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Morphological and molecular analysis of genetic variability within isolates of *Corynespora cassiicola* from different hosts

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Abstract Twenty-two isolates of *Corynespora cassiicola* obtained from cucumber, papaya, eggplant, tomato, bean, Vigna, sesame and *Hevea* rubber (*Hevea brasiliensis*) were analysed by morphological features, the differences of the ribosomal DNA internal transcribed spacer (rDNA-ITS) region sequence and the inter simple sequence repeat (ISSR) technique. Variability of morphological features was observed among the isolates. Pathogenicity tests showed that isolates from different hosts attacked *Hevea* rubber. Sequences of two outgroup taxa, *C. proliferata* and *C. citricola*, were downloaded from GenBank. The phylogenetic trees were constructed by using the rDNA-ITS region sequences from 24 Corynespora spp. isolates. In this analysis, the 24 sequences grouped into two clusters (A and B). Cluster A consists of sequences from all isolates of *C. cassiicola*; whereas cluster B consists of the two outgroup taxa, *C. proliferata* and *C. citricola*. However, the ITS region is conservative, and is not fit for studying differences among isolates. A total of 114 DNA fragments was amplified with 16 ISSR primers, among which 102 were polymorphic (89.5%). A dendrogram was created by the unweighted pair-group method with arithmetic averaging (UPGMA) analysis, and 22 isolates grouped into three clusters (C, D and E). Cluster C is composed of all of the *Hevea* rubber isolates, whereas cluster D is composed of nine isolates: four from papaya, five from cucumber, eggplant, bean, vigna and sesame. Cluster E is composed of two isolates from cucumber and tomato. These analyses showed that the genetic diversity was very rich among the tested isolates. There are no correlations between the morphological characteristics or rDNA-ITS region sequences of the 22 isolates and their host or geographical origin, but there is a link between ISSR clusters and their host origins. ISSR markers appear to be useful for intra-species population study in *C. cassiicola*.

Keywords Morphology · Plant pathogen · Inter simple sequence repeat (ISSR) · *Corynespora cassiicola*

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Introduction

The plant-pathogenic fungus *Corynespora cassiicola* (Burk. & Curt.) Wei. usually causes target spot on leaves but also on stems, roots and flowers of more than 280 host plants from over 70 tropical and subtropical countries (Farr et al. 2007). The organism has been reported on a great number of economically important crops including tobacco (Fajola and Alasoadura 1973), cowpea (Olive et al. 1945), eggplant (Onesirosan et al. 1974), sesame (Stone and Jones 1960), tomato (Mohanty and Mohanty 1955), soybean (Seaman and Shoermaker 1964), cucumber (Blazquez 1967), cotton (Jones 1961) and *Hevea* rubber (Deighton 1936). The Corynespora Leaf Fall (CLF) disease caused by the fungus *C. cassiicola* was first identified on *Hevea* rubber (*Hevea brasiliensis*) in Sierra Leone in 1936 (Deighton 1936). Since then *C. cassiicola* is most notable for a devastating leaf disease of plantation rubber in other countries, including India, Malaysia, Nigeria, Indonesia, Brazil, Sri Lanka, Thailand, Bangladesh and Vietnam. In 2006, *C. cassiicola* was identified on *Hevea* rubber in most rubber nurseries and a few plantations in Hainan and Yunnan provinces, China (Pu et al. 2007).

At one time *C. cassiicola* was considered as a weak pathogen on *Hevea* rubber. Since the 1980s, however, the severity of CLF disease has been on the increasing trend in many rubber-growing countries. *C. cassiicola* is known to vary in its pathogenicity and the susceptibility of rubber clones to the pathogen differs in different geographic regions (Darmono et al. 1996; Saha et al. 2000; Silva et al. 2003; Atan and Hamid 2003; Romruensukharom et al. 2005; Nghia et al. 2008). There are many *Hevea* rubber clones in natural rubber-growing areas in China, many of which are highly susceptible. By the end of 2007, more than 200 ha of *Hevea* rubber were infected and their incidence rate reached 94%, due to lack of effective control measures.

The most economical way of controlling the CLF disease is the use of resistant cultivars. Newly bred rubber genotypes (clones) should be screened for resistance against the fungus prior to recommendation to growers (Othman et al. 1996). Therefore, thorough information on the inherent variability of the pathogen would prove useful in *Hevea* rubber breeding programmes.

To gain insight on the causal pathogen, more and more researchers have paid attention to CLF disease with its prevalence continuing to increase in China. However, research on the degree of genetic variation in the pathogen was practically nonexistent. In this study, the differences of morphological features, rDNA-ITS region sequences and ISSR molecular fingerprinting markers analyses were conducted to analyse 22 *C. cassiicola* isolates obtained from different hosts at diverse locations in China from 2006 to 2008.

Materials and methods

Isolates of *C. cassiicola*

A total of 22 *C. cassiicola* isolates, deposited in the Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, was used in this study (Table 1). Eleven isolates came from a previous study, which revealed *C. cassiicola* to be a causal agent of CLF disease in *Hevea* rubber (*Hevea brasiliensis*), among which all isolates were race 1 except for isolate CC-023 (Qi et al. 2009a). Except for isolates CC-002 and CC-003, the other 9 isolates were obtained from infected leaves of papaya, tomato, eggplant, bean, vigna and sesame showing typical leaf spot infection symptoms in some rubber-growing districts in Yunnan and Hainan provinces of China from 2006 to 2007. Prior to further analysis, pure cultures of *C. cassiicola* isolates were obtained from single-spore isolation and maintained on Potato Dextrose Agar (PDA) medium (potato, 200 g; dextrose, 20 g; agar, 18 g; and distilled water, 1,000 ml; pH 7.0).

