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Transposon Tn1721 distribution among strains of *Xylella fastidiosa*

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Abstract

Transposons are mobile genetic elements found within the genomes of various organisms including bacteria, fungi, plants and animals. Fragments of the transposon Tn1721 were found included in the genome of *Xylella fastidiosa* strain 9a5c. Regions from such fragments were PCR-amplified using specially designed primers (TNP_1 and TNP_2). In order to detect insertions of the Tn1721 element, both primers were used and one of them included a region of the transposon (TNP_1) and the other one had the right repeat and part of the bacterial chromosome (TNP_2). The PCR products obtained from strain 9a5c were used as a pattern for fragment size comparisons when DNA samples from other *X. fastidiosa* strains were used as template for the PCR assays. Differences were observed concerning the PCR products of such amplifications when some *X. fastidiosa* strains isolated from grapevine and plum were used. For the citrus-derived strains only the strains U187d and GP920b produced fragments with different sizes or weak band intensity. Such variations in the *X. fastidiosa* genome related to disrupted Tn1721 copies are probably due to the possibility of such a transposon element being still able to duplicate even after deletion events might have taken place and also because the bacterial strains in which the main differences were detected are derived from different host plants cultivated under different climate conditions from the one used as reference.

Keywords: Transposon Tn1721; PCR fragment; *Xylella fastidiosa*

1. Introduction

Citrus variegated chlorosis, also known as 'amarelinho', is caused by *Xylella fastidiosa*, a Gram-negative bacterium, and is presently one of the most important diseases of the current Brazilian citriculture [1].

This bacteriosis is transmitted by insects (sharpshooters, leafhoppers) that feed on xylem sap [2] and has been found on crops of agronomical importance such as citrus, grapevine, coffee and plum as well as on less important plants such as urban, forest and herbal ones [3].

X. fastidiosa genome is made up of a circular chromosome with 2 679 305 bp with a CG content of 52.7% and two plasmids with 51 158 and 1285 bp [4].

Through the sequencing of its genome (Genome Project/FAPESP), around 4.92% of mobile genetic elements were found to be inserted within the genetic material of this phytopathogenic bacterium [4].

Transposons are normal genome constituents found in many prokaryotic and eukaryotic species [5]. The transposable element named Tn1721 resembles the bacterial transposon Tn3 [6] and is a member of the Tn21 subgroup that includes Tn21 itself and the Tn501 element. The Tn1721 element harbors a 3.8-kb region which contains the following genes: the transposase (*tnpA*), the resolvase (*tnpR*) and a resolution site (*res*), which are important for its transposition [7], and when transposition takes place it generates 5-bp direct repetitions on the target DNA [8]. The Tn1721 element has a complete sequence of 11 200 bp [9,10].

The transposon multiple site insertion potential, together with the potential to change multiple loci causing structural and regulatory changes through a single event, has been proposed as one of the most current common mechanisms supporting fast evolution steps [11].

The genetic rearrangements induced by certain mobile elements allow a direct selection value to the organism or to its further descendants, giving a simple, straight and satisfactory answer towards the real biological function of these elements [11].

This work had the objective to verify the presence of the mobile element Tn1721 in different *X. fastidiosa* strains.

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Table 1
Strains of *X. fastidiosa*

Strain	Host	Disease	Source
9a5c	citrus	CVC	INRA [4]
B-14	citrus	CVC	CCSM
CVC#5	citrus	CVC	USDA
GP920b	citrus	CVC	CCSM
M2-1	citrus	CVC	CCSM
U161b	citrus	CVC	CCSM
U187d	citrus	CVC	CCSM
U195c	citrus	CVC	CCSM
U1575b	citrus	CVC	CCSM
10348	citrus	CVC	IAPAR
10438	citrus	CVC	IAPAR
11037	citrus	CVC	IAPAR
11038	citrus	CVC	IAPAR
11066	citrus	CVC	IAPAR
11067	citrus	CVC	IAPAR
11347	citrus	CVC	IAPAR
11348	citrus	CVC	IAPAR
11380	citrus	CVC	IAPAR
11399	citrus	CVC	IAPAR
11400	citrus	CVC	IAPAR
11834	citrus	CVC	IAPAR
11779	citrus	CVC	IAPAR
9746	plum	plum leaf scald	IAPAR
JAB14	coffee	coffee leaf scorch	UNESP
12288	coffee	coffee leaf scorch	IAPAR
8935	grapevine	Pierce disease	IAPAR
9713	grapevine	Pierce disease	IAPAR
9715	grapevine	Pierce disease	IAPAR

