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Wolbachia strains in cryptic species of the *Anastrepha fraterculus* complex (Diptera, Tephritidae) along the Neotropical Region

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ABSTRACT

Infection by *Wolbachia* was described previously in eleven species of *Anastrepha* fruit flies some of which are important pests of fruticulture. One such species is the nominal *Anastrepha fraterculus*, the South American fruit fly, which actually comprises a complex of cryptic species. The suggestions of using *Wolbachia* for the control of these pest species, make imperative a more precise characterization of the existing strains of the bacteria. In this study, population samples of the *A. fraterculus* complex from Brazil, Argentina, Peru, Ecuador, Colombia, Guatemala and Mexico were analyzed for *Wolbachia* infection. The bacteria were genotyped by the MLST and WSP Typing methodologies. All samples were infected with *Wolbachia* of supergroup "A". For each of the five MLST genes, unique as well as already known alleles were detected. Nineteen sequence types for the concatenated sequences of the five MLST genes, and twenty *wsp* alleles were found in the samples. Host-specific haplotypes, shared strains among distinct hosts, and more than one strain of *Wolbachia* were found in some population samples. Recombination among the MLST genes and intragenic recombination between *wsp* haplotypes was rare. Phylogenetic analysis showed a great similarity among the *Wolbachia* strains in the *A. fraterculus* complex. However, some strains of *Wolbachia* are found throughout the Neotropical Region and there are specific strains in determined geographical areas.

Introduction

The bacteria *Wolbachia* found in both somatic and reproductive tissues of many species of arthropods and nematodes, has been shown to be associated with several effects in the reproduction of their hosts, such as cytoplasmic incompatibility (CI), parthenogenesis, feminization of genetic males and male killing [9,60,61]. The relationship between *Wolbachia* and their hosts varies in some cases evolving from a parasitic to a mutualistic condition [43,52,61].

Among frugivorous tephritid flies hosts *Wolbachia* infection was found to induce CI as showed in transinfected *Bactrocera oleae* [1] and *Ceratitis capitata* [65,66], and most likely in *Rhagoletis cerasi* [38]. Hence, their use in programs for suppression of fruit fly populations was proposed and tested [1,9,65,66].

In fruit flies of genus *Anastrepha,* endemic to the Neotropical Region, *Wolbachia* was found infecting 12 species [10,12,27,28,50,62], many of which are of economic importance such as those that belong to five infrageneric taxonomic groups, *fraterculus, grandis, pseudoparalella, striata* and *serpentina* [32]. The *fraterculus* species group received particular attention in recent years because it includes many of the pest species. One of the most important is the "South American fruit fly", *Anastrepha fraterculus* (Wiedemann) [63]. This nominal species actually comprises a complex of cryptic species, the *A. fraterculus* complex (AF complex), which is currently being characterized [10,11,17,18,46–49,51,55,58]. Morphometric analyses disclosed eight morphotypes within the AF complex, "Mexican" (with samples from Mexico and Central America), "Venezuelan" (from lowlands of Venezuela), "Andean" (from highlands of Venezuela and Colombia), "Peruvian" (from lowlands of Ecuador and Peru), "Ecuadorian" (from highlands of Ecuador and Peru), and three Brazilian morphotypes, "Brazilian-1" (*A.* sp.1 from Brazil and

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Argentina), "Brazilian-2" (*A.* sp.2), and "Brazilian-3" (*A.* sp.3) [17,18].

Crosses between AF complex morphotypes resulted in low viability of hybrid progenies due to several pre-zygotic and/or post-zygotic incompatibilities [10,13,14,40,45,48,49,59]. Since Brazilian-1 and Brazilian-2 morphotypes were found to be infected by *Wolbachia*, it was suggested that *Wolbachia* could be involved in the zygotic inviability observed in laboratory crosses [49,50]. However, a posterior study involving samples from Brazil, Argentina and Peru, similarities of *Wolbachia wsp* gene led to the suggestion that the bacteria would not be involved in the observed zygotic inviabilities [10]. However, the observed similarities of *Wolbachia* was determined just by analysis of the highly conserved regions (CRs) of the *Wolbachia wsp* gene, excluding the hypervariable regions of the gene.

So far, *Wolbachia* infecting the morphotypes of the AF complex were characterized by analysis of gene *ftsZ* [25,50] but mostly by gene *wsp* [10,12,25]. Recently, *Wolbachia* was found in five population samples of Brazilian-1 (*A.* sp.1 *aff. fraterculus*) but no details were given about the existing *wsp* alleles in the samples [14]. As is already known, a more reliable identification of strains is achieved when an approach of the MLST type (Multi Locus Sequence Typing) is applied. Such methodology, developed for *Wolbachia* [6,7,8,34], was employed successfully in fruit flies species of genera *Bactrocera* and *Dacus* [20,22,30,31], *Rhagoletis* [2,3,41] and *Anastrepha* [27].

