed with distinct algorithms. The maximum likelihood was done using the Hasegawa-Kishino-Yano Model (HKY) model (Hasegawa et al. 1985) with

gamma distribution and 1000 bootstraps, run with PHYLM plugin and nearest neighbor interchange search. The maximum parsimony analysis was carried out on PAUP 4.0 (Swofford 2003) using random step addition as parameter (1000 replicates) with branch swapping and tree bisection-reconnection. The analysis by Bayesian inference was performed on MrBayes (Huelsenbeck and Ronquist 2001) plugin (version 3.2.2) in Geneious R8 with GTR (General time-reversible) substitution model (Tavaré 1986), with gamma distribution, 4,000,000 generations chain, subsampling each 100 and burn-in of 25%. Due their high levels of similarities, the trees topologies were compared using concatenated data from *EF*-1α and IGS-rDNA regions. Only the Bayesian inference of concatenated *EF*-1α and IGS-rDNA sequences is presented here. The bootstrap values of maximum likelihood, maximum parsimony from the concatenated sequence analysis were transferred to the Bayesian inference tree.

Identification of single nucleotide polymorphisms (SNPs) and haplotype determination in a collection of FOLac race 1 isolates from Brazil and from reference isolates of worldwide origin The concatenated *EF*-1α and IGS-rDNA sequences of 23 Brazilian FOLac race 1 isolates were aligned with 20 worldwide reference isolates from FOLac race 1 available at GenBank and two Dutch race 4 isolates (Table 1) using Megalign (DNASTAR Inc., Madison, WI, USA). SNPs were identified after sequences alignment. Haplotype analysis was made with the aid of DnaSP v.5 (Librado and Rozas 2009). This haplotype analysis included single base substitutions, deletions or insertions of single or multiple bases.

Results

PCR assays and amplicon profiles The *EF*-1α primers ('EF-1H' and 'EF-2 T') amplified fragments of 672 bp to 720 bp for all isolates. The IGS-rDNA primers 'CNL12' and 'CNS1' generated a product between 2382 bp and 2800 pb for all isolates. Internal primers were used to generate smaller fragments of the IGS-rDNA (between 400 and 600 bp) in order to allow the complete sequencing of this genomic region.

EF-1α gene and IGS-rDNA sequencing and phylogenetic analyses It was observed that the data set derived from the IGS-rDNA region contains more informative sites than that of the *EF*-1α gene. The assembly of the *EF-1α* and IGS-rDNA contigs and their alignment, after trimming based on sequence quality, yielded a sequence of 604 and 1859 characters, respectively. From those, 545 and 1295 were constant; 59 and 564 were variable; 29 and 220 were parsimony-informative for the *EF*-1α and IGS-rDNA, respectively. The concatenated sequence with *EF*-1α and IGS-rDNA, generated a data set of 2461 characters, of which 1835 were constant, 375 variables, and 251 parsimony-informative. The maximum parsimony (MP) analysis for the concatenated sequence with *EF*-1α and IGS-rDNA presented values of 916 for tree length; 0.7555 for the consistency index (CI) and 0.7528 retention index (RI) for all sites. For the IGS-rDNA region the tree length was 822; CI was 0.7683 and RI 0.7497, whereas the *EF*-1α the tree length was 65; CI 0.9692 and RI 0.9835. The consensus tree is shown at Fig. 1 with geographic origin information (represented by colored blocks) for the collection of Brazilian FOLac race 1 isolates and four non-pathogenic *F. oxysporum* along several worldwide references. Twenty-three Brazilian isolates from FOLac race 1 (viz. Fus-171, Fus-172, Fus-173, Fus-174, Fus-187, Fus-202, Fus-203, Fus-205, Fus-206, Fus-207, Fus-208, Fus-209, Fus-210, Fus-219, Fus-222, Fus-223, Fus-227, Fus-240, Fus-241, Fus-242, Fus-243, Fus-252, and Fus-253) grouped with other USA, Italy and Japan isolates of the same race (viz. BMP 1300, BMP 1301, BMP 1306, BMP 1307, BMP1308, BMP 1323, BMP 1324, BMP 1326, BMP 1331, BMP 1333, BMP 1363, BMP1370, BMP 1375, BMP1880, HL-1, HL-2, S-1, ATCCMya-3040, Fuslat 1.14, and Fuslat 3.14), with the two isolates of the novel FOLac race 4 (viz. *L. sativa* 04750888 and *L. sativa* 04750896) and with other three *formae speciales* of *F. oxysporum* (viz. *rhois, phaseoli,* and *matthiolae*, forming the clade 1) (Fig. 1).