CVC – citrus variegated chlorosis; INRA – Institut Nacional de la Recherche Agronomique, Bordeaux, France; IAPAR – Instituto Agronômico do Paraná, Londrina, PR, Brazil; CCSM – Centro de Citricultura Sylvio Moreira, Cordeirópolis, SP, Brazil; USDA – United States Department of Agriculture, Beltsville, MD, USA; UNESP – Universidade Estadual Paulista, Jaboticabal, SP, Brazil.

As a result of this endeavor it became possible to differentiate some *X. fastidiosa* strains from the one that has been sequenced.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

All strains of *X. fastidiosa* used in this work are listed in Table 1 and were kept in solid BCYE [12]. For DNA extraction each *X. fastidiosa* strain was grown in 3.0 ml of PW [13] in a rotary shaker set at 180 rpm during 10 days at 30°C. The DNA extraction method [14] involved the addition of 100 μl of RNase (100 μg ml^{-1}) to the TE buffer solution (10 mM Tris–HCl, pH 7.5; 1 mM EDTA, pH 8.0; 30 μl of 10% SDS and 20 mg ml^{-1} proteinase K). DNA samples were stocked at −20°C and quantification was made by spectrophotometry at 260 nm.

Besides *X. fastidiosa*, other bacterial strains of commercial and agronomical value were analyzed and are listed in Table 2. *Bacillus* species were grown in BHI (Biobrás) and the DNA was extracted following [15]. The other bacterial species had their DNA samples obtained using InstaGene TM matrix (Bio-Rad) following the manufacturer's instructions. They were grown on NA [16] except the species from the *Pseudomonas* genus that were grown on King B medium [17]. All species were grown at 30°C.

2.2. Primer design procedure

Two pairs of primers were used for the detection of the transposon Tn1721. These primers were designed based on regions of this transposable element that had high similarity to sequences of the genomes of *X. fastidiosa* strains 9a5c [4]. One of them included a region of transposon Tn1721 itself and the other included the right repeat and its insertion point within the chromosome of *X. fastidiosa*.

The software used to generate these primers was OLIGO 4.0 (Copyright© 1989–91 Wojciech Rychlik) and Gene Runner release 3.00 (Copyright© 1994 Hasting Software Inc.). Table 3 lists the primers used in this study, their sequences, and the PCR expected product size. The primers were synthesized by Life Technologies do Brasil Ltda.

2.3. PCR amplification

For the primers TNP_1-F and TNP_1-R the following reaction mixture was used: 2 mM $MgCl_2$, 300 μM of each dNTP, 1 U of *Taq* DNA polymerase (Gibco/BRL®), 0.1 μM of each primer (TNP_1-F and TNP_1-R), 50 ng of genomic DNA and sterile Milli-Q water to complete 20 μl.

Table 2
Other bacterial species

No.	Species	Host	Disease	Source
1	*A. radiobacter*	soil	–	EMBRAPA
2	*A. tumefaciens*	rose	crown gall	EMBRAPA
3	*B. cereus*	–	–	UNESP
4	*B. thuringiensis* var. *israelensis*	diptera	–	UNESP
5	*P. stewartii* subsp. *stewartii* SW2	corn	Stewart's disease or bacterial wilt	IAPAR
6	*P. syringae* pv. *tabaci*	tobacco	wild fire	EMBRAPA
7	*X. axonopodis* pv. *citri*	citrus	citrus canker	EMBRAPA
8	*X. arboricola* pv. *pruni*	plum	plum bacteriosis	EMBRAPA

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuéria, Londrina, PR, Brazil; UNESP – Universidade Estadual Paulista, Jaboticabal, SP, Brazil; IAPAR – Instituto Agronômico do Paraná, Londrina, PR, Brazil.