Pathogenicity of *C. cassiicola* on multiple hosts

Most *C. cassiicola* isolates produce few spores under eutrophic conditions but more spores under oligotrophic conditions. PDA plates, inoculated with mycelium plugs, were incubated at 28°C for 7 days. 12 mycelium plugs (5-mm diameter), cut from the margin of the colony of each plate, were transferred and incubated on sterilized wet filter paper placed in the bottom of a petri dish. After 5 days, the spore suspension was prepared by washing each plate with 30 mL of sterile water. The spores from each plate

Table 1 Twenty-two isolates of *C. cassiicola* from China used in the study

Code	Isolate No.	Host	Location	Year	rDNA-ITS region GenBank accession number
1	CC-001	RRIM 600 (Rubber clone)	Danzhou, Hainan	2006	EF198115
2	CC-002	Cucumber	Xinxiang, Henan	2005	EF198116
3	CC-003	Cucumber	Xinxiang, Henan	2005	EF198117
4	CC-004	Rubber seedling	Hekou, Yunnan	2006	EU822310
5	CC-005	Papaya	Haikou, Hainan	2006	EU735060
6	CC-006	Papaya	Hekou, Yunnan	2006	EU735061
7	CC-007	Eggplant	Danzhou, Hainan	2006	EU735063
8	CC-008	Papaya	Wenchang, Hainan	2006	EU735062
9	CC-009	Papaya	Danzhou, Hainan	2006	EU735064
10	CC-016	Rubber seedling	Ledong, Hainan	2007	EU822309
11	CC-021	Rubber seedling	Danzhou, Hainan	2007	EU822311
12	CC-023	Rubber seedling	Changjiang, Hainan	2007	EU935735
13	CC-024	Rubber seedling	Qiongzhong, Hainan	2007	EU822313
14	CC-032	Rubber seedling	Baoting, Hainan	2007	FJ179260
15	CC-042	Tomato	Danzhou, Hainan	2007	EU822318
16	CC-043	Bean	Danzhou, Hainan	2007	EU822319
17	CC-044	Vigna	Lingao, Hainan	2007	EU735065
18	CC-045	Sesame	Lingao, Hainan	2007	EU735066
19	CC-087	Yunyan 77-2 (Rubber clone)	Mengman, Yunnan	2007	EU822314
20	CC-088	Rubber seedling	Jinghong, Yunnan	2007	EU822315
21	CC-090	Rubber seedling	Mengxing, Yunnan	2007	EU822316
22	CC-091	GT1 (Rubber clone)	Mengbang, Yunnan	2007	EU822317

were counted under bright field microscopy and adjusted to 2- 000 spores per ml.

Detached, young leaves of papaya, tomato, eggplant, and *Hevea* rubber clones with 10~14 days of uniform maturity (brownish to pale green) after bud burst were surface sterilized in 0.8% sodium hypochlorite and rinsed in sterile water three times. All of the detached leaves were slightly wounded crosswise at the lateral veins of the upper surface with a needle and treated with 10-μl drops of the spore suspension. The trial was repeated 10 times.

The inoculated leaves were placed under fluorescent light in an air-conditioned laboratory (28°C). The development of the fungus was assessed after 5 days of inoculation. An infection was considered to occur when the leaf tissue at the site of inoculation was necrotic. The severity of infection was scored as follows: 0, no visible lesion; 1, small dark discolouration below droplet; 2, prominent large lesion without mycelium; 3, prominent large lesion with mycelium (Ismail and Jeyanayagi 1999).

Colony morphology

All of the isolates shown in Table 1 were used for this experiment. A 5-mm diameter mycelial plug was cut from the margin of a 5-day-old culture of *C. cassiicola* on PDA and was centrally cultured in a 7-cm petri dish. Cultures were incubated at 28°C. The experiment was arranged in a completely randomized design (CRD) with 3 replications for each isolate. Colony characteristics such as colour, shape and size were recorded. The colony diameter was measured daily by taking the average length of two diameters at right angles for a period of 5 days. Data were subjected to analysis of variance (ANOVA) using SPSS software version 10.0 (SPSS Inc., Chicago, USA).

Conidial morphology

Spores were collected and fixed onto glass slides for observation. Fifty conidia were used to record shapes

and sizes (length and width), and then photographed by using a Leica DFC 420 Digital Camera system (Leica Microsystems Ltd., Heerbrugg, Switzerland) fixed on a Leica DMLB Microscope (Leica, Bensheim, Germany) and measured using Leica Application Suite version V 2.7.1 software (Leica Microsystems Ltd., Heerbrugg, Switzerland). Data were subjected to analysis of variance (General Linear Model Analysis, GLM) using SPSS software version 10.0 (SPSS Inc., Chicago, USA).

Isolation of DNA from *C. cassiicola*

The 22 isolates of *C. cassiicola* were grown on PDA plates for 5 days at 28°C. Mycelia were harvested by scraping the fungal colonies with a sterile glass slide. Genomic DNA was extracted as described by Qi et al. (2005) with minor modifications of NaCl concentration (up from 1.4 mol/l to 1.6 mol/l). DNA was dissolved in TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0) and stored frozen at −20°C for further use.

Molecular identification of *C. cassiicola* isolates

Identification of the 22 isolates of *C. cassiicola* from *Hevea* rubber and other hosts was achieved using the ribosomal DNA internal transcribed spacer (rDNA-ITS) region sequence. Polymerase chain reaction (PCR) was performed as described previously (Qi et al. 2009b) using the universal primers ITS1 and ITS4 (White et al. 1990). Purification of the ITS-PCR products was achieved using the Gel DNA purification kit (Upenergy Biotek Co. Ltd., Shenzhen, China). The purified PCR fragments were ligated into plasmid vector PMD18-T, using the PMD18-T Vector System Kit (TaKaRa Bio Inc., Shiga, Japan) and transformed into *Escherichia coli* JM109 competent cells. Screening of selected colonies was performed using the PCR protocol of Kilger and Schmid (1994), and amplified DNA was separated by electrophoresis. Five clones per isolate that corresponded with the expected insertion size were selected, purified using the Wizard Miniprep DNA purification system (Promega Corp., Madison, WI) and sequenced in both directions by the China South Gene Centre.

