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DNA fingerprinting detects genetic variability in the pearl millet downy mildew pathogen (*Sclerospora graminicola*)

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Abstract Genetic variability in six host genotype-specific pathotypes of pearl millet downy mildew pathogen *S. graminicola* was studied at the molecular level using mini- and micro-satellites. Our results indicated that microsatellites $(GAA)_6$, $(GACA)_4$, and especially $(GATA)_4$ were quite informative and showed high levels of polymorphism among the pathotypes. The six pathotypes could be classified into five groups based on the cluster analysis of their genetic similarities, thereby confirming the existence of distinct host genotype-specific virulence in *S. graminicola* pathotypes. We demonstrate, for the first time, the use of DNA fingerprinting to detect genetic variation in downy mildew fungus of pearl millet.

Key words Downy mildew · Microsatellites
DNA fingerprinting · Pearl millet

Introduction

Downy mildew, which is caused by *Sclerospora graminicola* (sacc.) Schroet, is an economically important disease of pearl millet (*Pennisetum glaucum*). The crop is grown both for food and forage (Rachie and Majumdar 1980) on about 26 million hectares (FAO 1983) in the arid and semi-arid tropical and subtropical environments of Asia and Africa. *S. graminicola* induces a systemic infection in pearl millet that manifests itself as foliar symptoms and as a green ear disease on panicle. The disease is a serious threat to the full exploitation of the high yield potential of F_1 hybrids because of their high susceptibility (Singh et al. 1993). The pathogen is both seed- and soil-borne, thus rendering crop rotation less effective in controlling the disease. *S. graminicola* is heterothallic (Michelmore et al. 1982), and the existence of variable pathotypes is known (Ball and Pike 1984). To develop breeding strategies for cultivars with stable and durable host resistance to downy mildew it is important to generate information about the pathogen populations and to characterize them at the molecular level.

Molecular approaches such as endogenous genomic and mitochondrial restriction fragment length polymorphism (RFLP) probes and random amplified polymorphic DNA (RAPD) markers have been successfully used to estimate the genetic diversity in fungal pathogens (Anderson et al. 1992; Milgroom et al. 1992; Levy et al. 1991). DNA fingerprinting facilitates the use of mini- and micro-satellites, which are hypervariable and dispersed in the form of long arrays of short tandem repeat units throughout the genome (Tautz and Renz 1984; Jeffreys et al. 1985).

The utility of DNA fingerprints is well-established in animal systems and has been reported to be extremely useful in detecting polymorphisms in higher plants (Weising et al. 1991a; Ramakrishna et al. 1994, 1995; Gupta et al. 1994). Recently, DNA fingerprinting using oligonucleotides such as $(GATA)_4$, $(GTG)_5$, $(CA)_8$, and $(TCC)_5$ has been reported to detect variation among isolates of *Ascochyta rabiei* (Weising et al. 1991b). In the case of filamentous fungi such as *Penicillium*, *Aspergillus*, and *Trichoderma*, oligonucleotide probes such as $(GATA)_4$ along with the M13 minisatellite probe have revealed informative DNA fingerprint patterns (Meyer et al. 1991). Human minisatellite probes 33.6 and 33.15 have been successfully used to distinguish pathotypes of *Colletotrichum gloeosporioides* (Braithwaite and Manners 1989). The development of polymerase chain reaction (PCR) fingerprinting using minisatellite and microsatellite core sequences as primers has proven to be a useful tool in detecting genetic variation (Meyer et al. 1993). In the investigation described here, we have examined the potential of several approaches, including a reliable and versatile technique of DNA fingerprinting,

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to study the extent of genetic variation in the pearl millet downy mildew pathogen *S. graminicola*.

Materials and methods

Fungal material

The six host-specific pathotypes of *S. graminicola* used were: path-1 (from host NHB 3), path-2 (BJ 104), path-3 (MBH 110), path-4 (852 B), path-5 (700651), and path-6 (7042 S) (Thakur et al. 1992). These pathotypes were maintained on the respective host genotypes by repeated inoculation with asexual spores in isolation chambers in a greenhouse.

