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Microsatellite Markers Reveal Genetic Variation within *Sclerotinia sclerotiorum* Populations in Irrigated Dry Bean Crops in Brazil

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Abstract

Microsatellites are powerful markers to infer population genetic parameters. We used 10 microsatellite loci to characterize the genetic diversity and structure of 79 samples of *Sclerotinia sclerotiorum* isolated from four Brazilian dry bean populations and observed that eight of them were polymorphic within populations. We identified 102 different haplotypes ranging from 6 to 18 per locus. Analyses based on genetic diversity and fixation indices indicated variability among and within populations of 28.79% (F_{ST} = 28793) and 71.21%, respectively. To examine genetic relatedness among *S. sclerotiorum* isolates, we used internal spacer (ITS1-5.8S-ITS2) restriction fragment length polymorphism (PCR-RFLP) and sequencing analysis. PCR-RFLP analysis of these regions failed to show any genetic differences among isolates. However, we detected variability within the sequence, which does not support the hypothesis of clonal populations within each population. High variability within and among populations may indicate the introduction of new genotypes in the areas analysed, in addition to the occurrence of clonal and sexual reproduction in the populations of *S. sclerotiorum* in the Brazilian Cerrado.

Introduction

The ubiquitous plant pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary infects 278 genera of crops and weed species (Boland and Hall 1994) and consequently has considerable economic importance as a pathogen of food crops (Purdy 1979). Among the soilborne fungi causing white mould on irrigated dry bean crops (*Phaseolus vulgaris* L.), *S. sclerotiorum* is one of the most harmful pathogens and causes great losses (Peres et al. 2002). According to Nasser and Sutton (1995), the most significant losses of bean yield since the 1980's in Brazil have been caused by *S. sclerotiorum*, especially in the Cerrado region, where irrigated beans have been increasingly cultivated.

Control of plant diseases caused by *S. sclerotiorum* is difficult (McQuilken et al. 1995) and requires the integration of several methods (Tu 1997). Survival of this fungus is facilitated by the production of vegetative sclerotia, resistant structures that can survive in the soil for periods up to 4–5 years (Adams and Ayers 1979). Longevity of sclerotia and its potential to infect the host over successive growing seasons represent challenges to successful disease control.

Sclerotinia sclerotiorum has a haploid somatic phase in which clonality is the result of both asexual reproduction by means of sclerotia and sexual reproduction by self-fertilization (Kohn 1995) with the expectation that intraclonal variation is due to mutation (Carbone et al. 1999; Carbone and Kohn 2001). Population studies on *S. sclerotiorum* have revealed a predominantly clonal mode of reproduction (Kohli et al. 1995; Cubeta et al. 1997; Kohli and Kohn 1998) with some evidence of outcrossing contributing to the population structure in a few regions (Kohli and Kohn 1998; Atallah et al. 2004; Sexton and Howlett 2004).

Simple sequence repeat (SSR) markers, or microsatellites, are hypervariable and dispersed in the form of long arrays of short tandem repeat units throughout the genome. These SSRs provide codominant Mendelian markers, much more powerful than dominant markers, and can be used to determine population genetic structure, kinship, reproductive mode, and genetic isolation. Several studies have shown the importance of using microsatellites or SSRs to understand epidemiological processes in plant pathogenic fungi (Giraud et al. 1999; Sirjusingh and Kohn 2001; Meinhardt et al. 2002; Atallah et al. 2004; Peever et al. 2004; Sexton and Howlett 2004; Mert-Türk et al. 2007).

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The structure and dynamics of the populations of *S. sclerotiorum* represent an essential part of understanding how the underlying mechanisms are involved in the pathogen history and distribution along geographical areas and different hosts (Carbone and Kohn 2001). Extensive crop damage and a general difficulty of controlling diseases caused by *S. sclerotiorum* have been the impetus for sustained research on this pathogen. This study aimed to better understand the biology, epidemiology, and genetics of *S. sclerotiorum* in irrigated dry bean crop areas. Our objective was to use molecular techniques – microsatellite and ITS1-5.8S-ITS2 region analyses – to examine the genetic variation within and between the populations of this pathogen.