Analyses of DNA sequences

ITS sequences of 24 Corynespora spp. isolates were aligned using Clustal X version 1.8 (Thompson et al. 1997). Phylogenetic analyses were done by the

Table 2 Summary information for ISSR analysis of 22 isolates of *C. cassiicola* from China

Primer	Sequence (5′ – 3′)	Annealing temperature (°C)	Amplified fragments	Polymorphic fragments
UBC807	AGAGAGAGAGAGAGAGT	52	9	9
UBC809	AGAGAGAGAGAGAGAGG	52	8	7
UBC811	GAGAGAGAGAGAGAGAC	52	12	11
UBC816	CACACACACACACACAT	52	4	4
UBC826	ACACACACACACACACC	50	6	5
UBC827	ACACACACACACACACG	50	7	6
UBC834	AGAGAGAGAGAGAGAGYT	50	6	6
UBC835	AGAGAGAGAGAGAGAGYC	50	7	6
UBC841	GAGAGAGAGAGAGAGAYC	50	8	6
UBC842	GAGAGAGAGAGAGAGAYG	48	8	8
UBC855	ACACACACACACACACYT	48	4	3
UBC856	ACACACACACACACACYA	48	7	5
UBC857	ACACACACACACACACYG	48	7	7
UBC888	BDBCACACACACACACA	48	7	6
UBC889	DBDACACACACACACAC	48	7	7
UBC890	VHVGTGTGTGTGTGTGT	48	7	6
Total number of fragments			114	102

Nomenclature: Y=C/T, B=G/C/T, D=A/G/T, V=A/G/C, H=A/C/T

neighbor-joining (NJ) method using the MEGA version 4.1 (Kumar et al. 2001) software package. The DNA sequences of the rDNA-ITS region of *Corynespora proliferata* (Accession No. FJ852596) and *C. citricola* (Accession No. FJ852594) were downloaded from GenBank and used as outgroups. We used bootstrap analysis to assess the confidence level of each node of the NJ trees with 1,000 bootstrap replicates.

ISSR analyses

Of the 16 ISSR primers (UBC primer set No. 9, Biotechnology Laboratory, University of British Columbia, Vancouver, Canada, http://www.ubc.ca/) chosen for ISSR-PCR of all DNA samples (Table 2), 12 were successfully applied to *C. cassiicola* from *Hevea* rubber (Qi et al. 2009a). All reactions were carried out in a final volume of 25 μl containing 2.5 μl of 10×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 0.4 mM each of primer, 1.0 U of Taq polymerase and 1.0 μl of genomic DNA per reaction. The final volume was adjusted to 25 μl with sterile Milli-Q water. Amplification was performed in thin-walled PCR tubes. The ISSR-PCR amplification was performed in a PTC 200 DNA thermal cycler (TM Research) with modifications of the annealing temperature to optimize the reaction conditions for individual primers.

Amplicons were separated on 1.5% agarose gels with 1×TAE buffer at 5 V/cm. Band patterns were photographed under UV light (302 nm) after staining with ethidium bromide (EB). The sizes of amplified fragments were estimated by comparison to DNA marker DL 2000 (Takara, Biotechnology Co. Ltd., Danlian, China).

Analysis of ISSR data

Bands were analyzed manually as binary data with 1 indicating the presence and 0 indicating the absence of a band at a given location in a lane. ISSR bands within

Table 3 Pathogenic host range on papaya, tomato, eggplant and three *Hevea* rubber clones (PR107, RRIM 600 and Dafeng 95) of 22 isolates of *C. cassiicola* included in this study

Isolate No.	Host	Pathogenic host range[a]					
		Papaya	Tomato	Eggplant	PR107	RRIM 600	Dafeng 95
CC-001	Rubber	v	v	v	1	3	1
CC-002	Cucumber	av	v	v	2	1	1
CC-003	Cucumber	av	v	v	2	1	2
CC-004	Rubber	v	v	v	3	3	2
CC-005	Papaya	v	v	av	1	1	1
CC-006	Papaya	v	v	av	1	1	1
CC-007	Eggplant	av	v	v	2	1	1
CC-008	Papaya	v	v	av	1	1	1
CC-009	Papaya	v	v	av	1	1	1
CC-016	Rubber	v	v	v	1	3	1
CC-021	Rubber	v	v	v	3	3	2
CC-023	Rubber	v	v	v	3	1	1
CC-024	Rubber	v	v	v	2	3	1
CC-032	Rubber	v	v	v	1	3	3
CC-042	Tomato	v	v	v	2	2	3
CC-043	Bean	av	v	v	1	1	1
CC-044	Vigna	av	v	v	1	1	1
CC-045	Sesame	av	v	av	1	1	1
CC-087	Rubber	v	v	v	2	3	1
CC-088	Rubber	v	v	v	3	3	2
CC-090	Rubber	v	v	v	3	3	1
CC-091	Rubber	v	v	v	3	3	1

[a] Pathogenic host range: av, avirulent; v, virulent. 1, small dark discolouration below droplet; 2, prominent large lesion without mycelium; 3, prominent large lesion with mycelium (Ismail and Jeyanayagi 1999)

isolates were scored as missing if they were poorly resolved on the gel or if the template DNA did not amplify well. The experiments were repeated twice for each isolate to confirm the repeatability. A dendrogram was constructed using the TREE procedure by the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) computer program version 2.1 (Rohlf 1998) based on Jaccard's similarity coefficient (Jaccard 1908) using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Bootstrap analysis was also used to assess the confidence level of each node of the dendrogram with 1,000 bootstrap replicates.

Results

Pathogenicity of *C. cassiicola* on multiple hosts

A large number of host-specific strains has been reported for *C. cassiicola* based on pathogenicity testing (Onesirosan et al. 1974; Chee 1988; Silva et al. 2003). In this study, 22 isolates of *C. cassiicola* were tested for virulence on papaya, tomato, eggplant and three *Hevea* rubber clones (Table 3).

All of the *C. cassiicola* isolates produced water soaked-lesions 48 h after inoculation on tomato (Table 3). These lesions later became necrotic and the heavily infected leaves rotted after 5 days. Four isolates originally from papaya (CC-005, CC-006, CC-008 and CC-009) were avirulent on eggplant, consistent with the findings of Silva et al. (2003), whereas all papaya isolates attacked tomato. In addition, six of the isolates (CC-002, CC-003, CC-007, CC-043, CC-044 and CC-045) were avirulent on papaya.