EF-1α and IGS-rDNA analysis The maximum parsimony, maximum likelihood, and Bayesian inference analyses of the single regions (*EF*-1α or IGS-rDNA) displayed tree topologies very similar to the concatenated analysis with elevated boostrap and posterior probability levels (≥60 and ≥0.96, data not shown). However, the tree generated from *EF*-1α data included six other *formae speciales* than *lactucae* on clade I: *cepae*, *heliotropii*, *fabae*, *rhois*, *phaseoli,* and *matthiolae*. For the IGS-rDNA region the results were similar to the concatenated and only three *formae speciales* of *F. oxysporum* (viz. *rhois, phaseoli,* and *matthiolae*) are on clade 1 (data not show).

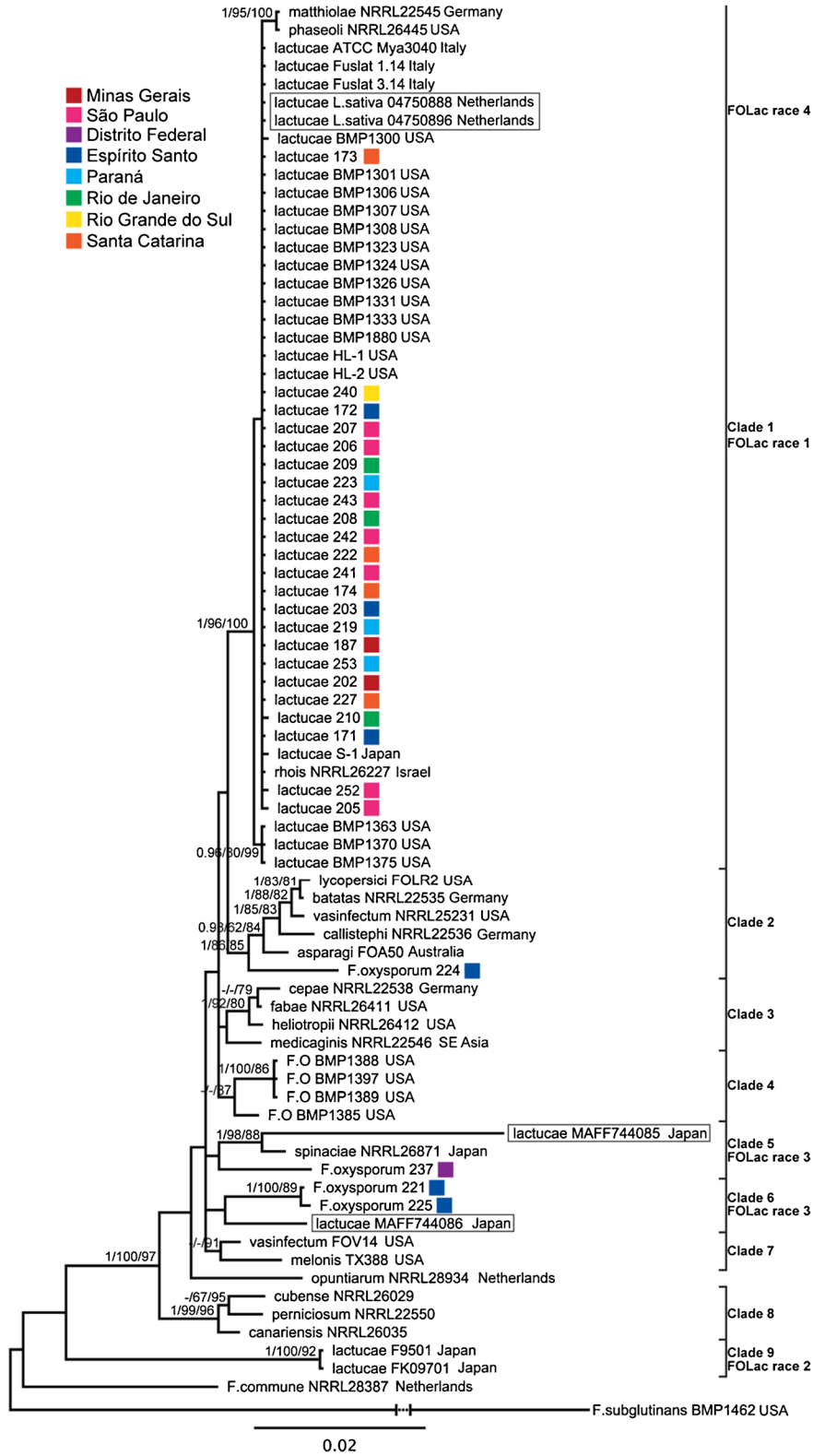

Fig. 1 Phylogenetic tree of concatenated sequences of the *EF*-1á and IGS-rDNA regions of 23 Brazilian *Fusarium oxysporum* f. sp. *lactucae* (FOLac) race 1 isolates, four non-pathogenic *F. oxysporum* isolates and 49 reference sequences from Genbank, among these, *F. subglutinans* and *F. commune* that were employed as outgroups. Values on branches correspond to posterior probability of Bayesian inference followed by maximum likelihood and maximum parsimony bootstraps, those without values are below acceptable posterior probability/ bootstrap (≤0.96 bayesian, ≤60 maximum likelihood and maximum parsimony). The Brazilian isolates were identified with color blocks corresponding to the a specific State from where the isolate was originated