Materials and Methods

Sclerotinia sclerotiorum isolates and growth conditions

We recovered 79 *S. sclerotiorum* isolates from dry bean plants showing typical symptoms in four irrigated areas located in the Brazilian Cerrado region in 2001. Using a hierarchical sampling regime allowed us to compare variations at different levels, i.e. within a single irrigation area and between irrigation areas in different locations. The isolates were sampled randomly from within the field, at least 25 m apart. The approximate distance between irrigation areas was 10–50 km.

Sclerotia were surface-sterilized by immersion in 70% ethanol for 2 min followed by three washes in sterile reverse-osmosis water. Each isolate was derived from a single sclerotium, with only one isolate obtained from each plant. All cultures were grown and maintained on potato dextrose agar (PDA) at 20°C with a 12-h photoperiod. For DNA extraction, isolates were grown in potato dextrose broth (PDB). All cultures were stored on PDA slopes at 4°C until use.

Genomic DNA extraction

Lyophilized mycelia were used for genomic DNA extraction, which was carried out using a small-scale DNA isolation method according to the protocol described by Zolan and Pukkila (1986).

Microsatellite data analyses

Primer pairs for amplification and polymorphism of 10 microsatellite regions described by Sirjusingh and Kohn (2001) were tested on 79 isolates of *S. sclerotiorum*. Eight primer pairs, which gave the most consistent amplification of polymorphic products, were chosen for further analysis.

Polymerase chain reaction (PCR) amplification of microsatellite markers and detection were performed according to Sirjusingh and Kohn (2001). Haplotypes were created after assigning allele numbers to each locus in each individual. Genetic data analysis (GDA) programme was used to calculate allele frequencies and gene diversity (Lewis and Zaykin 2001). Arlequin, integrated software for population genetics data analysis, was employed for calculating haplotype frequencies, FST estimator theta (Weir and Cockerham 1984), and a hierarchical analysis of molecular variance (AMOVA) between and within samples.

ITS-RFLP and sequencing

The ITS1-5.8S-ITS2 regions were amplified by PCR utilizing the universal oligonucleotides ITS5 (5′GGA AGT AAA AGT CGT AAC AA-3′) and ITS4 (5′TCC TCG CTT ATT GAT ATG A-3′) (White et al. 1990). The system consisted of 0.25 μM of each oligonucleotide, 0.2 μM of each dNTP, 1.5 μM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 8.4, 2.5 U *Taq* DNA polymerase (~~Invitrogen~~), 10 ng of genomic DNA, and Milli-Q water to a final volume of 30 μl. Amplification was carried out in a PTC-100 thermocycler, programmed for a cycle of 94°C for 3 min, followed by 40 cycles at 92°C for 1.5 min, 42°C for 1.5 min, 72°C for 2.5 min, and a final extension at 72°C for 5 min.

We digested 3 μg of the PCR-amplified ITS1-5.8S-ITS2 regions with each of the following restriction endonuclease enzymes: *Msp* I, *Hae* III, *Taq* I, *Hinf* I, and *Hha* I, following the manufacturer's instructions. The restriction fragments were separated in 3% MetaPhor agarose gels, stained with ethidium bromide (0.5 μg/ml), and visualized under UV light. The size of the fragments was estimated based on the size markers 1- kb ladder (~~Promega~~).

The PCR-amplified ITS1-5.8S-ITS2 region was sequenced directly according to the manufacturer's instructions using a MegaBace 1000 automatic sequencer (Amersham Biosciences, Piscataway, NJ, USA). Each reaction mixture comprised 1 μl of ITS-PCR product (100 ng/μl), 4 μl of DYEnamic kit ET DYE (Terminator Cycle Sequencing for MegaBace; Pharmacia Biotech, Denver, CO, USA), 0.5 μl of oligonucleotide (0.5 μM), and Mili-Q water to a final volume of 10 μl. The amplification reactions were performed in a 96-well GeneAmp PCR System 9700 thermocycler (Applied Biosystem, Foster City, CA, USA) under the following conditions: an initial denaturation step at 96°C for 2 min, 35 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The quality of the chromatograms obtained was assessed using the base-calling program Phred (Ewing and Green 1998). We compared the sequences generated with existing data available in the GenBank using the similarity search program basic local alignment search tool (BLAST) (Altschul et al. 1997), thereby exploring all data banks of available DNA sequences.