In the present report, we describe results of the characterization of *Wolbachia* strains in samples of the *A. fraterculus* complex collected along the Neotropical Region using the MLST and the WSP Typing methodologies. The data show the existence of strains already known in the WSP data base, of several novel strains, the rare presence of putative recombinant sequences and a close phylogenetic relationship among the strains within this species complex.

Material and methods

Fruit fly samples

Samples of the AF complex from 48 locations along the Neotropical Region, including Brazil, Argentina, Peru, Ecuador, Colombia, Guatemala and Mexico (Fig. 1), were screened for *Wolbachia* infection. The analyzed adult flies from Brazil were obtained from infested fruits brought to the laboratory. After emergence, adult flies were preserved in ethanol at −20 °C. The samples from other localities along the Neotropical Region were obtained from batches of flies previously employed in morphometric analysis of the AF complex, which were preserved in ethanol [17]. Localities where the host fruits were collected are listed in Table 1.

DNA extraction, amplification and sequencing of gene fragments

DNA was extracted from single flies according to method previously used for *Anastrepha* [12], and *Wolbachia* infection was screened by the MLST and WSP Typing methodologies [6,7]. Amplification of the five MLST genes was made with the primers described by Baldo et al. [6]. The PCR cycles were of 2 min at 94 °C, 37 cycles (30 s at 94 °C, 45 s of annealing at 54 °C for *ftsZ, gatB, hcpA coxA*, and 59 °C for *fbpA*), 1 min 30 s at 72 °C, followed by10 min at 72 °C. For the *wsp* gene, the primers 81F and 691R [67], were used. The PCR steps were as follow: one cycle of 2 min at 95 °C, followed by 35 cycles (1 min at 95 °C, 1 min at 55 °C, 1 min at 75 °C), and a final cycle of 10 min at 72 °C. Infected samples of *C. capitata* [39] were used as positive control of amplification. As control for negative amplification the DNA templates were tested by amplification of gene 28S rDNA with primers and PCR conditions described by Werren et al. [62]. Samples that continued to be negative were discarded only after new DNA concentrations for amplification were tested, and in cases of persistent negative results new extractions were made on the samples. The amplified fragments were sequenced using primers DF2 and DR2 and the BigDye 2.0 kit for automated sequencing in an ABI Prism Automatic Sequencer (Applied Biosystems). Inspection of the electropherogram of each amplified fragment with the web tool *Electropherogram Quality Analysis* [57] showed no sequences with signals of slippage or double peaks. Furthermore, when identical sequences were obtained in two–five individuals in a given sample, the sequences were considered as free of PCR errors. In the samples where single sequences were obtained from single individuals, the PCR was repeated on the same DNA template to confirm the sequence data [44]. Furthermore, the conceptual amino acid translations of the sequences without interruptions also indicated that the sequences had no PCR artifacts [64].

Analyses of sequences, recombination and phylogenetic inferences

The obtained sequences aligned using the Clustal Omega [53] were adjusted manually and the haplotypes identified by the DnaSP 5.10 software [23]. The concatenated five genes used in the MLST methodology (*gatB, coxA, hcpA, ftsZ* and *fbpA*) were analyzed according to Baldo et al. [6], and the *wsp* gene according to the WSP Typing protocol [7]. Blast of the sequences to the NCBI and to the *Wolbachia* Database (http://www.pubmlst.org/wolbachia/) identified sequences already described in other insects. Sequences that had no matches were submitted to the curators for assignment of novel identification numbers. The sequences were deposited in the *Wolbachia* Database and may be accessed by their MLST and wsp codes or by the identification numbers (ID 1777–1803), and in the GenBank (accession numbers KX129130–KX129171). Evidences for recombination between sequences were tested by three methods, Maxchi [29], Geneconv [33] and Chimaera [35] implemented in the RDP3.5 software [26]. The intragenic recombination between *wsp* sequences was also analyzed by the HVRs amino acid profiles according to the WSP Typing methodology [7]. Gene *wsp* is highly variable and is organized in four hypervariable regions (HVRs) which are separated one from the other by conserved nucleotide sequences (CRs). Alleles of the *wsp* gene are identified by a code composed of four numbers each one related to the amino acid sequence of each HVR plus half of its contiguous CR [7].

The phylogenetic analyses using Maximum-Likelihood (ML) implemented in the PhyML 3.1 program [15] were conducted for a concatenated data set of the five MLST genes and for *wsp* gene apart. The percentage of replicate trees in which the associate strain clustered together was measured by boostrap tests (1000 replicates). The best evolutionary model was chosen according to the AICc (Akaike Information Criterion, corrected) implemented in MEGA6 [56]. Sequences of *Wolbachia* strains infecting other insects (outgroups), may be assessed in the *Wolbachia* Database (isolates/search) by their ID codes. The ID identify simultaneously the strains based on the allelic codes of the five MLST genes and of gene *wsp*: *Anastrepha striata* (ID307), *Bactrocera decurtans* (ID560), *Bactrocera tryoni* (ID559), *Drosophila bifasciata* (ID5), *Drosophila orientacea* (ID9), *Drosophila simulans* (ID11), *Ephestia kuehniella* (ID13), *Solenopsis invicta* (ID2) and *Agelenopsis longistyla* (ID179).