Determination of FOLac races 1 and 4 haplotypes derived from analyses of the EF-1α gene and IGS-rDNA region SNPs were detected only within six sites of the IGS-rDNA region of the FOLac isolates (Table 3). The *EF*-1α gene segments were identical for all FOLac isolates. Intrarace variation was not observed among the Brazilian isolates. All FOLac race 1 isolates from Brazil and Italy as well as a subset of race 1 isolates from the United States were classified into a single haplotype based upon the six single nucleotide polymorphisms (SNPs) detected in the IGS-rDNA region (Table 3). Four haplotypes were identified among the FOLac race 1 isolates. The 23 Brazilian FOLac race 1 isolates and 15 isolates from USA, Italy (BMP-1301, BMP-1306, BMP-1307, BMP-1308, BMP-1323, BMP-1324, BMP-1326, BMP-1331, BMP-1333, BMP-1880, HL-1, HL-2, ATCCMya-3040, Fuslat 1.14, Fuslat 3.14) as well as race 4 isolates from the Netherlands (*L. sativa* 04750888 and *L. sativa* 04750896) were arbitrarily classified as haplotype I. The isolate BMP-1300 (from the USA) was the only isolate classified as haplotype II. The haplotype III group was composed by three isolates from the USA (BMP-1363, BMP1370, and BMP-1375), whereas only the Japanese isolate S-1 (DQ831863) was classified as haplotype IV.