Lesions of different sizes were produced 72 h after inoculation and *C. cassicola* isolates from different host origins were virulent on all three *Hevea* rubber clones. A visual rating was recorded according to the size and appearance of the lesions (Table 3).

Table 4 Visual characteristics of 22 *C. cassiicola* cultures after 5 days of incubation

Isolate No.	Color		Texture	Shape	Growth rate (mm/d) [1]	Size (mm) [1]
	Top	Bottom			Mean ± SE	Mean ± SE
CC-001	Green	Black	Thick	Polygon	9.0^{efg}±0.34	44.8^{e}±1.68
CC-002	Grey	Red brown	Thick	Polygon	10.0^{cde}±0.15	50.2^{bcd}±0.75
CC-003	White	Black	Thick	Polygon	10.0^{cde}±0.13	50.3^{bcd}±0.67
CC-004	Green	Dark grey	Thick	Round	9.3^{ef}±0.21	46.7^{e}±1.05
CC-005	Grey	Dark grey	Thick	Polygon	7.5^{h}±0.1	37.7^{g}±0.49
CC-006	Grey	Black	Thick	Round	7.3^{h}±0.12	36.7^{h}±0.61
CC-007	White	White	Thick	Round	9.2^{ef}±0.19	46.2^{e}±0.95
CC-008	Grey	Black	Thick	Round	7.4^{h}±0.12	36.8^{h}±0.6
CC-009	Grey	Black	Thick	Round	7.4^{h}±0.12	37.2^{g}±0.6
CC-016	Green	Dark brown	Thin	Polygon	8.5^{g}±0.04	42.3ef±0.21
CC-021	Green	Black	Thick	Round	8.0^{gh}±0.06	39.8^{efg}±0.31
CC-023	Pale green	Black	Thick	Round	10.0^{sde}±0.19	49.8^{de}±0.95
CC-024	Dark green	Black	Thick	Polygon	11.4^{b}±0.30	57.2^{b}±1.49
CC-032	White	Pale brown	Thin	Round	12.5^{a}±0.05	62.4^{a}±0.26
CC-042	Green	Black	Thick	Polygon	10.3^{cd}±0.15	51.5^{bcd}±0.76
CC-043	Grey	Pale brown	Thick	Polygon	10.5^{cd}±0.1	52.7^{bc}±0.49
CC-044	Green	Black	Thick	Polygon	10.2^{cd}±0.12	51.0^{bcd}±0.58
CC-045	Green	Black	Thick	Polygon	9.8^{cde}±0.10	49.2^{de}±0.48
CC-087	Green	Black	Thick	Polygon	8.2^{gh}±0.30	40.8^{efg}±1.49
CC-088	Green	Black	Thick	Polygon	8.5^{g}±0.12	42.8^{ef}±0.6
CC-090	Green	Black	Thick	Polygon	9.7^{cde}±0.09	48.5^{de}±0.43
CC-091	Green	Black	Thick	Round	10.6^{c}±0.31	53.2^{bc}±1.54

[1] The data are the average of three replicates. Means in the same column with the same letter are not significantly different at $P<0.05$ based on the Duncan multiple range test

Colony morphology

The mycelia of all the isolates grew uniformly in all directions on PDA, and produced colonies with concentric growth rings and abundant aerial mycelia. The colonies were white, grey or green coloured in the middle and appeared brighter at the edge either with thin or thickly haired, effused mycelia, which were mostly branched, septate, subhyaline to pale brown and smooth walled. The colony morphology of the fungus showed variation among the isolates. The differences were found either in mycelium colour (white to grey or green), texture (thin to thick, observed from the top), or in colony colour (white to pale brown, red brown, dark brown, dark grey or black, observed from the bottom of the Petri dishes) as well as in the shape of the cultures (round to slightly polygonal) (Table 4, Fig. 1).

The mean growth rates calculated over 5 days and the colony sizes after 5 days of incubation differed significantly among isolates (Table 4). The mean separation analysis using the Duncan multiple range test divided the isolates into 8 groups for these parameters. Isolate CC-032, obtained from Baoting, Hainan on a *Hevea* rubber seedling in 2007, showed the fastest growth rate (12.5 mm/day) and had the largest colony size (62.4 mm in diameter). Isolate CC-006 showed the slowest growth rate (7.3 mm/day) and smallest colony size (36.7 mm). These two isolates and isolate CC-008 (36.7 mm in diameter) were distinct from the others in the Duncan analysis.

Conidial morphology

A high degree of variability in conidial morphology was observed among isolates (Fig. 2). Differences were observed in shape (oval, obclavate, cylindrical or Y; curved or straight; Fig. 3), size (10.1–277.2 μm long; 1.3–17.1 μm wide) and the number of pseudosepta (0–18) (Table 5).

The variability in conidial shape within isolates was observed and the ratios of these shapes differed significantly among isolates. The oval-shaped conidia were dominant in isolate CC-032 but were minor in others. The obclavate- and cylindrical-shaped conidia appeared in all isolates. The Y-shaped conidia only appeared in all four isolates from papaya and two isolates from *Hevea* rubber (CC-004 and CC-016). A similar observation was noted for contour (curved or straight) of conidia (Fig. 3). The longest spore and the highest number of pseudosepta were observed in