Results

Six of the eight morphotypes of the AF complex [17,18] were employed in the analyses: Brazilian-1 (*A.*sp.1 and Argentina), Brazilian-2 (*A.* sp.2), Brazilian-3 (*A.* sp.3), Peruvian [Ecuador (*A.* sp.4) and Peru], Andean (Colombia), and Mexican (Guatemala and Mexico). Out of 260 individual flies of the AF complex screened for

Fig. 1. Approximate location of samples collection. [Brazil]: 1. Uberlândia, 2.Mirassol, 3. Araraquara, 4. Botucatu, 5. Piracicaba, 6. Itapetininga, 7. Ibiúna, 8. Santa Isabel, 9. Jacareí, 10. Salesópolis, 11. Jambeiro, 12. Taubaté, 13. São Luís do Paraitinga, 14. Lorena, 15. Três Rios, 16. Ubatuba, 17. Ilhabela, 18. São Sebastião, 19. Bertioga, 20. Itanhaém, 21. Peruíbe, 22. Pedro de Toledo, 23. Ilha Comprida, 24. Paranaguá, 25. Caiobá, 26. Guaratuba, 27. Guaramirim, 28. Rio Negrinho, 29. Guarapuava, 30. Irati, 31. Imbituva, 32. Sengés, 33. Itararé. [Argentina]: 34. Misiones, 35. Concordia, 36. Tucumán, 37. Horco Molle. [Perú]: 38. La Molina, 39. Piura. [Ecuador]: 40. Guayaquil. [Colombia]: 41. Tolima.[Guatemala]: 42. Guatemala City. [México]: 43. Tapachula, 44. San Vicente, 45. Quintana Roo, 46.Teocelo, 47. Apazapan, 48. La Jicayana.

Wolbachia, 255 were found to be infected (Tables 2 and 3). Only one individual in each of four samples from Mexico (Jicayana, Teocelo, Tapachula and San Vicente), and in the sample from Concordia (Argentina) were not infected.

MLST genotyping

The MLST analysis showed the existence of seven *gatB*, four *coxA*, six *hcpA*, eight *ftsZ* and nine *fpbA* alleles. The data also shows the presence of new alleles for the five loci: five for *gatB*, three for *coxA*, five for *hcpA*, six for *ftsZ* and eight for *fpbA*. Moreover, the analysis identified 19 sequence types (STs) determined by the combination of alleles of the five MLST genes among the morphotypes of the AF complex (Table 2), and that *Wolbachia* strains infecting the AF morphotypes belong to supergroup A. Two sequence types, ST-1 and ST-13, previously known for *Wolbachia* infecting many insect species, and 17 new strains not found in the MLST database were detected (Table 2). ST-1 was the most common strain found in 69% (176 out 255) individuals, occurring in 17 samples of Brazilian-1, 11 samples of Brazilian-2, 10 samples of Brazilian-3, and also in one sample of Peruvian (Piura) and Mexican (Tapachula) morphotypes. ST-13 was found in 6.2% (16 out 255) of the individuals, from three Brazilian-1 samples (Itapetininga, Mirassol, Salesópolis), and one sample of Brazilian-2 (Taubaté). Out of the 17 new STs, only four were found as single sequence from single analyzed flies, ST-329 (Jacarei) and ST-330 (Salesópolis) found in Brazilian-1, ST-332 in Brazilian-3 (Salesópolis), and ST-336 found in Mexican (Apazapan) morphotypes. The majority of samples were infected by single strains, and two morphotypes, Brazilian-1 (Sengés) and Brazilian-3 (Salesópolis), shared a same strain (ST-331). Moreover, infections by two distinct strains (in distinct individuals) were found in Brazilian-1 (Jacarei: ST-1, ST-329; Salesópolis: ST-13, ST-330), in Brazilian-3 (Salesópolis: ST-1, ST-332), and in Mexican (Apazapan: ST-335, ST-336) morphotypes (Table 2). The differences among strains for the five genes are due to scattered synonymous and nonsynonymous nucleotide substitutions (Table S1) without any nucleotide gaps. Variability of the each MLST genes is low, from 0.0005 (*coxA*) to 0.0019 (*hcpA* and *ftsZ*) (Table S2), as well as the variability of the strains within each morphotype, from 0.0001 (Brazilian-2) to 0.0027 (Peruvian). The overall genetic distances between the morphotypes are lower than 1% (Table S3).

WSP Typing

Genotyping of *wsp* was made by the WSP Typing methodology [7] revealing the presence of nine HVR1, eight HVR2, three HVR3 and 13 HVR4 variants. Within these variants eight HVR1, seven HVR2, two HVR3 and eleven HVR4 were new haplotypes. The analysis confirms the results of the MLST data by showing that the *wsp* sequences belong to *Wolbachia* of supergroup A. The HVR analy-

Table 1
Localities, codes, geographic coordinates and sampled host fruits or laboratory colonies in the Neotropical Region.