Discussion

Distinct genomic regions have been employed to estimate the genetic relationships among populations and *formae speciales* within members of the *F. oxysporum* species complex (FOSC). So far, the information employed in phylogenetic studies of FOSC is derived from the sequence analyses of the mitochondrial small ribosomal subunit-DNA (mtSSU rDNA) and from the rDNA intergenic spacer (IGS-rDNA) regions as well as from genes coding for polygalacturonases, phosphate permease, nitrate reductase, β-tubulin, translation elongation factor 1α (*EF*-1α), and two mating-type idiomorphs (*MAT*1–1 and *MAT*1–2) (Mbofung et al. 2007; Hirano and Arie 2009; Wulff et al. 2010). The partial *EF*-1α gene sequence (Mbofung et al. 2007; Amatulli et al. 2010) in combination with the entire IGS-rDNA sequence are the two most phylogenetic informative genomic regions for the genus *Fusarium*, and, therefore, more comprehensive and fast-increasing databases are currently available (Kawabe et al. 2005; Llorens et al. 2006; Mbofung et al. 2007; Enya et al. 2008; Dissanayake et al. 2009; O'Donnell et al. 2009; Srinivasan et al. 2011; Gilardi et al. 2017a).

Phylogenetic approaches are useful not only to study the geographical origin of isolates but also for investigating overall diversity among isolates of plant pathogenic fungi. To estimate the relationships among closely related individuals (such as races and isolates of FOLac), it is mandatory to employ highly informative genomic regions with sufficient phylogenetic signal. In the present study, genetic information derived from the sequence analysis of the *EF*-1α gene and IGS-rDNA region was employed aiming to clarify the phylogenetic relationships among FOLac race 1 isolates collected in major lettuce-producing regions of Brazil. The isolates used in this work have been previously characterized at *forma specialis* and race levels by using molecular and biological tests (Cabral et al. 2014). Of the chosen genomic regions (*EF*-1α and IGS-rDNA), the last one revealed more variation among FOLac isolates.

Nationwide surveys are indicating the exclusive presence of FOLac race 1 isolates in all major lettuce-producing regions of Brazil. The genetic relationships of these Brazilian FOLac isolates with other of worldwide origin might elucidate if they were either introduced into the country via contaminated seeds or if they may represent potentially endemic fungal populations. One approach to this question would be the molecular characterization of these FOLac isolates employing of the most comprehensive databases available for comparative analyses of *Fusarium* isolates: the combination of the *EF*-1α gene and IGS-rDNA sequence information. In this context, the 23 isolates obtained from infected lettuce plants across major producing region in Brazil were submitted to more extensive comparative analyses with a worldwide set of FOLac isolates using genetic information derived from the partial *EF*-1α gene and the entire IGS-rDNA region.

The phylogenetic analysis based upon the IGS-rDNA and *EF*-1α genomic regions confirmed previous results obtained by Mbofung et al. (2007), in which, isolates of *F. oxysporum* f. sp. *lactucae* race 1 and race 2, were discriminated into two different genetic lineages. Fujinaga et al. (2005), analyzing IGS-rDNA region, revealed that FOLac isolates from races 1, 2, and 3 are genetically dissimilar and could have different origins. Isolates of FOLac races 1, 2, and 3 were found occurring in Japan and a considerable diversity was found among these isolates (Fujinaga et al. 2003). Like in previous studies (Fujinaga et al. 2005; Mbofung et al. 2007), isolates from race 1, 2

Table 3 Haplotypes defined by six single nucleotide polymorphisms (SNPs) detected within the rDNA intergenic spacer region (IGS-rDNA) sequence in a collection of 45 *Fusarium oxysporum* f. sp. *lactucae* (FOLac) race 1 and 4 isolates from Brazil, Japan, Italy, USA, and Netherlands