Table 3
PCR primers used in this study

Primer	Sequence	PCR expected size (bp)
TNP_1-F	5′-GCCGCCAAGGATGTGCTCGAC-3′	
TNP_1-R	5′-CGCGGGTATCGGAAGAAAACA-3′	611
TNP_2-F	5′-GGGCCACAGCAACCTGAAATA-3′	
TNP_2-R	5′-GCCGGTAATCCCACCAACTGA-3′	1248

The same reaction mixture was used for the other set of primers except for the $MgCl_2$, which was reduced to 1 mM.

The amplifications were carried out in a PTC-100 thermocycler (MJ Research, Inc.). The amplification cycles for the TNP_1 primers were: a single denaturation step of 2 min at 94°C, 30 cycles (each one consisting of a denaturation step of 1 min at 95°C, annealing at 51°C for 1 min and extension at 72°C for 1 min), and an extra extension step of 5 min at 72°C. The amplification conditions for the TNP_2 primers were similar except for the annealing temperature, which was set at 52°C.

2.4. PCR product restriction

Using the software OLIGO 4.0 it was possible to choose the restriction endonucleases that had restriction sites on the amplified regions. The restriction reactions and EB-AGE were carried out according to [18].

After the amplifications, 10 μl of each PCR reaction product obtained using the TNP_1 primers was submitted to restriction using 0.5 U *Hin*dIII (Gibco/BRL®), 1× buffer and sterile Milli-Q water up to a total of 20 μl, kept at 37°C, and for the amplifications using the TNP_2 primers, 0.5 U of *Sma*I (Gibco/BRL®), 1× buffer and sterile Milli-Q water up to a total of 20 μl, at 30°C, were used. Both restriction reactions lasted for 60 min.

3. Results and discussion

The results allowed to detect signs of the presence of the transposon Tn1721 within the genome of *X. fastidiosa* strain 9a5c (Fig. 1); however, the sequences are not complete probably due to genetic recombination by rearrangements. The later statement is sustained by the fact that the normal antibiotic resistance to tetracycline (Tet^r) harbored by the complete transposon was not found through antibiogram assays performed with the *X. fastidiosa* strains analyzed in this work.

For the identification of these fragments the FASTA sequences found were compared to those already deposited in the Genebank (NCBI) and submitted to similarity search on the *X. fastidiosa* database using the alignment tool Clustal-W.

The Clustal-W alignments of the detected sequences served as a basis for the elaboration of the specific primers for PCR amplifications (Fig. 1). The PCR-amplified sequences had 89% identity with those of the transposon Tn1721 from the *X. fastidiosa* genome.

Fig. 1. Clustal-W alignment of the transposon Tn1721 found on the genome of *X. fastidiosa*.

3.1. Strains with primer TNP_1-amplified sequences

After the improvement of the PCR reactions using the pair of TNP_1 primers with DNA from *X. fastidiosa* strain 9a5c, a fragment of the 611-bp part of the Tn1721 transposon was produced. The detected band (611 bp) as well as its *Hin*dIII-restricted fragments (215 and 417 bp) corresponded to the expected fragments based on the software OLIGO 4.0 prediction (Fig. 2A,B).

It was possible to demonstrate that the 28 *X. fastidiosa* DNA samples in which an amplified band had been produced when the TNP_1 primer was used had the same pattern in almost all of them, with the exception of the DNA samples from strains U187d (citrus), 9746 (plum), and 8935, 9713 and 9715 (grapevine). DNA from strain U187d (citrus) produced an extra band of around 450 bp without *Hin*dIII restriction.

The transposon Tn1721 was probably able to replicate and transpose [19] and such rearrangements could have happened [20] even after an end deletion event occurred with possible full end elimination [21,22]. Since this was the only instance in which such a change took place, the possibility that a genetic rearrangement might have really occurred was considered. For the DNA from sample 9746 the amplified pattern was completely different and resembled the one typical of the grapevine DNA samples (Fig. 2B). Consistently with this, it was found that all the grapevine strains exhibited the same amplification pattern without *Hin*dIII restriction sites (Fig. 2B).