Localities		Codes	Coordinates	Host fruits
Brazil	Uberlândia	Ube	18°56′46”S, 48°13′55”W	Guava *(Psidium guajava)*
	Mirassol	Mir	20°49′07”S, 49°30′30”W	Guava
	Araraquara	Ara	21°47′38″S, 48°10′ 33″W	Guava
	Botucatu	Bot	22°56′18”S, 48°18′25”W	Guava
	Piracicaba	Pir	22°43′30”S, 47°38′56”W	Guava
	Itapetininga	Itp	23°35′31”S, 48°03′10”W	Guava
	Ibiuna	Ibi	23°39′21”S, 47°13′22”W	Guava
	Santa Isabel	Isa	23°19′00”S, 46°13′25”W	Guava
	Jacarei	Jac	23°18′18”S, 44°41′16”W	Guava
	Salesópolis	Sal	23°31′51”S, 45°50′30”W	Guava
	Jambeiro	Jam	23°15′14”S, 45°07′30”W	Guava
	Taubaté	Tau	23°01′33”S, 45°33′31”W	Guava; Orange (*Citrus* sp.)
	São Luiz do Paraitinga	Slp	23°13′24”S, 45°18′47”W	Guava
	Lorena	Lor	22°43′51”S, 45°07′30”W	Guava
	Três Rios	Tri	22°07′21”S, 43°12′47”W	Guava
	Ubatuba	Uba	22°46′24”S, 45°41′52”W	Tropical almond *(Terminalia cattapa)*
	Ilhabela	Ilh	23°48′22”S, 45°21′54”W	Guava
	São Sebastião	Sse	23°45′22”S, 45°24′54”W	Tropical almond; orange
	Bertioga	Ber	23°51′14”S, 46°08′20”W	Tropical almond
	Itanhaém	Ita	24°10′58”S, 46°47′20”W	Tropical almond
	Peruibe	Per	24°19′12”S, 46°59′52”W	Tropical almond
	Pedro de Toledo	Pto	24°16′30”S, 47°13′58”W	Guava; Orange
	Ilha Comprida	Ico	24°44′27”S, 47°32′24”W	Tropical almond
	Paranaguá	Par	25°31′12”S, 48°30′32”W	Tropical almond
	Caiobá	Cai	25°49′04”S, 48°32′34”W	Tropical almond
	Guaratuba	Gut	25°52′58”S, 48°34′30”W	Tropical almond
	Guaramirim	Gum	26°28′23”S, 49°00′10”W	Guava
	Rio Negrinho	Rne	26°15′14”S, 49°31′04”W	Guava
	Guarapuava	Gua	25°23′42”S, 51°27′28”W	Guava
	Iratí	Ira	25°28′10”S, 50°39′30”W	Guava
	Imbituva	Imb	25°13′48”S, 50°36′16”W	Guava
	Sengés	Sen	24°16′46”S, 49°27′50”W	Guava
	Itararé	Itr	24°16′54”S, 49°20′27”W	Guava
Argentina	Misiones	Misi	27°23′59”S, 55°56′01”W	Guava
	Concordia	Conc	31°23′13”S, 58°01′12”W	Guava
	Tucumán	Tucu	27°02′18”S, 65°19′13”W	Guava
	Horco Molle	Hmol	26°46′37”S, 65°19′49”W	Guava
Peru	La Molina	LMol	12°14′15”S, 76°31′50”W	Lab colony, cherimola *(Annona cherimola)*
	Piura	Piur	07°40′23”S, 79°12′40”W	Guava
Ecuador	Guayaquil	4Guy	02°12′13”S, 79°53′50”W	Guava
Colombia	Tolimá	Toli	04°26′11”N, 75°11′29”W	Lab colony, coffe *(Coffea arabica)*
Guatemala	Guatemala City	Guat	14°36′51”N, 90°32′22”W	Guava
Mexico	Tapachula	Tapa	14°53′47”N, 92°24′31”W	Guava; loquat *(Eriobotrya japônica)*
	San Vicente	Vice	16°24′16”N, 92°10′30”W	Guava
	Quintana Roo	QRoo	19°37′39”N, 88°38′56”W	McPhail trap (adult flies)
	Teocelo	Teoc	19°23′14”N, 96°57′23”W	Guava
	Apazapan	Apaz	19°17′00”N, 96°36′59”W	McPhail trap (adult flies)
	La Jicayana	Jica	19°21′44”N, 96°39′23”W	Guava