FOLac isolate	Country	Race #	Haplotype #	SNP position[1]					
				253	332	728	826	847	935
FUS 171	Brazil	1	I	–	G	G	T	G	G
FUS 172	Brazil	1	I	–	–	–	–	–	–
FUS 173	Brazil	1	I	–	–	–	–	–	–
FUS 174	Brazil	1	I	–	–	–	–	–	–
FUS 187	Brazil	1	I	–	–	–	–	–	–
FUS 202	Brazil	1	I	–	–	–	–	–	–
FUS 203	Brazil	1	I	–	–	–	–	–	–
FUS 205	Brazil	1	I	–	–	–	–	–	–
FUS 206	Brazil	1	I	–	–	–	–	–	–
FUS 207	Brazil	1	I	–	–	–	–	–	–
FUS 208	Brazil	1	I	–	–	–	–	–	–
FUS 209	Brazil	1	I	–	–	–	–	–	–
FUS 210	Brazil	1	I	–	–	–	–	–	–
FUS 219	Brazil	1	I	–	–	–	–	–	–
FUS 222	Brazil	1	I	–	–	–	–	–	–
FUS 223	Brazil	1	I	–	–	–	–	–	–
FUS 227	Brazil	1	I	–	–	–	–	–	–
FUS 240	Brazil	1	I	–	–	–	–	–	–
FUS 241	Brazil	1	I	–	–	–	–	–	–
FUS 242	Brazil	1	I	–	–	–	–	–	–
FUS 243	Brazil	1	I	–	–	–	–	–	–
FUS 252	Brazil	1	I	–	–	–	–	–	–
FUS 253	Brazil	1	I	–	–	–	–	–	–
BMP1301	USA	1	I	–	–	–	–	–	–
BMP1306	USA	1	I	–	–	–	–	–	–
BMP1307	USA	1	I	–	–	–	–	–	–
BMP1308	USA	1	I	–	–	–	–	–	–
BMP1323	USA	1	I	–	–	–	–	–	–
BMP1324	USA	1	I	–	–	–	–	–	–
BMP1326	USA	1	I	–	–	–	–	–	–
BMP1331	USA	1	I	–	–	–	–	–	–
BMP1333	USA	1	I	–	–	–	–	–	–
BMP1880	USA	1	I	–	–	–	–	–	–
HL-1	USA	1	I	–	–	–	–	–	–
HL-2	USA	1	I	–	–	–	–	–	–
ATCCMya-3040	Italy	1	I	–	–	–	–	–	–
Fuslat 1.14	Italy	1	I	–	–	–	–	–	–
Fuslat 3.14	Italy	1	I	–	–	–	–	–	–
L.sativa 04750896	Netherlands	4	I	–	–	–	–	–	–
L.sativa 04750896	Netherlands	4	I	–	–	–	–	–	–
BMP1300	USA	1	II	–	–	T	–	–	–

Table 3 (continued)

FOLac isolate	Country	Race #	Haplotype #	SNP position[1]					
				253	332	728	826	847	935
BMP1363	USA	1	III	–	–	–	C	A	A
BMP1370	USA	1	III	–	–	–	C	A	A
BMP1375	USA	1	III	–	–	–	C	A	A
S-1	Japan	1	IV	C	A	–	–	–	–

Only parsimony-informative SNPs were included in the table

C, cytosine; T, thymine; A, adenine; G, guanine

[1] SNP number indicated the nucleotide number within the rDNA intergenic spacer region (IGS-rDNA) sequence that displayed an informative polymorphism among isolates. (−) = identical nucleotide

and 3 were placed in different clades, revealing a dissimilar origin for all three races and showing that the *forma specialis lactucae* is polyphyletic.