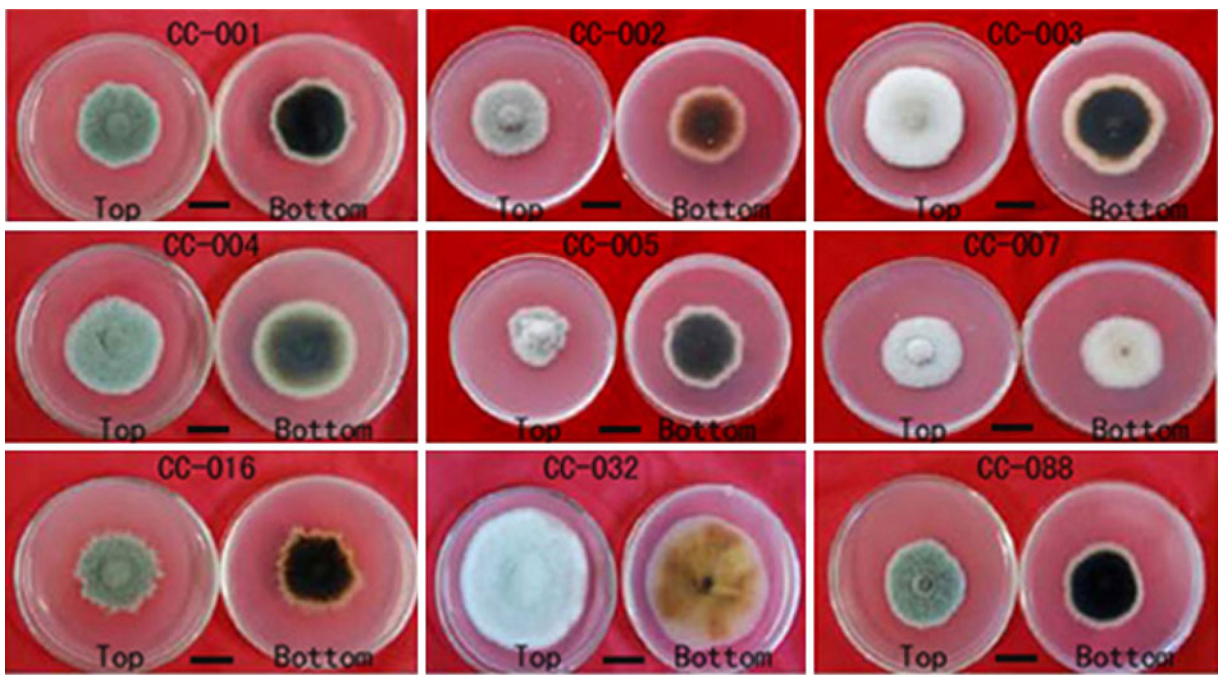

Fig. 1 Variability in colony morphology among 22 isolates of *C. cassiicola* after 5 days of incubation on PDA observed from the top and bottom of the Petri dishes. The name of the isolate is indicated on the top of each picture. Scale bars: 25 μm

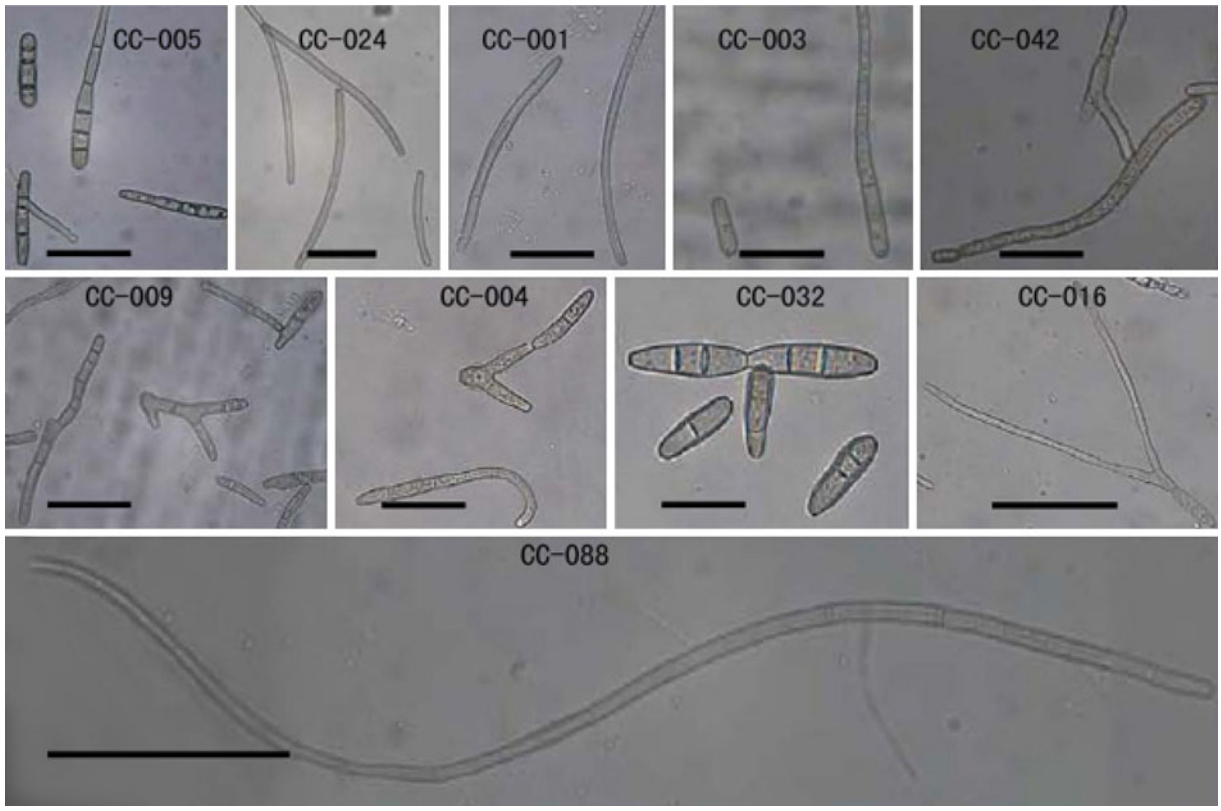

Fig. 2 Variation in conidia shapes and sizes among and within 22 isolates of *C. cassiicola* after 5 days of incubation on PDA observed from the top and bottom of the Petri dishes. The name of the isolate is indicated on the top of each picture. Scale bars: 50 μm

isolate CC-088 obtained from Jinghong, Yunnan on a *Hevea* rubber seedling in 2007 (277.2 μm and 18 respectively).The widest spore was found in isolate CC-043 obtained from Danzhou, Hainan on bean in 2007 (17.1 μm). Isolate CC-088 contained the longest average conidial length (125.2 μm) and the highest average number of pseudosepta (18), whereas the widest average conidial width (9.5 μm) was observed