Different from the results in which the transposition frequency of the insertion sequence IS1086 was 1000 times greater at 37°C compared to that at 30°C, the Tn3 translocation varied between 26 and 30°C [23]. Above 30°C the frequency dropped drastically up to null when the temperature was 37°C [24]. The molecular basis for such effects is not known yet but a number of papers seem to suggest that the molar concentration of the transposase protein is lower at 42°C than at 30°C probably as a consequence of the high turnover for such an enzyme [25].

Based on these data it is possible to speculate a correlation between the differences detected for the grapevine and plum *X. fastidiosa* strains due to the fact that these crops are regularly cultivated under colder temperatures as compared to the citrus samples, and since the transposon Tn1721 belongs to the same group as the Tn3 a higher transposition frequency might have taken place, resulting in at least two bands being detected for the temperate climate plants. As a consequence, bacteria with such higher amounts of insertion sequences might have greater genetic variability and this fact might result in different genetic arrangements, a situation that explains the differences in the banding pattern described in this work.

This suggests that apart from the existence of variations within the genome of *X. fastidiosa* strain, in relation to the copies of transposon Tn1721, the bacteria isolated from the plum and grapevine trees still retain their pathogenicity.

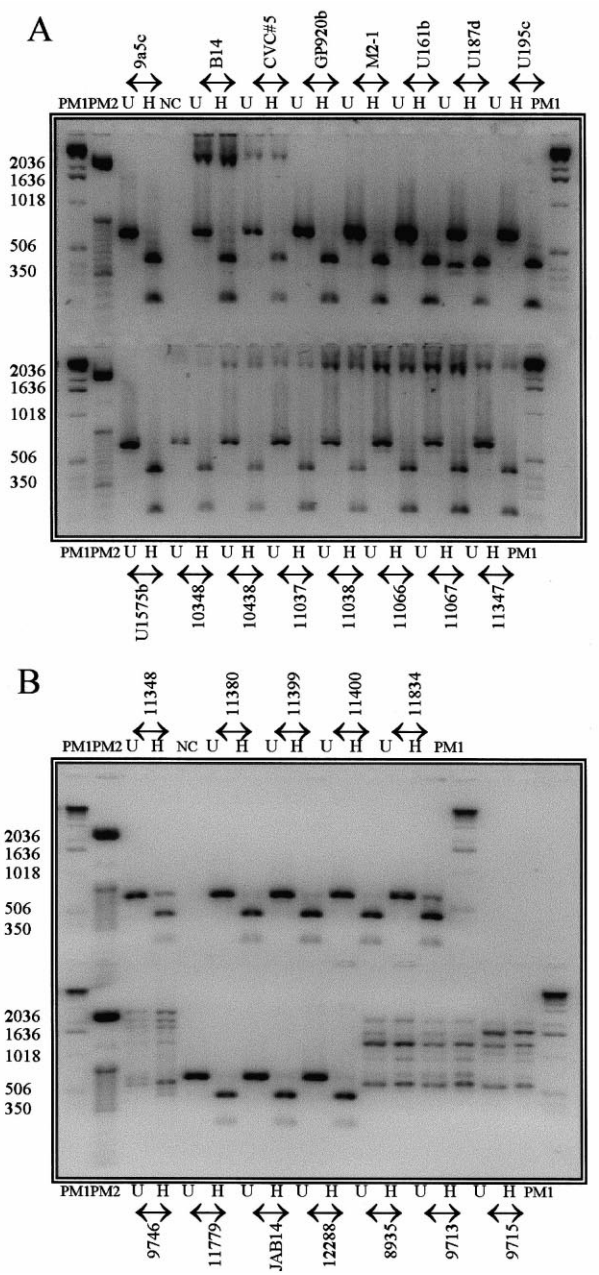

Fig. 2. Electropherogram of amplified genetic material using primers TNP_1-F and TNP_1-R. Part A shows citrus strains; part B shows citrus strains (upper lanes) and the strains from plum, citrus, coffee and grapevine (lower lanes). The numbers on each lane refer to *X. fastidiosa* strain denominations. PM1: 1-kb DNA ladder; PM2: 50-bp DNA ladder; NC: negative control; U: unrestricted DNA samples; H: *Hin*dIII-restricted DNA samples.