The polyphyletic origin of *formae speciales* of *F. oxysporum* has been already confirmed among members within the FOSC (Abo et al. 2005; Lievens et al. 2008). In the present work, isolates of *F. oxysporum* f. sp. *rhois, F. oxysporum* f. sp. *phaseoli*, and *F. oxysporum* f. sp. *matthiolae* were more closely related to FOLac race 1 isolates, being placed in the same clade. Studies conducted by Bogale et al. (2006), employing three molecular approaches, including the sequence of the *EF*-1α gene, observed that 18 *formae speciales* from *F. oxysporum* are not separated neither by host nor by geographic region, indicating that pathogenicity is not necessarily correlated with phylogenetic groupings. As mentioned by Correll (1991), the phylogenetic groups usually display low correlation with pathogenic groups, because the determination of *formae speciales* is based upon phenotypic characteristics (pathogenicity in specific plant or set of plants), which expression might be affected by environmental factors (e.g. temperature, host age, and inoculation methods). In addition, there are evidences that FOSC isolates may acquire specific host specialization in a somewhat fortuitous way (Correll 1991; Baayen et al. 2000). Several mutations and transposition events may lead to the independent acquisition of pathogenicity factors for the same host in genetically distant FOSC isolates (Baayen et al. 2000). Besides that, other mechanism may be involved in the acquisition of pathogenicity, as parasexuality and can contribute for the horizontal gene transfer from isolates distantly related (Van der Does et al. 2008). Ma et al. (2010) showed that non-pathogenic isolates of *F. oxysporum* f. sp. *lycopersici* can be easily converted into pathogenic isolates, via the transfer of either specific genes or entire chromosomes. These biological properties could explain the polyphyletic origin of some *formae speciales* and the emergence of new pathogenic lineages inside the FOSC.

The analysis of the concatenated sequences suggests that FOLac race 1 isolates are phylogenetically different from other reference isolates, while presenting a homogeneous population. The 23 race 1 Brazilian isolates grouped with other USA, Italy, and Japan isolates of the same race as well as with the two isolates of the novel FOLac race 4 (*L. sativa* 04750888 and *L. sativa* 04750896). They also clustered with other three *formae speciales* of *F. oxysporum* (*rhois, phaseoli,* and *matthiolae*), forming the clade 1. The recent work carried out by Gilardi et al. (2017a) pointed out that the genetic information derived from the *EF*-1α gene and the entire IGS-rDNA region is not able to provide sufficient resolution to discriminate FOLac race 1 from FOLac race 4 isolates, requiring the combination of pathogenicity tests as well as additional molecular marker assays (Gilardi et al. 2017a). In this context, it is important to highlight that all the 23 FOLac race 1 isolates from Brazil were unable to induce disease in the cultivar 'Costa Rica No. 4', but were highly virulent to 'Banchu Red Fire' (Cabral et al. 2014), which is in sharp contrast with FOLac race 4 isolates (Gilardi et al. 2017a).

Conflicting results have been obtained with the employment of the partial *EF*-1α gene sequence plus the entire IGS-rDNA region as a tool for detection of intra-race variability within members of the FOSC. For example, the IGS-rDNA region was found to be one of the few available tools for identification of genetic variability between races of

FOLac (Mbofung et al. 2007) and *F. oxysporum* f. sp. *dianthi* (Canizares et al. 2015). Likewise, Dissanayake et al. (2009) were able to identify four IGS-rDNA haplotypes within a population of 30 *F. oxysporum* isolates from onions in Japan. Similarly, Srinivasan et al. (2012) reported four different clusters among 31 isolates of *F. oxysporum* f. sp. *raphani* in Italy. On the other hand, Fujinaga et al. (2005) were unable to discriminate FOLac race 1 isolates from Japan into sub-clades employing the information of *EF*-1α and IGS-rDNA regions. Mbofung et al. (2007) and O'Donnell et al. (2009) also used IGS-rDNA sequences, but they observed some discrepancies of the phylogenetic relationships among FOLac isolates. According to these studies, the low IGS-rDNA haplotype diversity within *F. oxysporum* implies either low frequency or complete absence of sexual reproduction in this fungus.