All the sequences belonging to the isolates classified as *S. sclerotiorum* that had a base-call accuracy of 99% (quality score of $Q \geq 20$) were aligned using the CLUSTAL W program, and the genetic distance was calculated based on the Kimura coefficient (Kimura 1983). The phylogenetic relationships among the nucleotide sequences of the isolates were determined using Phylogeny Inference Package (PHYLIP) version 3.5 (Serb et al. 2001). Confidence levels for the groupings generated were determined by a bootstrap of 1000 repetitions (Yap and Nelson 1996).

Results

In this study, the genetic variability of 79 *S. sclerotiorum* populations isolated from four irrigated dry bean crop areas in the Brazilian Cerrado region was estimated using the microsatellite markers previously described by Sirjusingh and Kohn (2001).

We observed 102 different alleles, ranging from 6 to 18 alleles per locus, indicating an average of 10.2 alleles per locus. The allelic diversity within each locus ranged from 0.65 to 0.91. Some loci presented a large number of alleles and a high level of diversity, as locus 7-2 with 14 alleles and diversity of 0.91 and locus 13-2 with 18 alleles and diversity of 0.90 (Table 1).

Among the 10 microsatellite primer sets used in this study and in isolates from North America, mainly from Canada (Sirjusingh and Kohn 2001), eight have also been analysed in 154 *S. sclerotiorum* isolates from four Australian canola fields (Sexton and Howlett 2004), six in 167 isolates from four potato fields in the Columbia Basin of Washington, USA (Atallah et al. 2004), eight in 105 isolates from canola fields in Australia (Sexton et al. 2006), and three in a population of 36 isolates of oilseed rape fields in Turkey (Mert-Türk et al. 2007). In Table 1, the number of alleles observed in each locus in our study is compared with the results found in the previous researches. These data indicate that the isolates collected from irrigated dry bean plants in the Brazilian Cerrado region presented higher numbers of alleles in all the analysed loci, except for locus 106-4, in which we observed nine alleles, whereas in the isolates from North America, Sirjusingh and Kohn (2001) observed 10 different ones. Another interesting result refers to locus 36-4, which presented nine different alleles and variability of 0.82, and is monomorphic in the USA and Australia (Atallah et al. 2004; Sexton and Howlett 2004).

Table 1
Number of alleles/gene diversity for each microsatellite marker set in the *Sclerotinia sclerotiorum* populations used in the present study compared with previous reports

Locus	Repetitive sequence	Alleles (no.)	Allele frequency > 0.05 s	Allele size (bp)[a]	Alleles previous reports (no.)
7-2	$(GA)_{14}$	14	0.91	160–172	4[a], 3[b], 6[d], 10[e]
8-3	$(CA)_{12}$	9	0.83	251–271	4[a], 7[d], 12[e]
9-2	$(CA)_9(CT)_9$	8	0.76	358–382	4[a], 5[d], 7[e]
12-2	$(CA)_9$	9	0.79	215–225	4[a], 2[b], 3[c], 5[d], 5[e]
13-2	$(GTGGT)_6$	18	0.90	284–304	2[a], 2[b], 5[d], 10[e]
36-4	$CA_6(CGCA)_2$ CAT_2	9	0.82	415–429	2[a], 1[d], 1[e]
42-4	GA_9	6	0.65	410–414	3[a]
92-4	$(CT)_{12}$	8	0.86	374–378	3[a], 2[b], 4[d], 5[e]
106-4	$(CATA)_{25}$	9	0.87	491–571	10[a], 3[b], 6[c]
114-4	$(AGAT)_{14}$ $(AAGC)_4$	12	0.88	351–391	8[a], 4[b], 9[c]

[a]Sirjusingh and Kohn (2001).
[b]Atallah et al. (2004).
[c]Mert-Türk et al. (2007).
[d]Sexton and Howlett (2004).
[e]Sexton et al. (2006).

Using the GDA progam (Lewis and Zaykin 2001), we generated data on the allelic diversity per locus according to Nei (1973). Our data indicated high variability among loci, an average of 0.83, ranging from 0.65 in locus 42-4 to 0.91 in locus 7-2 (Table 1).