3.2. Strains with primer TNP_2-amplified sequences

Considering the TNP_2 primers the *X. fastidiosa* strains that produced different bands when compared to the pattern generated by the strain 9a5c, only the strain GP920b

(citrus) listed in Fig. 3A and the strain 9746 (plum) were different from the strains from grapevine listed in Fig. 3B.

In all repetitions that were carried out, the strain GP920b produced weak bands as seen in Fig. 2A. The weak band amplification is explained to be due to genetic polymorphism within the particular region of the genome that was under consideration for these experiments and not due to bad DNA quality since the DNA samples were the same as those used for amplification with the TNP_1 primers.

The strain 9746 (plum) presented a similar amplification pattern, as did the grapevine ones, all of them being different from the sequenced strain 9a5c. The above reasoning also explains the presence of only two fragments when *Sma*I was used to restrict the PCR products of the strain as opposed to the three expected fragments of 892, 215 and 136 bp (Fig. 3B).

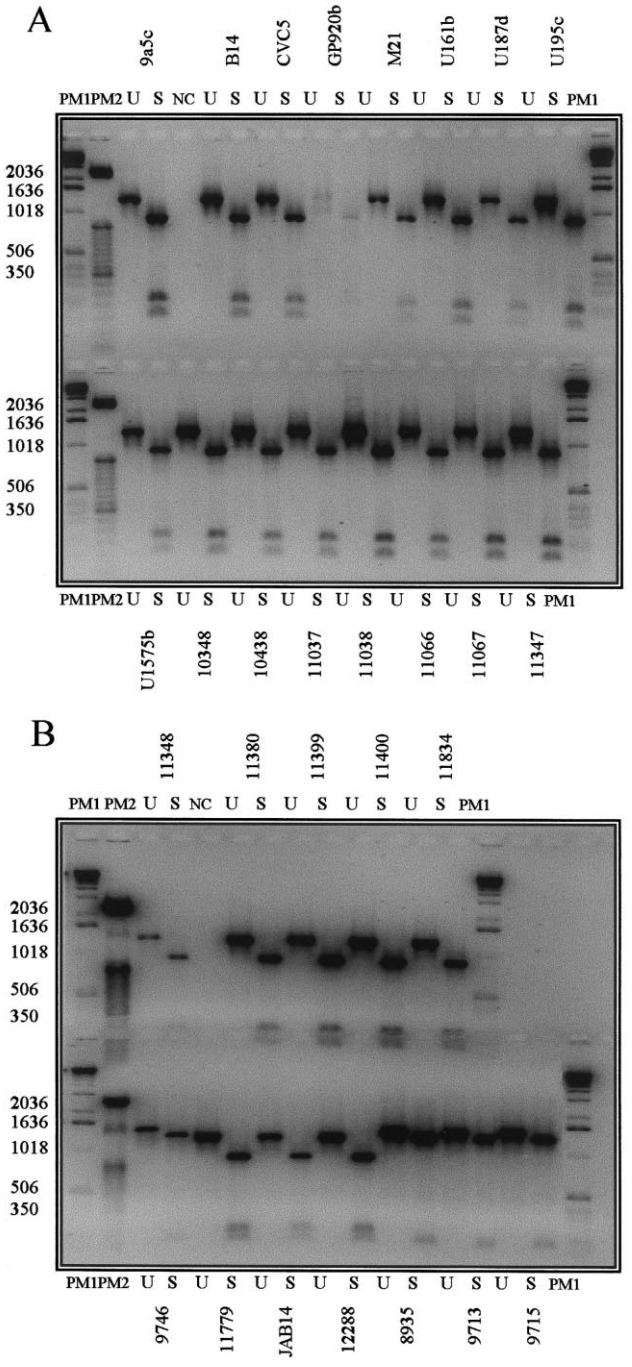

Fig. 3. Electropherogram of amplified genetic material using primers TNP_2-F and TNP_2-R. Part A shows the citrus strains; part B shows citrus strains (upper lanes) and the strains from plum, citrus, coffee and grapevine (lower lanes). The numbers on each lane refer to *X. fastidiosa* strain denominations. PM1: 1-kb DNA ladder; PM2: 50-bp DNA ladder; NC: negative control; U: unrestricted DNA samples; H: *Sma*I-restricted DNA samples.