sis (amino acid haplotypes) showed the existence 20 WSP alleles (Table 3) in the samples of the AF complex. Two alleles, wsp-31 and wsp-23, were already found in the *Wolbachia* Database. *Wolbachia* with wsp-31 (similar to wMel) was found in 69% (176 out 255) of the individuals, being 76 in Brazilian-1, 36 in Brazilian-2, 39 in Brazilian-3, nine in Peruvian, three in Andean, and 13 in Mexican morphotypes. Bacteria harboring allele wsp-23 occurred in 4.8% of the individuals, and it was found in 11 individuals from three sampled populations of Brazilian-2 (Guaratuba, Pedro de Toledo, Ubatuba), and in three individuals of Brazilian-3 (Pedro de Toledo). Eighteen alleles not previously described, were found in *Wolbachia* infecting seven sampled populations of Brazilian-1, one in Brazilian-2, four in Brazilian-3, and six in samples from Mexican morphotypes. No double infection was found in single flies, but two *Wolbachia* harboring distinct *wsp* alleles were found in five sampled populations: two from Brazilian-3 (Pedro de Toledo: wsp-23, wsp-646; Salesópolis: wsp-31, wsp-647), and three from Mexican (Apazapan: wsp-650, wsp-651; San Vicente: wsp-31, wsp-652; Tapachula: wsp-31, wsp-654) morphotypes (Table 3). Single synonymous and nonsynonymous nucleotide substitutions (Table S4) and very few gaps characterize the variability of the *Wolbachia wsp* gene in the morphotypes. Nucleotide variability of gene *wsp* within each morphotype was low, ranging from no substitution in Brazilian-3 to 0.0151 in the Mexican samples. Overall gene *wsp* showed a low variability in the *Wolbachia* samples from the neotropics (Table S2), reflecting in a low genetic distances of *Wolbachia* infecting the morphotypes, as showed in Table S3.

Recombination between sequences

Search for signatures of recombination was made between sequences of the five MLST genes and for gene *wsp*. The MLST genes were concatenated in order, *gatB, coxA, hcpA, ftsZ* and *fbpA* [4,6], generating sequences of 2079 positions. A case of putative recombination was detected involving STs 329, 342 and 346 ($P \ll 0.001$ detected by Maxchi, Geneconv and Chimaera), as shown in Fig. 2.

Table 2
Number of individuals, allelic codes of the five MLST genes, STs and localities where *Wolbachia* infecting the AF complex morphotypes in the Neotropical Region were sampled.

Morphotypes	N	Alleles					ST	Localities of collection
		gatB	*coxA*	*hcpA*	*ftsZ*	*fbpA*		
Brazilian-1	76	1	1	1	1	1	1	1Ara, 1Bot, 1Ibi, 1Itr,1Jac, 1Jam, 1Lor, 1Pir, 1Isa, 1Slp, 1Tri, 1Ube, 1Gua, 1Imb, 1Ira, 1Gum, 1Rne
	12	1	1	1	3	1	13	1Mir, 1Sal
	1	199	1	214	1	1	329[a]	1Jac
	1	200	190	1	3	257	330[a]	1Sal
	5	1	1	1	162	1	331[a]	1Sen
	3	1	1	1	1	261	342[a]	Conc
	5	1	1	1	167	262	343[a]	Hmol
	2	1	1	1	168	263	344[a]	Tucu
	2	203	1	1	1	264	345[a]	Misi
Brazilian-2	48	1	1	1	1	1	1	2Ber, 2Ilh, 2Ico, 2Ita, 2Pto, 2Per, 2Sse, 2Uba, 2Cai, 2Gut, 2Par
	4	1	1	1	3	1	13	2Tau
Brazilian-3	45	1	1	1	1	1	1	3Ber, 3Ico, 3Jam, 3Pto, 3Per, 3Sal, 3Tau, 3Cai, 3Gut, 3Rne
	5	1	1	1	162	1	331[a]	3Sal
	1	1	1	1	162	258	332[a]	3Slp
	4	1	191	1	1	1	333[a]	3Uba
Peruvian	2	1	1	1	1	1	1	4Guy
	2	1	1	217	1	260	341[a]	LMol
	5	1	1	1	1	261	342[a]	Piur
Andean	3	23	1	86	166	1	340[a]	Toli
Mexican	5	1	1	1	1	1	1	Tapa
	4	1	1	1	164	1	335[a]	Apaz
	1	201	1	215	164	1	336[a]	Apaz
	8	1	1	1	165	1	337[a]	Vice, Qroo
	4	1	1	216	164	259	338[a]	Teoc
	3	202	192	1	1	1	339[a]	Guat
	4	201	1	1	164	1	346[a]	Jica

[a] Indicates new STs.

Table 3
Number of individuals, peptide code of HVRs, *wsp* alleles, and localities of collection of *Wolbachia* infecting the AF complex morphotypes in the Neotropical Region.