We developed the analysis of variance of genic frequencies (Cockerham 1969) based on the assumption that the *S. sclerotiorum* populations under study originated from the same ancestral population, allowing us to estimate the inbreeding coefficient. The fixation index (F_{ST}) (Weir and Cockerham 1984) indicates the distribution of genetic variability among populations based on probability of allele identity. According to Yeh (2000), estimates of F_{ST} lower than 0.050, between 0.051 and 0.150, and between 0.151 and 0.250 represent low, medium, and high levels of genetic differentiation, respectively. Fixation index (F_{ST}) calculated for the isolates used in this study was 0.28793 (Table 2), a value above the established standard of high level of genetic differentiation, indicating the occurrence of high variability among the populations of *S. sclerotiorum* analysed. The variance test (AMOVA) showed higher genetic variability within the populations (71.21%) than among them (28.79%), indicating that there is still a gene flow among these populations (Table 2).

Our findings showed a moderate to high level of genotypic diversity of *S. sclerotiorum* populations collected from irrigated dry bean plants (Table 3). In addition to the high genetic variability observed in the isolates, we found eight private alleles in populations A1, A2, and A3 and five in population Am (Table 3), which can be considered a large number, and observed 29 private alleles (28.43%) in a total of 102 alleles obtained.

ITS1-5.8S-ITS2 regions analysis

The ITS1-5.8S-ITS2 regions of the rDNA of these isolates produced an amplicon of approximately 600 bp in length, which was digested with the restriction enzymes *Msp* I, *Hae* III, *Taq* I, *Hinf* I, and *Hha* I, respectively, and none of the enzymes revealed any difference among the isolates (data not shown).

To determine whether the large genetic diversity observed in this study is sustained by separation within the species, 25 isolates were characterized by ITS1-5.8S-ITS2 region sequencing. These isolates were chosen on the basis of geographical origin and SSR analyses based on their high genotypic diversity.

Table 2
AMOVA test comparing isolates of four *Sclerotinia sclerotiorum* populations (A1, A2, A3, and Am) collected from irrigated dry bean crop areas in the Brazilian Cerrado in 2001

Source of variation	Sum of squares	Variation (%)
Among populations	6613	28.79
Within populations	18 400	71.21

Fixation index (F_{ST}) 0.28793.

Table 3
Sample sizes and indicators of genotype diversity of four Brazilian *Sclerotinia sclerotiorum* field populations collected from irrigated dry bean (*Phaseolus vulgaris*) crop areas in the Brazilian Cerrado in 2001

Population/area	Population size	Genotype diversity[a]	Private allele (no.)[b]
A1	19	0.711572	8
A2	20	0.70995	8
A3	20	0.69253	8
Am	20	0.641017	5

[a]Genotype diversity measure as described by GDA.
[b]Total number of private alleles = 29.

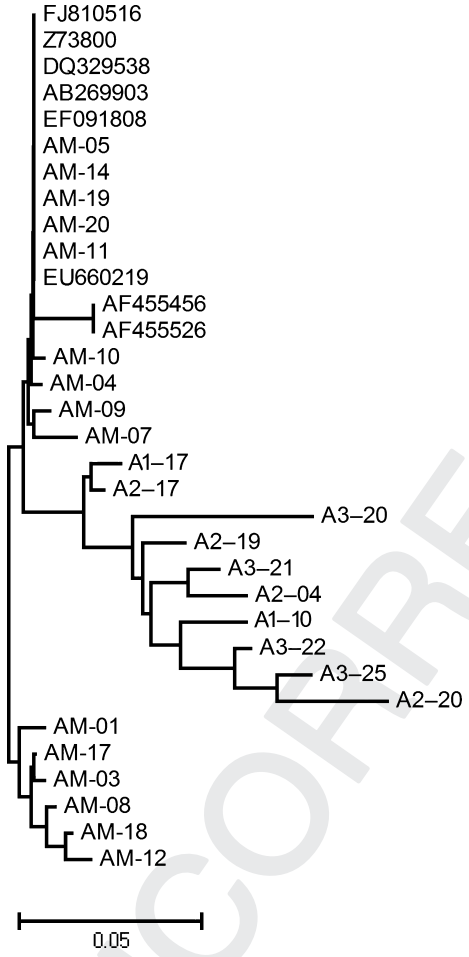

Fig. 1 Phylogram based on the ITS1-5.8S-ITS2 regions of the genomic rRNA gene of the 33 *Sclerotinia sclerotiorum* isolates resulting from DNA distance-based and neighbour-joining analysis of the data