3.3. Control bacterial strain analysis with primers TNP_1 and TNP_2 amplification products

The Tn3 family is a group of known transposons that are able to colonize a large set of Gram-negative and Gram-positive bacteria [25].

When the pair of TNP_1 primers was used, it was possible to observe amplification products in all control DNA samples except *Bacillus thuringiensis* var. *israelensis* (Fig. 4). The absence of amplified material might suggest that *B. thuringiensis* var. *israelensis* could have had an evolutionary diversion from the line that all the other bacteria seem to share because they might have evolved latter.

For *Agrobacterium radiobacter* (Beijerinck and van Delden 1902) Conn 1942 and *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942 there has been

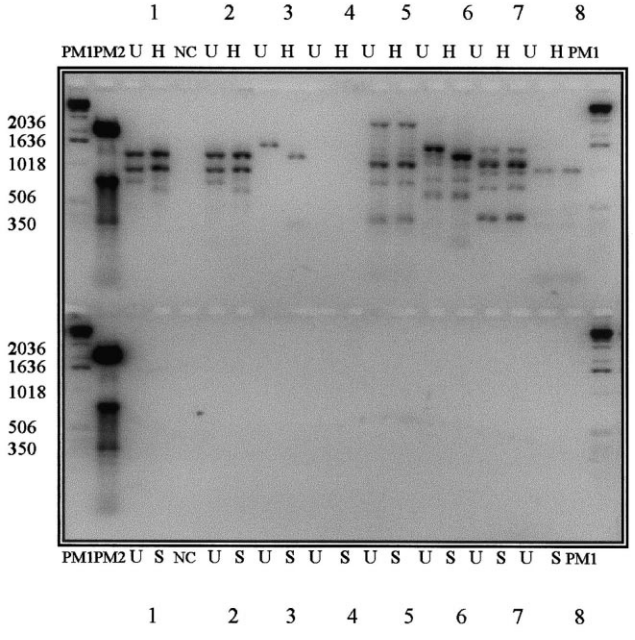

Fig. 4. Electropherogram of amplified genetic material using primers TNP_1-F and TNP_1-R (upper lanes) and TNP_2-F and TNP_2-R (lower lanes). 1: *A. radiobacter*; 2: *A. tumefaciens*; 3: *B. cereus*; 4: *B. thuringiensis* subsp. *israelensis*; 5: *P. stewartii* subsp. *stewartii*; 6: *Pseudomonas angulata*; 7: *X. axonopodis* pv. *citri*; 8: *Xanthomonas citri* pv. *pruni*. PM1: 1-kb DNA ladder; PM2: 50-bp DNA ladder; NC: negative control; U: unrestricted DNA samples; H: *Hin*dIII-restricted DNA samples; S: *Sma*I-restricted DNA samples.

the same amplification product with two quite strong bands. These amplification products demonstrate that the transposon Tn1721 is present in the genomes of such species with at least two copies.

For *Bacillus cereus* and *Pseudomonas syringae* pv. *tabaci* (Wolf and Foster 1917; Young, Dye and Wilkie 1978) there was a pattern of amplification similar to that of the strain 9a5c from *X. fastidiosa*, however with larger fragments. This variation denotes some degree of genetic polymorphism between these bacterial strains that was revealed by the analysis of the transposon Tn1721 insertion site for each bacteria.

The bacteria *Pantoea stewartii* subsp. *stewartii* SW2 (Smith 1898) Mergaert, Verdonck and Kersters 1993 presented similar characteristics to those exhibited by *Xanthomonas axonopodis* pv. *citri* (Hasse 1915) Vauterin, Hoste, Kersters and Swings 1995. The difference in the amplification patterns presented by the species of *X. axonopodis* pv. *citri* and *Xanthomonas arboricola* pv. *pruni* (Smith 1903) Vauterin, Hoste, Kersters and Swings 1995 can also be related to climate differences since one of them affects the citrus-originated strain and the other the strain that came from plum. None presented *Hin*dIII-restricted fragments.

There was no amplification with the primer TNP_2 for any of the control bacterial samples since this primer was formally designed based on *X. fastidiosa* sequenced material and the sequence in which the transposon Tn1721 is inserted within the genome is not shared with the other control bacteria, Gram-positive or Gram-negative.

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