Morphotypes	N	Peptide codes				wsp allele	Localities of collection
		HVR1	HVR2	HVR3	HVR4		
Brazilian-1	76	1	12	21	24	31	1Ara, 1Bot, 1Ibi, 1Itr, 1Jac 1Jam, 1Lor, 1Mir, 1Pir, 1Sal, 1Isa, 1Slp, 1Tri, 1Gua, 1Gum, Conc, Hmol, Tucu
	5	218	252	21	24	640[a]	1Ita
	4	1	255	21	278	641[a]	1Ube
	5	1	225	21	278	642[a]	1Sen
	5	1	12	21	245	643[a]	1Rne
	2	225	12	21	19	656[a]	Misi
	5	226	253	21	277	658[a]	1Imb
	5	1	12	21	278	659[a]	1Ira
Brazilian-2	11	1	12	21	19	23	2Pto, 2Uba, 2Gut
	36	1	12	21	24	31	2Ber, 2Ilh, 2Ico, 2Ita, 2Per, 2Sse, 2Tau, 2Uba, 2Par
	5	1	12	21	279	645[a]	2Cai
Brazilian-3	3	1	12	21	19	23	3Pto
	39	1	12	21	24	31	3Ber, 3Ico, 3Jam, 3Per, 3Sal 3Slp, 3Tau, 3Uba, 3Cai
	2	1	12	21	280	646[a]	3Pto
	1	1	12	21	281	647[a]	3Sal
	5	220	12	21	24	648[a]	3Gut
	5	1	256	21	24	649[a]	3Rne
Peruvian	9	1	12	21	24	31	4Guy, Lmol, Piur
Andean	3	1	12	21	24	31	Toli
Mexican	13	1	12	21	24	31	Vice, Qroo, Tapa, Guat
	4	1	12	21	282	650[a]	Apaz
	1	1	12	257	283	651[a]	Apaz
	1	221	12	21	284	652[a]	Vice
	4	222	257	21	289	653[a]	Jica
	2	223	258	21	286	654[a]	Tapa
	4	224	12	21	19	655[a]	Teoc

[a] Indicates new *wsp* alleles.

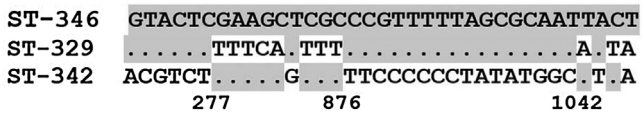

Fig. 2. Recombination detected among sequences of *Wolbachia* MLST genes infecting the AF complex. Only the polymorphic sites are shown in the alignment. Gray shaded parts of sequences are polymorfisms shared with the top sequence in the alignment. The numbers below the alignment indicate the approximate nucleotide position of the breakpoints detected by the three methods (Maxchi, Geneconv, Chimaera).

ST-329 was suggested as the putative recombinant, ST-346 as the major parent, and ST-342 the minor parent. Since three break points were detected two events of recombination may have occurred. Shuffling of a long segment from 5′ end of gene *gatB* to the break point at position 1142 within gene *hcpA* giving origin to the recombinant wsp-329, and two breaks, at position 277 (gene *gatB*) and 876 (gene *ftsZ*), interchanged a smaller segment between wsp-342 and the recombinant wsp-329.

Intragenic recombination between *wsp* alleles was tested following a procedure developed by Baldo et al. [7]. Fig. 3 shows the aligned amino acid sequences arranged according to similarities of HVR1, but differing in the other HVRs; three blocks of sequences were formed: one with novel sequences similar to wsp-31, one with new sequences similar to wsp-23, and a third one with a single novel sequence, wsp-653. As previously known, wsp-23 is an ancestral allele and differ from wsp-31 in their HVR4 [5,7]. Allele wsp-653 may be a recombinant having HVRs 2, 3 and 4 similar to those of wsp-23, but a distinct HVR1 of unknown origin. As wsp-653 is a new sequence detected in the present analysis it was not possible to determine its putative second parental sequence. Moreover, analysis of the nucleotide sequences by the three statistical methods of recombination detection did not indicate events of interchange between wsp-23, wsp-31 and the novel alleles, most probably due to the low divergence between them, a factor that precludes detection of recombination events according to the RDP3 manual. However, the analysis suggested, although not with strong statistical support (P = 0.01), that wsp-653 may be a putative recombinant sequence.

Phylogenetic analyses

In the phylogenetic analyses that follow the putative recombinant sequences were not included since the Maximum Likelihood (ML) methodology requires sequences with the same evolutionary history [42,64]. Phylogenetic inference based on the concatenated set of the five MLST genes (Fig. 4) shows that *Wolbachia* strains detected in the samples of the six AF morphotypes grouped in a large cluster. Within this cluster no significant internal branching was observed indicating a close relationships among *Wolbachia* strains infecting the AF complex. This cluster is isolated from the strains of *Wolbachia* infecting other species of insects, including the fruit flies *B. decurtans* and *B. tryoni*. All these *Wolbachia* strains belong to supergroup A while *Wolbachia* infecting another species of *Anastrepha, A. striata*, is of supergroup B, as previously described [27]. Phylogenetic analysis based on the *wsp* gene revealed a similar set of results for *Wolbachia* infecting the AF complex morphotypes (Fig. 5). However, subgrouping could be further defined in future analyses based on whole genome basis, as is being pursued [24,36,37].