Sequences obtained were submitted to GenBank and accession numbers from GU229794 to GU229818 were respectively assigned to isolates AM-01, AM-09, AM-17, AM-10, AM-18, AM-03, AM-11, AM-19, AM-04, AM-12, AM-20, AM-05, AM-14, AM-07, AM-08, A1-17, A1-10, A3-20, A3-21, A3-22, A3-25, A2-04, A2-17, A2-19, and A2-20.

The calculation of the genetic distance among the isolates, based on these data and carried out using PHYLIP (Serb et al. 2001), permitted the formation of groups that are represented in a dendrogram (Fig. 1). The neighbour-joining analysis demonstrated that some *S. sclerotiorum* isolates from distinct geographical origins and various hosts showed little genetic variability. In general, the sequences corresponding to ITS1-5.8S-ITS2 regions of 25 isolates used in this analysis showed 99–98% identity to reported sequences (FJ810516.1, Z73800.1, EF091808.1, DQ329538.1, AB269903.1, EU660219.1, AF455456.1, and AF455526.1) obtained from public databases (GenBank). The isolates were grouped into two major clusters, and the first major cluster was separated into two subclusters: the first one consisted of AM-05, AM-14, AM-19, AM-20, AM-11, AM-10, AM-04, AM-09, AM-07, and GenBank sequences and the second one consisted of A1-17, A1-10, A3-20, A2-19, A3-21, A2-04, A1-10, A3-22, A3-25, and A2-20. The remaining isolates in the second major cluster consisted of AM-01, AM-17, AM-03, AM-08, AM-18, and AM-12.

None or very little intraspecific variation was found in the ITS1-5.8S-ITS2 regions of *S. sclerotiorum* isolated from bean crops in Brazil (AM-05, AM-14, AM-19, AM-20, and AM-11), ATCC MYA-4521 (FJ810516.1), *Lactuca* sp. in Canada (Z73800.1), vegetable crops in Alaska (EF091808.1 and DQ329538.1) (Winton et al. 2006), blueberry (*Vaccinium corymbosum* L.) in Japan (AB269903.1) (Umemoto et al. 2007), or *Argyranthemum frutescens* in Italy (EU660219.1) (Garibaldi et al. 2008). *Sclerotinia* (AF455456.1 and AF455526.1) isolated from the nasal mucus of patients suffering from chronic rhinosinusitis in Austria (Buzina et al. 2003) were placed within a cluster separated from other isolates.

No tendency was observed between geographical distribution of the isolates and degree of similarity in the region of rDNA analysed. This was evidenced in the subgroup that comprises isolates from A1, A2, and A3 populations; although sharing high similarity, they are from distant geographical regions and were placed within the same clusters (Fig. 1). Therefore, the variability observed within the sequence does not support the hypothesis of clonal populations within each region.

Discussion

Our results showed high level of genotypic diversity in *S. sclerotiorum* populations collected from irrigated dry bean plants. AMOVA indicated higher genetic variability within the populations than among them, which is consistent with the findings in Australian canola fields (Sexton and Howlett 2004). Results demonstrate gene flow among the four populations because – as populations in general become more isolated and gene flow is reduced – genetic drift tends to manifest by fixation of different alleles, resulting in loss of intrapopulational genetic variability and increased interpopulation divergence.

Previous studies on populations of *S. sclerotiorum* in canola and cabbage fields in Canada and North Carolina (USA) indicated the presence of strong clonal patterns, even in populations that were 2000 km (Kohn et al. 1991; Kohli et al. 1992; Kohn 1995; Kohli and Kohn 1996; Cubeta et al. 1997). Earlier studies of genetic analyses using different markers, such as RFLPs, nuclear and mitochondrial probes, demonstrated that each sample from a group presenting mycelial compatibility in isolates from North America was genetically uniform, with limited variability in regions 18S and 28S of rDNA (Kohn et al. 1991; Carbone et al. 1999). On the other hand, studies on *S. sclerotiorum* population structure have shown genotype diversity and provided evidence of both clonal and sexual reproduction (Carpenter et al. 1999; Sun et al. 2005; Malvárez et al. 2007; Arbaoui et al. 2008).