Oospore and zoospore isolates

The isolates were collected from different pearl millet growing areas of the Indian sub-continent (Thakur and Shetty 1993). The sexual spores (oospores) are formed in leaves infected with thalli of compatible mating types. Such oospore isolates were collected from Coimbatore (designated as CB OS1), Mysore (MYS OS1), Hissar (HS OS6), and ICRISAT (IC OS1). The asexual single zoospore isolates were obtained from sporangia produced on seedlings inoculated with the above oospore isolates. Individual zoospores released from germinating sporangia on 0.75% water agar were used to inoculate seedlings in pots in order to induce infection. PT2 and PT3 are opposite mating-type isolates and can be maintained asexually on highly susceptible pearl millet genotype 7042 S.

DNA isolation

Sporangia were harvested from sporulating infected leaves in ice-cold sterile distilled water, and the suspension was centrifuged at 4000 g for 20 min at 5 °C in Sorvall RC centrifuge. The sporangial pellet was powdered in liquid nitrogen in a mortar and pestle, and the powder extracted with 5 volumes of extraction buffer (50 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, 0.5 *M* NaCl, 1.0% SDS) and kept at 65 °C for 20 min. An equal volume of chloroform:phenol (1:1) was added to the slurry, mixed gently, and then centrifuged for 10 min at 12 000*g* in a Sorvall RC 2C centrifuge. The aqueous layer was removed, and an equal volume of chloroform:Isoamyl alcohol (24:1) was added, mixed well and centrifuged for 10 min. The aqueous phase was removed, and nucleic acids were precipitated by adding 0.6 volumes of isopropanol. DNA was spooled with a glass rod, washed twice with 70% ethanol, and suspended in $T_{50}E_{10}$ buffer (50 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0). The DNA solution was treated with RNase (50 µg/ml) at 37 °C for 2 h. An equal volume of phenol:chloroform was added to the solution at the end of incubation, mixed well for 5 min, and centrifuged. The aqueous phase was transferred to another tube, and DNA was precipitated by adding 2.5 volumes of absolute ethanol. The DNA pellet was washed twice with 70% ethanol, vacuum dried, and resuspended in $T_{10}E_1$ buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0).

Restriction enzyme digestion of DNA and agarose gel electrophoresis

Various restriction enzymes, such as *Alu*I, *Bam*HI, *Bgl*I, *Eco*RV, *Hae*III, *Hinf*I, *Hin*dIII, *Hpa*II, *Msp*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, and *Taq*I, were used to digest genomic DNA. About 8–10 µg of DNA was digested with the restriction enzymes (according to supplier's instructions), and electrophoresis was carried out on 0.8–1.2% agarose gels in TPE buffer (90 m*M* Tris-phosphate, 2 m*M* EDTA, pH 7.5). Complete restriction enzyme digestion of DNA was ensured by using higher concentrations of the enzyme. The gels were stained in ethidium bromide, photographed, and dried in a gel dryer.

DNA probes and Southern hybridization

The human minisatellite probe, pV47, isolated from the human chromosome 6-specific library, was cloned in pUC18 and propagated in JM 101. A 282-bp fragment containing nine tandem repeats of a 15-bp core sequence from M13mp 18RF was obtained by digesting the latter with *Hae*III and *Cla*I. Both these probes had been labelled with [α-^{32}P]-dCTP by the random priming method described by Sambrook et al. (1989) and were used for hybridization in 30% formamide, 5 × SSPE, 0.1% SDS, 5 × Denhardts, 0.1 × BLOTTO at 42 °C. The filters were washed twice in 1 × SSPE, 0.1% SDS for 15 min at room temperature and at 55 °C for 10 min for M13; and for pV47 as described above except that the hot wash was given at 60 °C for 15 min. Oligonucleotides were synthesized on a gene assembler plus (Pharmacia), desalted on a NAP column, and purified on a 20% denaturing polyacrylamide gel. Oligonucleotide probes were 5'-end-labelled by T4 polynucleotide kinase as described by Sambrook et al. (1989). Dry gels were denatured, neutralized, and hybridized at their $T_m - 5$ °C, i.e., 35 °C for $(GATA)_4$, 43 °C for $(GGAT)_4$ and $(GACA)_4$, 45 °C for $(CAC)_5$, and 55 °C for $(TG)_{10}$, and subjected to stringent washing as described by Schafer et al. (1988). The blots dry gels were exposed to X-ray films (Kodak, USA) for a specific period, the length of time depending on the intensity of the hybridization signal. Dry gels were reused after stripping the probe by treatment with a denaturation solution (0.5 *M* NaOH, 0.15 *M* NaCl) followed by a neutralization solution (0.5 *M* Tris-HCl pH 8.0, 0.15 *M* NaCl) for 30 min.