Even with these limitations, we could identify worldwide FOLac intra-race 1 variation based upon six single nucleotide polymorphisms (SNPs) detected only in the IGS-rDNA region. All 23 Brazilian FOLac race 1 isolates and 15 isolates from USA, Italy (BMP-1301, BMP-1306, BMP-1307, BMP-1308, BMP-1323, BMP-1324, BMP-1326, BMP-1331, BMP-1333, BMP-1880, HL-1, HL-2, ATCCMya-3040, Fuslat 1.14, and Fuslat 3.14) as well as two race 4 isolates from the Netherlands (*L. sativa* 04750888 and *L. sativa* 04750896), were discriminated into the haplotype I. The isolate FOLac race 1 isolate BMP-1300 (from the USA) was the only one classified as haplotype II. The haplotype III group was composed by three isolates from the USA (BMP-1363, BMP1370, and BMP-1375), whereas only the Japanese isolate S-1 (DQ831863) was classified as haplotype IV. Therefore, the FOLac race 1 haplotype I encompassed 38 out 43 analyzed isolates (from Brazil and from worldwide origin). Perhaps the other USA haplotypes could be considered as haplotype I variants, with exception of haplotype IV (isolate S-1). Srinivasan et al. (2012) reported that during search for SNPs, sequence and/or alignment errors as well as defective algorithms could result in the annotation of false polymorphisms, resulting in the report of false and/or inexistent haplotypes.

It has been recommended the combination of other loci in order to increase the discrimination power of *F. oxysporum formae speciales* like: α-tubulin (tub2) (O'Donnell et al. 1998), endo-exo-polygalacturonase genes (Hirano and Arie 2009; Canizares et al. 2015), and SIX (secreted in xylem) genes (Thatcher et al. 2012). In our analysis, the IGS-rDNA region could not discriminate, for example, the FOLac race 1 and race 4 isolates from three other *formae speciales* of *F. oxysporum* (Fig. 1). In fact, more efficient detection of intra-race variability will demand in further studies the employment of other analytical techniques (e.g. RAPD, RFLP, IRAP, and SAAP-AFLP) that are more suitable in highlighting possible differences among FOLac isolates.

The high homogeneity levels and relative low number of SNPs and haplotypes found within clade 1, reinforces the notion that FOLac isolates race 1 have monophyletic origin (Gilardi et al. 2017a). Besides that, the 23 Brazilian race 1 isolates formed a single haplotype with FOLac race 1 isolates from USA and Italy (tentatively named as haplotype-1). The majority of genetic marker studies have been used with success to distinguish indigenous from introduced pathogens. Low genetic variation of fungal populations is a strong indication of a recent pathogen introduction in a given area (Engelbrecht et al. 2007). When a single pathogen variant is recently introduced in a given area, only a limited number of mutations are observed even when a large collection of isolates is analyzed (Harrington et al. 2003). Therefore, the low diversity levels and the presence of only a single haplotype across the entire country are strong indications that Brazilian FOLac race 1 isolates are result of recent introduction event(s). This fast and widespread distribution of FOLac race 1 in Brazil has occurred more likely via importation and planting of contaminated seeds (Davis et al. 2006; Garibaldi et al. 2004a; Mbofung and Pryor 2010; Gullino et al. 2014).

In conclusion, the analyses employing the *EF*-1α gene and IGS-rDNA sequence information indicated a consistent single-cluster pattern of the Brazilian isolates of FOLac race 1 and isolates of the same race from California-USA, Arizona-USA, Italy and Japan, reinforcing the notion of the common origin of this pathogen variant (Gilardi et al. 2017a). All isolates causing lettuce wilt in Brazil were classified into a single haplotype, suggesting that the FOLac race 1 isolates were more likely result of recent and widespread introduction event(s), probably through contaminated seed lots, which has been reported as a very efficient propagation vehicle of this pathogen (Davis et al. 2006; Garibaldi et al. 2004a; Mbofung and Pryor 2010; Gullino et al. 2014).

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Compliance with ethical standards

Conflict of interest The authors do not have any conflict of interest.

Research involving human participants and/or animals Not applicable.

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