Comparing our results with isolates from Australia (Sexton and Howlett 2004), the USA (Atallah et al. 2004), and Turkey (Mert-Türk et al. 2007), using the same group of microsatellite primers, the high genetic variability found in *S. sclerotiorum* isolated from the irrigated dry bean plants in the Brazilian Cerrado region suggests the occurrence of both clonal and sexual reproduction. Although genotypic diversity has been observed to some extent in most of the studies carried out in different countries and continents, it is possible that the genotypes may not share identical alleles (Mert-Türk et al. 2007).

In addition to the significant differences in terms of climatic factors in countries such as Brazil, the USA, Canada, Australia, and Turkey, host species, agricultural practices, and conditions also affect the disease cycle and epidemiology of *S. sclerotiorum* in different continents. Factors supporting high standing genetic diversity in Brazilian isolates of *S. sclerotiorum* could include a mild climate lacking a hard winter, lack of crop rotation to non-hosts, highly diverse crops with many potential hosts, poor weed control, and a diversified life cycle with both myceliogenic and carpogenic germination, probably during the same crop cycle (Malvárez et al. 2007).

Private alleles are present in populations with restricted gene flow and may contribute to their characterization. The occurrence of a large number of exclusive alleles in the populations of *S. sclerotiorum* isolated from four irrigated dry bean crop areas analysed herein may indicate that the isolates present in these populations originated from the insertion of different genotypes in cultivated areas at different moments through contaminated seeds, agricultural implements, or even animals. This hypothesis can be corroborated by the sequence of the ITS region presented in our study, indicating that the alterations found are likely to result from the insertion of different genotypes and not only due to a high level of sexual reproduction.

In summary, this report provides new information on the genetic variability of *S. sclerotiorum* isolates from four irrigated dry bean crop areas in the Brazilian Cerrado region, detected through the use of microsatellite markers and ITS sequence. Local environmental conditions and crop rotation may be favouring sexual recombination in these populations, in addition to the use of contaminated seeds, which can enhance the number of isolates presenting different genotypes.

Our data suggest that the level of intrapopulation gene flow was high, thus providing the opportunity for spread of rare alleles, possibly including those that may confer character traits such as drug resistance and virulence.

Although sexual reproduction and recombination may contribute to the diversity of populations of *S. sclerotiorum*, population genetic data do not rule out seed as a source of primary inoculum. Further experimentation will be needed to definitely determine the relative importance of windborne ascospores and seedborne asexual inoculum in epidemics of *S. sclerotiorum* in the Brazilian Cerrado.

This study demonstrated the significance of these microsatellite loci to assess gene flow and reproductive system of this important pathogen. Also, it showed that white mould in irrigated dry beans in the Brazilian Cerrado is caused by genetically diverse populations of *S. sclerotiorum* and that this pathogen is highly variable within small geographical areas (centre-pivot irrigation). Regardless of geographical location, the major source of genetic variation came from differences among isolates within populations. Such information has implications for disease management. Moreover, further research is needed to investigate the potential impacts of outcrossing on epidemics and disease management. To the best of our knowledge, this has been the first report on the use of microsatellite markers for molecular discrimination of dry bean crop pathogen *S. sclerotiorum* in irrigated areas.

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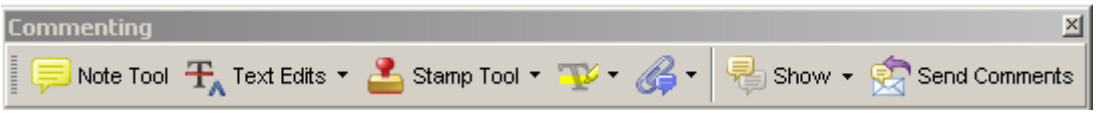
USING E-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required Software