Discussion

The present study showed that *Wolbachia* infection was found in 98% of flies belonging to the screened morphotypes of the *A. fraterculus* complex. This result, in line with previous report on infection in Brazilian morphotypes [12], places these species among those with high rate of infection [19,20,61,64,68]. In the six morphotypes of the AF complex here studied, 34 MLST alleles and 20 WSP alleles of *Wolbachia* were found. Hence, about 8.8 alleles/morphotype, against 137 alleles found in 42 species of other insects (3.7 alleles/species) [4] evidenced that, on average, the variability of *Wolbachia* in the AF complex is among the highest so far detected in insects.

In all samples of infected *Anastrepha* studied in this work, *Wolbachia* belonged to supergroup “A”, as previously reported [10,12]. To date, only one sample of nominal *A. fraterculus* of unknown origin from South America were doubly infected with strains of supergroups “A” and “B” [10], and a sample of *A. striata* from Mexico was infected with *Wolbachia* supergroup “B” [27]. Hence, the data suggest that *Anastrepha* species seems to be more susceptible to infection by *Wolbachia* of supergroup “A”, similar to what has been found in other dipteran species [54].

Variability of *Wolbachia* strains herein described shows two contrasting relationships with the regions along the Neotropical Region where the AF morphotype hosts were collected [17]. The most abundant strain, ST-1, was found spread from Brazil/Argentina through Mexico (except in the single sample from Colombia), clearly not exhibiting correlation with the geographic locations or the hosts, similarly observations on many dipteran species from distinct worldwide regions [54]. On the other hand, specific *Wolbachia* strains were found in most of the morphotypes indicating

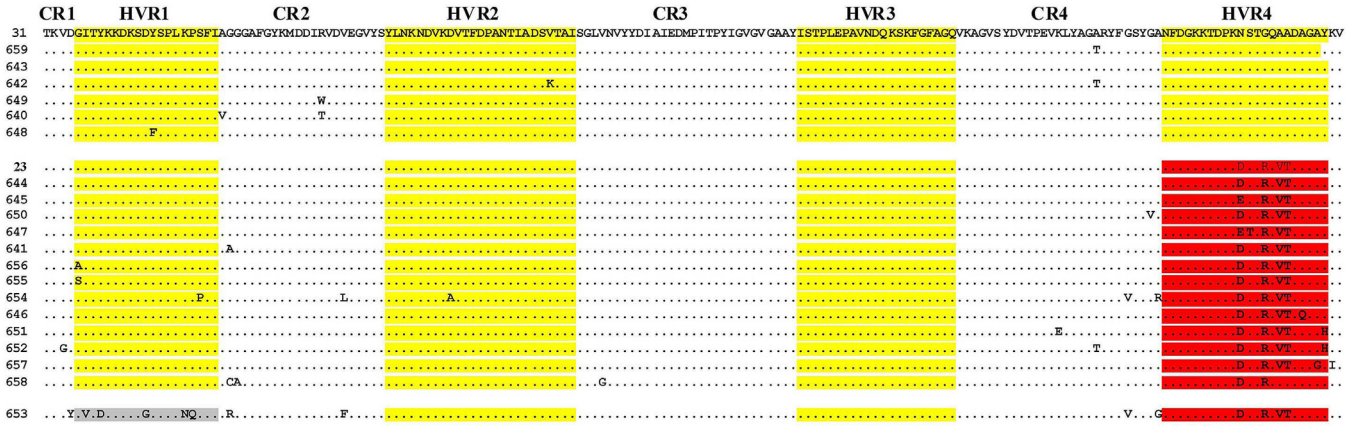

Fig. 3. Amino acid motifs of *Wolbachia wsp* alleles infecting the AF complex. The alleles were arranged into three groups according to similarity of polymorphism and taking HVR1 of wsp-31, wsp-23 and wsp-653 as the reference for grouping. Each group of WSP alleles has unique combination of HVRs indicating shuffling of HVRs.

Fig. 4. Maximum Likelihood analysis of concatenated sequences of the five MLST genes (2079 bp). The tree with highest log is shown (-2613.1528). Bootstrap values are indicated at the nodes (only those >50%). The analysis involved 28 nucleotide sequences. Supergroups A and B are indicated. Strains are indicated by the ST code from MLST database, followed by the morphotype and the sample codes. *Indicates that ST-1 comprised 40 samples, being 17 from Brazilian-1, 11 from Brazilian-2, 10 from Brazilian-3, one from Peruvian and one from Mexican morphotypes (see Table 2).