Polymerase chain reaction

Amplification reactions were performed using 50 ng genomic DNA and 200 µM dNTPs (NEB, England) with the addition of 1.85 KBq of [α-^{32}P]-dCTP, 20 ng of primer $(GATA)_4$, and 1 unit of *Taq* polymerase (Bangalore Genei, India) in 25 µl of reaction mix using a MJR Thermocycler (MJ Scientific, USA) programmed for 35 cycles with a temperature regime of 93 °C for 1 min, 35 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplified products were analyzed by electrophoresis on nondenaturing 8% polyacrylamide gels in 1 × TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA, pH 8.0) and detected by overnight exposure to X-ray film (Kodak, USA).

Statistical analysis

The relatedness of the six pathotypes was estimated by means of scorable bands from the different probe-enzyme combinations. Differences in banding pattern were scored on the basis of absence or presence of a band. Similarity indices expressing the probability that a fragment in one pathotype is also found in another for all pair-wise comparisons were made (Wetton et al. 1987). Cluster analysis of data was carried out using the statistical software package SYSTAT 5.1. A dendrogram showing the mean similarities between groups of different pathotypes was generated.

Results

Repetitive DNA sequences from a genomic library of the most virulent strain path-6 constructed in lambda gt11 vector (selected on the basis of strong intensity of hybridization signal with total DNA) were used to detect polymorphism among the six host genotype-specific pathotypes. These probes showed a continuous smear with five to six strong bands that were monomorphic.

Minisatellites as markers

The M13 minisatellite probe, which is known to be useful in detecting high levels of polymorphism in humans and animals (Vassart et al. 1987), did not show any polymorphism among the six pathotypes. On the other hand, the human minisatellite probe, pV47, known to be very effective in detecting genetic polymorphisms in humans and plants (Longmire et al. 1990; Ramakrishna et al. 1995), was found to be useful in detecting polymorphism among the DNA digests of the six pathotypes of *S. graminicola* with *Pvu*II, and this proved to be more informative than other restriction enzyme digests. Path-4 was clearly distinguishable from the other five pathotypes on the basis of a prominent band of 3 kb, suggesting that this locus was present only in path-4 (data not shown).

Microsatellites as markers

The simple repetitive DNA sequences such as $(GATA)_4$, $(GACA)_4$, $(GAA)_6$, $(CAC)_5$, $(GGAT)_4$, and $(TG)_{10}$ were tested for their ability to detect polymorphisms in *S. graminicola* pathotypes using several restriction enzymes. Microsatellites, $(TG)_{10}$ $(GGAT)_4$, and $(CAC)_5$ largely produced a smear containing bands that were mostly monomorphic. We obtained different levels of polymorphisms and complexities with the $(GATA)_4$, $(GACA)_4$, and $(GAA)_6$ probes. Highly polymorphic hybridization patterns unique for each pathotype were obtained with the $(GATA)_4$ microsatellite with *Msp*I (lanes 1–6) and *Taq*I (lanes 7–12) (Fig. 1). Five to eight distinct polymorphic bands that varied from 1 to 3 kb in size were observed for each pathotype (Fig. 1). Path-3, path-4, and path-5 (lanes 2, 4, and 6, respectively, with *Msp*I and lanes 8, 10, and 12, respectively, with *Taq*I) showed diverse fingerprint patterns with both these enzymes. Path-1 and path-2 (lanes 3 and 1) showed similar hybridization patterns in *Msp*I-digested DNA, whereas they could be distinguished from each other using *Taq*I (lanes 9 and 7). Probes $(GACA)_4$ and $(GAA)_6$ could also distinguish path-1, path-3, and path-4 from the others (data not shown). The average similarity indices among the pathotypes with probes $(GATA)_4$, $(GACA)_4$, and $(GAA)_6$ were estimated to be 0.26, 0.71, and 0.74, respectively. This clearly shows the hypervariable nature of probe $(GATA)_4$ and its ability to detect highly polymorphic regions in the genome.