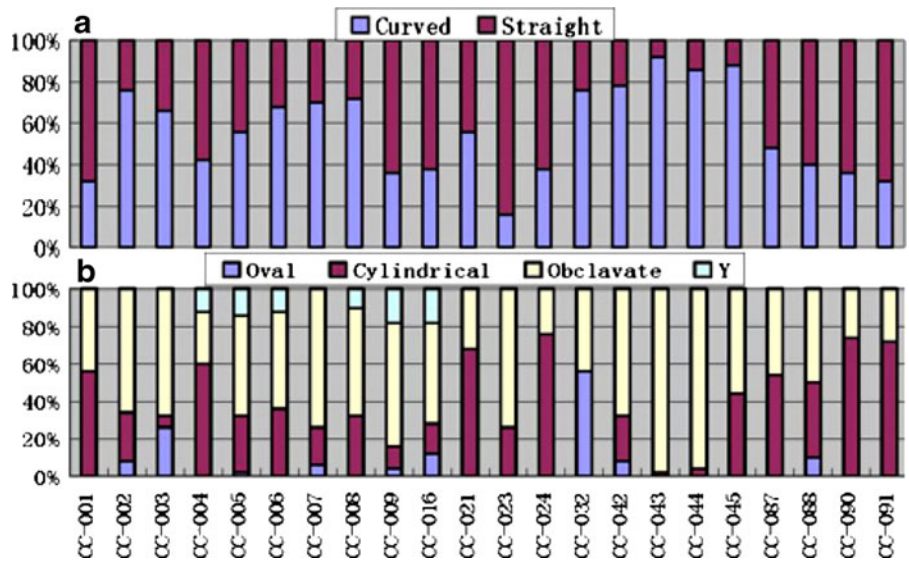

Fig. 3 Distribution in percentage (%) of conidium contour (**a**) and shape (**b**) of 22 isolates of *C. cassiicola* from which 50 conidia/isolate were observed

Table 5 Conidium size and number of pseudosepta of 22 isolates of *C. cassiicola* from which 50 conidia/isolate were observed

Isolate no.	Length (μm)			Width (μm)			No. of pseudosepta		
	Min	Max	Mean±SE	Min	Max	Mean±SE	Min	Max	Mean±SE
CC-001	37.3	185.8	109.6^{c}±4.88	5.6	12.4	7.9 ^{cd}±0.22	0	5	2.3^{de}±0.15
CC-002	16.2	129.1	54.9^{efg}±3.22	3.8	11.2	7.3^{de}±0.24	0	9	3.3^{bc}±0.37
CC-003	25.4	126.4	45.7^{gh}±4.44	6.3	8.8	7.9 ^{cd}±0.12	0	4	1.9^{efg}±0.15
CC-004	13.2	145.5	58.6^{ef}±4.42	2.2	8.3	5.1^{hi}±0.17	0	6	2.3^{de}±0.16
CC-005	13.9	168.9	48.5^{gh}±4.25	4.7	14.9	7.8 ^{cd}±0.29	0	10	3.2^{bc}±0.35
CC-006	21.6	246.7	65.4^{def}±4.38	6.1	12.4	9.1^{b}±0.21	0	6	3.4^{bc}±0.28
CC-007	14.9	175.5	50.1^{efg}±4.99	4.9	15.8	8.2^{c}±0.32	0	10	3.2^{bc}±0.35
CC-008	27.2	271.6	68.8^{de}±5.07	6.7	13.1	9.5^{a}±0.24	0	8	3.8^{b}±0.31
CC-009	15.3	248.6	64.2^{def}±5.70	1.3	14.8	8.0 ^{cd}±0.36	0	18	3.4^{bc}±0.51
CC-016	11.2	106.6	33.7^{j}±2.59	2.6	9.3	5.3 ^{h}±0.20	0	9	2.4^{de}±0.28
CC-021	22.1	145.1	63.0^{def}±4.93	2.7	12.4	6.4 ^{g}±0.20	0	11	3.4^{bc}±0.31
CC-023	18.9	109.6	45.3^{ghi}±5.35	5.4	8.9	6.5 ^{g}±0.21	0	8	1.1 h±0.23
CC-024	13.0	152.6	67.9^{de}±5.68	2.2	6.5	4.2^{u}±0.13	0	9	3.1^{bcd}±0.29
CC-032	11.2	85.9	28.3^{k}±2.32	2.9	8.5	5.8^{gh}±0.18	0	9	1.6^{gh}±0.23
CC-042	19.3	112.9	46.2^{gh}±5.76	5.6	8.5	7.0^{ef}±0.29	0	10	1.2 ^{h}±0.31
CC-043	10.1	212.6	56.8^{efg}±2.65	4.9	17.1	7.8 ^{cd}±0.12	0	10	2.4^{de}±0.25
CC-044	22.9	164.0	64.9^{def}±6.08	4.3	9.2	7.1^{ef}±0.19	0	5	2.1^{def}±0.27
CC-045	16.7	36.2	59.2^{ef}±6.11	4.0	7.0	6.8^{efg}±0.2	0	9	2.8 ^{cd}±0.34
CC-087	14.9	152.5	62.4^{def}±3.42	2.5	8.6	5.6^{h}±0.13	0	7	2.1^{def}±0.14
CC-088	34.2	277.2	125.2^{a}±8.46	2.3	10.1	6.8^{efg}±0.21	1	15	5.9^{a}±0.48
CC-090	25.9	153.9	65.8^{def}±2.95	8.6	9.7	9.2^{b}±0.06	0	2	1.1 ^{h}±0.13
CC-091	39.6	246.8	115.9^{b}±6.63	4.9	10.4	6.4^{g}±0.14	0	4	1.2 ^{h}±0.16

The data are the average of three replicates. Means in the same column with the same letter are not significantly different at $P<0.05$ based on the means separation analyses

in isolate CC-008. Based on the means separation analysis, the isolates were separated into 11 groups for spore length, 8 groups for number of pseudosepta, and 9 groups for spore width, with isolate CC-008 as the stand-alone (Table 5).