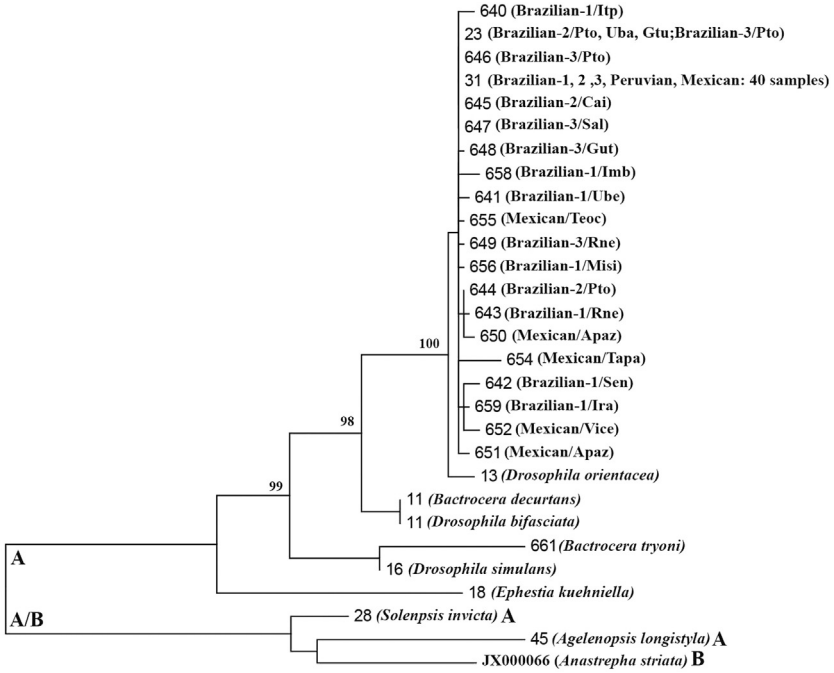

Fig. 5. Maximum Likelihood analysis of the *wsp* profiles (576 positions). The tree with highest log is shown (−2658.3202). Bootstrap values are indicated at the nodes (only those >50%). The analysis involved 29 nucleotide sequences. Supergroups A and B are indicated. Sequences of *wsp* are identified by the allele codes from *Wolbachia* database. Between brackets are indicated the morphotypes and the sample codes. *Indicates that wsp-31 comprises 44 samples being 18 from Brazilian-1, nine from Brazilian-2, nine from Brazilian-3, three from Peruvian, one from Andean and four from Mexican morphotypes (see Table 3).

a close relationship between strain types and their fly hosts. This type of association was also found, for example, in fig wasps from Panama and Australia [16], in ants from the New and Old World [41], in the *testacea* complex of *Drosophila* from North America, Europe and Asia, and in two chloropid flies from North America [54]. The cause of the continental wide distribution of some *Wolbachia* strains within the AF complex is unknown. It may be considered that the wide spread *Wolbachia* strains in the AF com-

plex are extant forms of ancient origins, while the unique, specific strains might have evolved by mutational events and/or horizontal transmission. The low variability existing in the strains suggests that the differences could be the result of mutations, similarly to the case of strains in *Rhagoletis pomonella* [44]. However, the presence of identical strains in zones of host sympatry, for example, ST-13 in Brazilian-1 (1Mir, 1Sal) and Brazilian-2 (2Tau) morphotypes, suggested that horizontal transmission may not be excluded. Evidences of this kind of transmission was found for *Wolbachia* infecting species of *Bactrocera* [30], and between species of *Anastrepha* and of parasitoid braconid wasps [28]. Another scenario would be to suppose that these strains were relics of ancient forms before the splitting of the *fraterculus* complex morphotypes.

The results reinforce that phylogenetic reconstruction based on single genes do not represent accurately the relationship between the bacteria strains. Moreover, the MLST methodology efficiently characterize *Wolbachia* strains, as shown in other groups of insects, including the fruit flies as previously demonstrated [2,3,8,20,27,30,31,44]. However, the results show that analysis of *wsp* is very useful to reveal groups of close related individual strains, as usually observed in other insects [5,21,54]. This is relevant since the mosaic nature of genes coding for surface proteins, such as the *wsp*, may be responsible for the appearance of new phenotypes that could lead to distinctive interactions of parasitic strains with their insect hosts [5,6,7,52,61]. Novel haplotypes resulting from inter or intragenic recombination may also increase the number of *Wolbachia* strains [3,7,21]. Even though the present data show that recombination events were not frequent within the bacteria infecting the AF complex, the results suggested the presence of a putative recombinant allele, wsp-653, among the 18 novel described *wsp* haplotypes, and a single signature of putative intergenic recombination involving the MLST genes. This is in line with previous surveys showing that recombination between MLST genes and intragenic recombination of gene *wsp* are found in *Wolbachia* infecting many insect species [4,6].

In conclusion, and additionally to previous observations [10,12], the results of the present analyses show that the morphotypes of the *A. fraterculus* complex actually harbor distinct strains of *Wolbachia.* Under the presumption that *Wolbachia* may induce cytoplasmatic incompatibility in *Anastrepha*, such as demonstrated in other fruit fly species [1,38,65,66], the detection of specific strains associated with distinct morphotypes leads to the suggestion that *Wolbachia* may have acted as a reinforcing factor in the diversification processes of the *A. fraterculus* complex.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2016.11.002.

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