Further, to study the stability of the $(GATA)_4$-derived DNA fingerprints, we examined the pathotypes after ten asexual generations, they were found to be identical (data not shown).

The highly polymorphic nature of oligonucleotide $(GATA)_4$ prompted us to use it as a primer for PCR fingerprinting in order to amplify the DNAs from the six pathotypes. Most of the bands were monomorphic, and polymorphisms were mainly limited to differences in the intensity of bands. Therefore, we concluded that PCR fingerprinting using $(GATA)_4$ as a primer was not as useful as actual hybridization with simple repeat motifs as reported in yeasts (Lieckfeldt et al. 1993).

Cluster analysis of pathotypes

A dendrogram based on the DNA fingerprints obtained with all three probes $(GATA)_4$, $(GACA)_4$, and $(GAA)_6$ clustered the six pathotypes into five groups (Fig. 2). Path-5, path-3, and path-4 formed distinct groups, whereas path-1, path-2, and path-6 together formed a separate group in which path-1 and path-2 clustered as one subgroup and path-6 as another.

Variability study in the oospore- and zoospore-derived isolates

Since the $(GATA)_4$ probe could decipher DNA fingerprints with host-specific pathotypes, we used this probe

Fig. 1 Autoradiogram of the DNA fingerprints of six host genotype-specific pathotypes of *S. graminicola*. Fungal DNA was digested with *Msp*I (*lanes 1–6*) and *Taq*I (*lanes 7–12*) and hybridized with $(GATA)_4$ as described in the Materials and methods. pathotypes: *lanes 1&7* Path-2 (BJ 104), *2&8* Path 3 (MBH 110), *3&9* Path 1 (NHB 3), *4&10* Path 4 (852 B), *5&11* Path 6 (7042 S), *6&12* Path 5 (700651). Molecular size of the markers in kb are indicated in the *right margin*

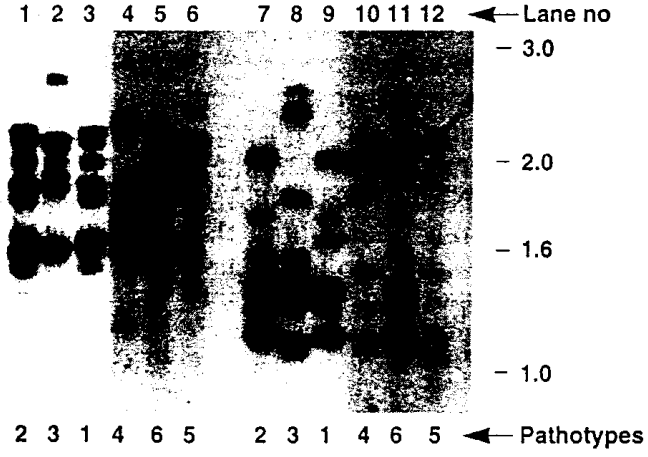
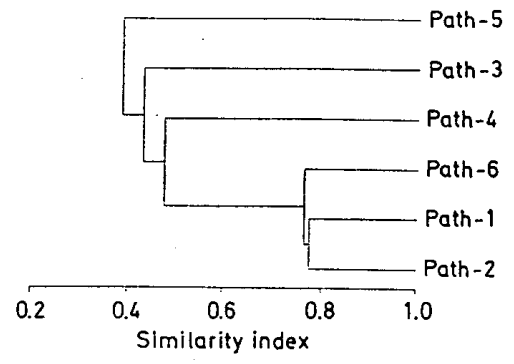


Fig. 2 Dendrogram of the six host genotype-specific pathotypes of *S. graminicola* with microsatellite probes. Cluster analysis of the data obtained from the hybridization of *Msp*I-and *Taq*I-digested fungal DNA with the microsatellite probes, $(GATA)_4$, $(GACA)_4$, and $(GAA)_6$ was done as described in the Materials and methods