Molecular identification of *C. cassiicola* isolates

To eliminate the errors of cloned sequences, a consensus was made by aligning ten rDNA-ITS region sequences of five clones from each isolate. By sequence analysis, the *C. cassiicola* rDNA ITS1 (180 bp), 5.8S (158 bp), and ITS2 (221 bp), total amplified-fragments length was 559 bp. The resulting rDNA-ITS region sequences were compared using the basic local alignment search tool (BLAST) with sequences deposited at the National Center for Biotechnology Information (NCBI). The similarity of the sequences from 22 *C. cassiicola* isolates to those deposited at the NCBI (data not shown) was 100%, and to each other was 99–100%. Only two unique sequences were identified among the 22 isolates. The 22 sequences from this study were also deposited at the NCBI with accession numbers EF198115-EF198117, EU735060-EU735066, EU822309-EU822311, EU822313-EU822319, EU935735 and FJ179260 (Table 1).

Analyses of DNA sequences

Sequences from two outgroup taxa, *C. proliferata* and *C. citricola*, were downloaded from GenBank. The molecular phylogenetic trees were constructed by the NJ method, based on the rDNA-ITS region sequences from 24 Corynespora spp. isolates (Fig. 4). The 24 sequences of the rDNA-ITS region grouped into two clusters (A and B). Cluster A consists of sequences from all *C. cassiicola* isolates, whereas cluster B consists of the two outgroup taxa, *C. proliferata* and *C. citricola*. Each branch in the NJ tree was strongly supported by high bootstrap values.

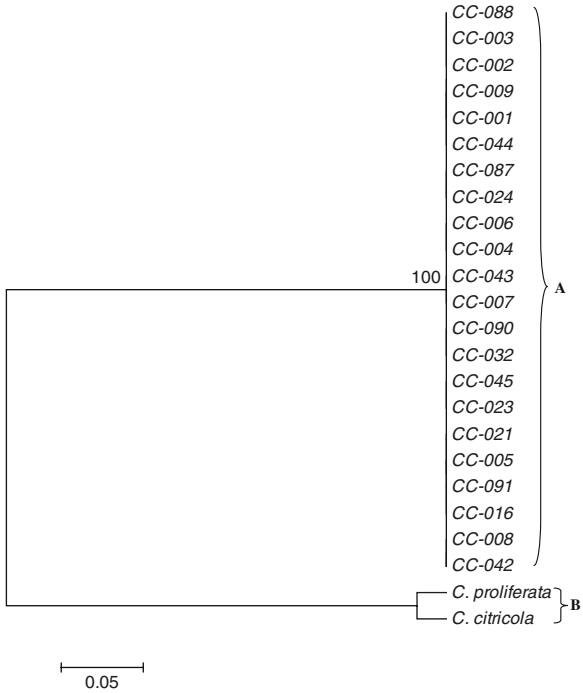

Fig. 4 Phylogenetic NJ tree based on 24 sequences of the rDNA-ITS region from Corynespora spp. isolates. The numbers above the nodes are supporting percentages by 1 000 bootstrap replicates. *C. proliferata* and *C. citricola* were regarded as outgroups

ISSR analyses

ISSR fingerprinting using the 16 informative primer combinations on 22 isolates of *C. cassiicola* from China yielded 114 reproducible amplification fragments, among which 102 (89.5%) were polymorphic. The highest level of polymorphism (100%) was obtained with primers UBC807, UBC816, UBC834, UBC842, UBC857 and UBC889, whereas the lowest was 71.4% and was obtained with primer UBC856 (Table 2).

The number of amplified ISSR bands generated by individual primers varied from a minimum of 4 (UBC816 and UBC855) to a maximum of 12 (UBC811) with a range in size from 250 to 3 000 bp (Fig. 5).

Analysis of ISSR data

The dendrogram constructed using the UPGMA method differentiated the 22 isolates of *C. cassiicola* into three major clusters C, D and E. Cluster C consists of all isolates from *Hevea* rubber. Cluster D consists of nine isolates, among which are four from papaya, and are each from cucumber, eggplant, bean, vigna and sesame. Cluster E consists of two isolates from cucumber and tomato. The isolates in cluster C showed 59.2% similarity. The isolates in cluster D showed 63.5% similarity, while isolates in cluster E showed 66.4% (Fig. 6). Each branch in the dendrogram was strongly supported by high bootstrap values.

Discussion

We confirmed that all the isolates in this study were *C. cassiicola* by conducting pathogenicity tests, morphological research and sequencing the rDNA ITS1-5.8S-ITS2 region.

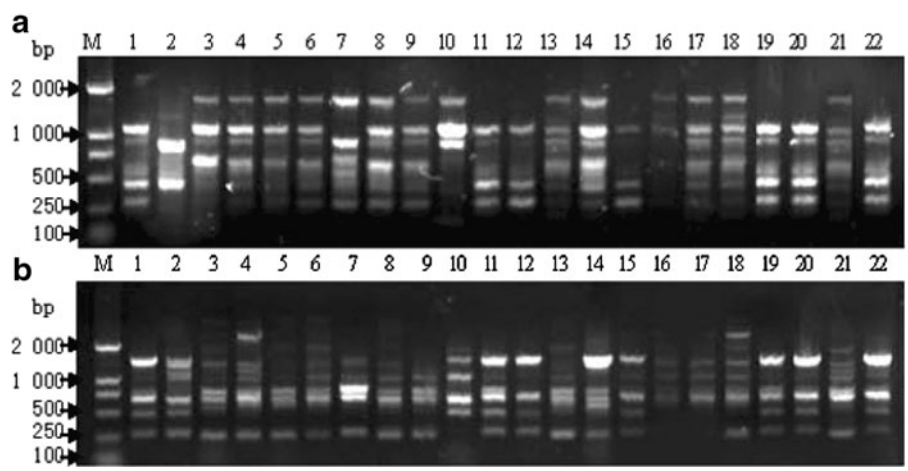

Fig. 5 The ISSR fingerprinting of 22 isolates of *C. cassiicola* using primers UBC807 (**a**) and UBC811 (**b**) M: DL 2000 DNA marker; 1: CC-001; 2: CC-CC-023; 13: CC-024; 14: CC-032; 15: CC-042; 16: CC-043; 17: CC-044; 18: CC-045; 19: CC-087; 20: CC-088; 21002; 3: CC-003; 4: CC-004; 5: CC-005; 6: CC-006; 7: CC-007; 8: CC-008; 9: CC-009; 10: CC-016; 11: CC-021; 12: CC-090; 22: CC-091