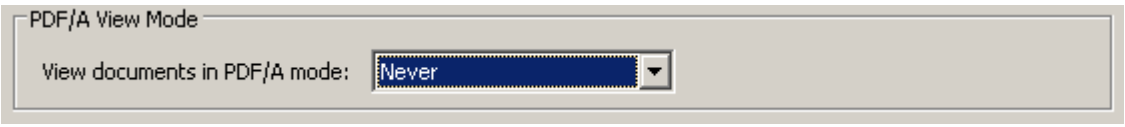
Adobe Acrobat Professional or Acrobat Reader (version 7.0 or above) is required to e-annotate PDFs. Acrobat 8 Reader is a free download: http://www.adobe.com/products/acrobat/readstep2.html

Once you have Acrobat Reader 8 on your PC and open the proof, you will see the Commenting Toolbar (if it does not appear automatically go to Tools>Commenting>Commenting Toolbar). The Commenting Toolbar looks like this:



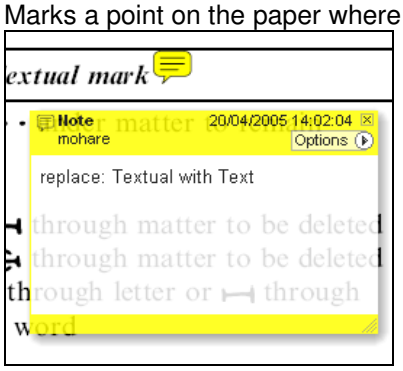
If you experience problems annotating files in Adobe Acrobat Reader 9 then you may need to change a preference setting in order to edit.

In the "Documents" category under "Edit – Preferences", please select the category 'Documents' and change the setting "PDF/A mode:" to "Never".



Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.

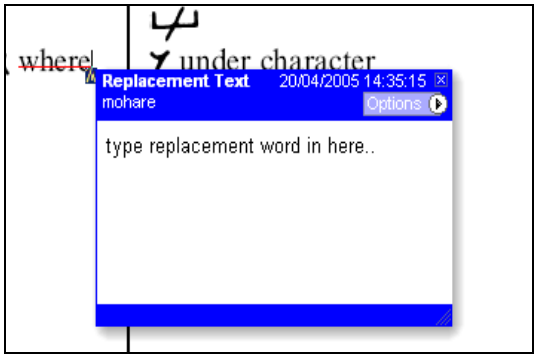


How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.

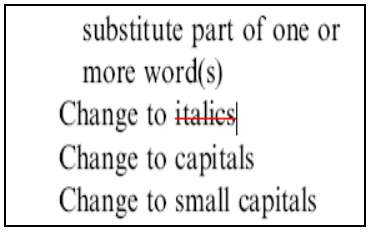


How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

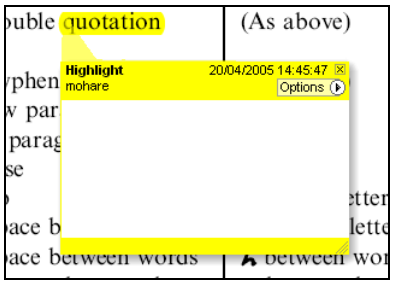
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

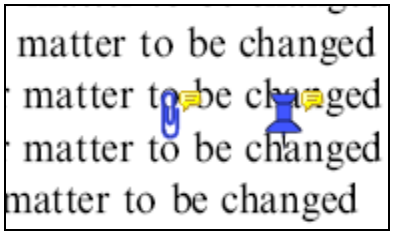


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

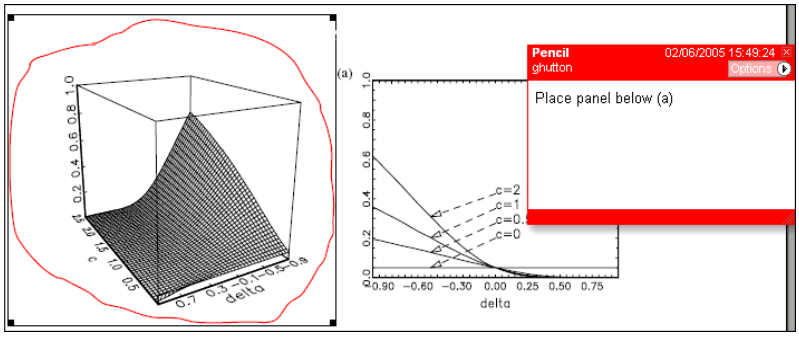


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

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