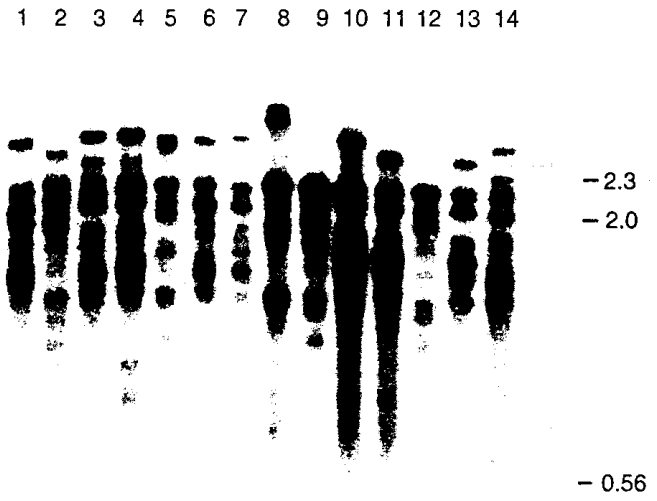


Fig. 3 Autoradiogram of the DNA fingerprints of oospore- and zoospore-derived isolates of *S. graminicola*. DNA of the oospore- and zoospore-derived isolates were digested with *Msp*I and hybridized to the microsatellite probe, $(GATA)_4$, as described in the Materials and methods. *Lanes 1* HS OS6, *2* IC OS5, *3* CB OS4, *4* CB OS1 Z-1, *5* CB OS1 Z-3, *6* CB OS1 Z-4, *7* JPR Z-11, *8* PT2, *9* PT3, *10* PT2 Z-1, *11* PT3 Z-2, *12* MYS OS1, *13* MYS Z-1, *14* MYS Z-4

futher to analyze the oospore- and zoospore-derived isolates. Figure 3 shows *Msp*I-digested DNAs from oospore and zoospore isolates hybridized with $(GATA)_4$. Isolates HS OS6, IC OS5, CB OS4, JPR Z-11, PT2, PT3, and MYS OS1 (lanes 1, 2, 3, 7, 8, 9, and 12, respectively) showed diverse DNA fingerprint patterns that were unique relative to those of the six host-specific pathotypes. Further, the variation/similarity between individual isolates and different zoospore isolates collected from the same location was also studied. All three zoospore isolates obtained from Coimbatore showed considerable variation (lanes 4, 5, and 6, respectively). On the other hand, the oospore isolate CB OS4 and zoospore isolate CB OS1 Z-1 (lanes 3 and 4, respectively) showed almost similar patterns. Contrary to this, striking differences in the hybridization patterns of oospore (lane 12) and zoospore isolates (lanes 13 and 14, respectively) from Mysore were observed. The two opposite mating-type isolates PT2 and PT3 (lanes 8 and 9, respectively) showed a high degree of variation. However, the zoospore isolates from Patancheru, PT2, Z-1 and PT3 Z-2, showed similar patterns, except for a single band (lanes 10 and 11, respectively). Interestingly, the PT3 isolate showed almost 100% similarity with Mysore oospore isolate MYS OS1 (lanes 9 and 12, respectively).

Discussion

Biological pathotyping indicates the existence of variation in the pathogenic populations of *S. graminicola* with reference to host specificity, aggressiveness, and virulence (Thakur 1993; Thakur and Shetty 1993). Pathotyping based on genome similarity adds one more dimension to these studies. Moreover, such pathotyping is more precise and devoid of the influences of environmental factors since the variation is studied at the DNA level. Genomic repetitive DNA sequences that are believed to be involved in speciation are the main source of polymorphism in eukaryotic systems as has been shown in *Magnaporthe griesa* by Hammer et al. (1989). In *S. graminicola*, however, repetitive probes form the genomic library were not able to bring out any polymorphism, indicating the highly conserved nature of these repeats in the fungal isolates.

Variability studies in host genotype-specific pathotypes

Although the human minisatellite pV47 could distinguish a particular pathotype, path-4, it was mostly the microsatellite-generated DNA fingerprints that were more effective in distinguishing between all of the *S. graminicola* isolates used in the present study. The DNA fingerprint patterns revealed differences in the relative abundance and organization of micro- and mini-satellites in the genome of fungal isolates. Polymorphism in the fungal isolates was much pronounced with $(GATA)_4$, $(GACA)_4$, and $(GAA)_6$, which clearly demonstrates the hypervariable nature of such simple repeats as $(GATA)_4$, which has been well-documented in a variety of eukaryotic systems (Epplen 1988; Vosman et al. 1992). The detection of a high level of polymorphism among the six pathotypes using $(GATA)_4$ was unique, and the other repeat probes described above did not bring about this level of polymorphism with any of the restriction enzymes used in our study. In general, the use of restriction enzymes with a four-base specificity can decipher more variability in *S. graminicola* than can hexacutter-specific enzymes, as has been observed in other systems (Kashi et al. 1990). Microsatellite $(GATA)_4$-derived DNA fingerprints were stable even after ten asexual generations, indicating the utility of this probe in DNA fingerprinting.

As shown in our results, the simple sequence repeats/microsatellites were highly informative in the development of pathotype-specific fingerprint patterns. The dendrogram constructed on the basis of fingerprints, classified the six pathotypes into five distinct groups. The dendrogram (Fig. 2) corresponds well with the characterization of the six host genotype-specific pathotypes into five major groups based on differential host reaction (Thakur 1993). The genetic distance among pathotypes can be explained based on their host specificity and genetic dissimilarities among host genotypes. This data is in good agreement with the groupings based on RAPD patterns of the genotype-specific pathotypes of *S. graminicola* with the A-8 primer (Sivaramakrishnan et al. 1995). On the basis of the dendrogram, path-1 (NHB 3) and path-2 (BJ 104) can be considered to be closely related, and this can be explained by the fact that the two host genotypes share a common male parent.

Variability studies in oospore- and zoospore-derived isolates

We used the same enzyme-probe combination mentioned above to test the usefulness of the probe in studying different oospore- and zoospore-derived isolates. These isolates did not show much homology with the six host genotype-specific pathotypes. They also showed much variation within the isolates collected form the same location and a lot of similarities between the isolates from different locations. This variation in the oospore isolates is natural owing to its demographic locations and adaption to the different pearl millet genotypes growing in that location. Heterogeneity in terms of virulence and aggressiveness was observed among oospore isolates (Thakur and Shetty 1993). The different zoospore isolates from the same field either represent diverse fungal isolates or they represent a clonal population of the same isolate with variations at the DNA level that could be detected with probe $(GATA)_4$. In the case of *Septoria tritici*, a wheat pathogen, a high level of variability among the isolates was found to exist in the same lesion or on the same leaf (Boeger et al. 1993). In *Magnaporthe grisea*, a high degree of genetic diversity was detected within locations in the same field (Xia et al. 1993). This supports the finding that differences at the DNA level are distributed along a very fine spatial scale in several fungi (Kohli et al. 1992; McDonald et al. 1994; Xia et al. 1993) that can be detected only with the help of a sensitive tool such as DNA fingerprinting. There are reports that oligonucleotide probes can detect variations even in clonal populations (Turner et al. 1992).

The combined influences of the introduction of new host genotypes, step-wise selection for virulence, and the possibility of other mechanisms for genetic reassortment may have contributed to the development of new virulent races. However, at the molecular level, there seems to be limited variation among the pathotypes, and this may be confined to a specific locus in the genome that can be detected only with the help of hypervariable probes. DNA fingerprinting demonstrates a very high level of genetic variation in natural populations, indicating that these populations probably contain sufficient genetic variability to allow for rapid selection of clones that are resistant to fungicides or virulent on pearl millet varieties having pathotype-specific resistance. These data provide some insight into population structure and clonal dynamics.

Thus, the use of simple sequence repeats such as $(GATA)_4$, which is highly polymorphic, may be very useful for the rapid and efficient identification and genetic analysis of the pathogen population. These fingerprint profiles of different pathotypes can be used as diagnostic tools to formulate breeding strategies targeting resistance to local population and for monitoring the emergence of new virulent races.

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