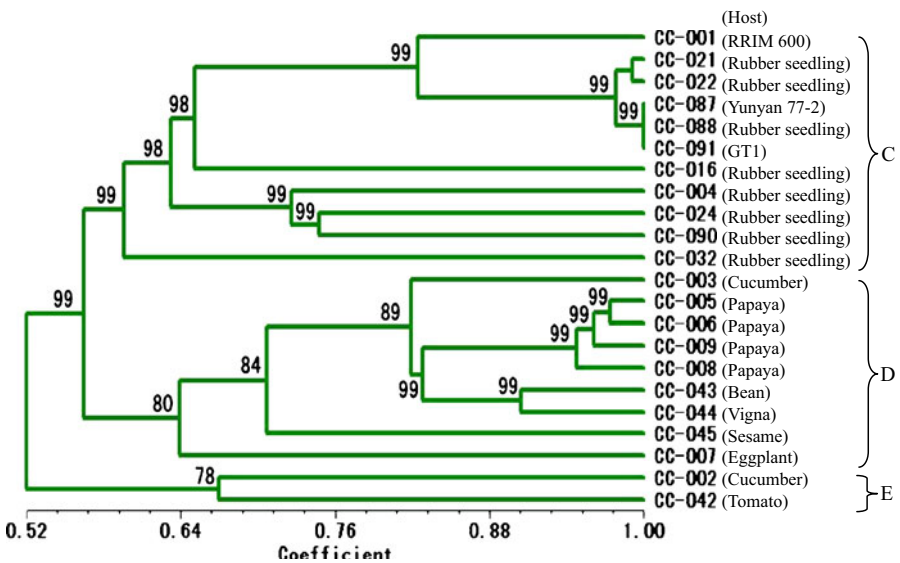

Fig. 6 Dendrogram based on ISSR bands. The numbers above the nodes are supporting percentages of 1 000 bootstrap replicates

Some researchers regarded *C. cassiicola* as a non-host specific pathogen (Ellis and Holliday 1971), while others remarked that *C. cassiicola* is host specific (Miller and Alfieri 1973; Onesirosan et al. 1974; Anonym 1984; Toshiko et al. 2008; Dixon et al. 2009). Onesirosan et al. (1974) analysed one Nigerian isolate from papaya that attacked only papaya. Dixon et al. (2009) also showed that *C. cassiicola* from papaya was specific to papaya. In this study, however, papaya isolates are pathogenic to other hosts. Whether this is real or is due to false positives from inoculations on detached leaves and in moisture chambers is not known.

Earlier works have described the variability in colour, texture of the fungal colonies, size and shape of the conidia not only among the isolates obtained from different hosts and geographical regions but also within a single isolate (Darmono et al. 1996; Chee 1988; Spencer and Walters 1969; Onesirosan et al. 1974; Duarte et al. 1981; Nghia et al. 2008). In this study, the differences in morphology of the studied *C. cassiicola* isolates were observed for mycelium colour, texture, or colony colour as well as the shape of the cultures, and in conidia contour, shape, size and the number of pseudosepta. Sato and Kitazawa (1980) previously found rarely Y-shaped conidia from soybean isolates. In this study, we also found a mass of Y-shaped conidia from two *Hevea* rubber isolates and all four papaya isolates from China. However, the 22 isolates of *C. cassiicola* from various host origins did not have any distinguishable morphological characteristics that could separate them into specific groups. Therefore, there was no correlation between morphological characteristics of isolates and their host origins. This finding warranted further work to differentiate the isolates of *C. cassiicola* from different host origins.

According to the molecular phylogenetic trees of the rDNA-ITS region constructed by the NJ method, all *Hevea* rubber isolates of *C. cassiicola* grouped together (cluster A) and each branch in the NJ tree has been strongly supported by high bootstrap values. However, ITS sequences were too conserved to be useful for studying differences among *C. cassiicola* isolates.

Molecular genetic techniques for the DNA fingerprinting of living organisms are proving to be important tools for increasing our understanding of the genetic diversity and epidemiology of fungal plant pathogens (Maclean et al. 1993). There is sufficient evidence to suggest that the virulence of this fungal pathogen may be associated with identifiable genetic variability at the molecular level (Nghia et al. 2008; Qi et al. 2009a). Consequently, molecular characterization of *C. cassiicola* isolates from different host plant species was undertaken to detect any genetic variability which may be present among isolates collected from different host origins. Though some other RAPD analyses have found no correlation (Darmono et al. 1996; Romruensukharom et al. 2005), several related records have reported a correlation between RAPD groups and the features of the

isolates, e.g., pathogenicity, geographical origin, or host plant genotype (Atan and Hamid 2003; Silva et al. 2003).

We chose to investigate the utility of ISSR markers to survey the genomes of 22 isolates of *C. cassiicola* from China. ISSR detects differences between SSRs (Simple Sequence Repeats) (Ziekiewicz et al. 1994). Compared with other molecular markers, ISSR can reveal high polymorphism, which helps to distinguish individuals at inter-and/or intra-species levels. Moreover, ISSR has specific advantages over other markers which include the non-requirement of sequence information, simple operation, high stability and low cost. Therefore, ISSR has been proposed as a more economical and reliable DNA marker system (Bornet and Branchard 2001).

Based on the UPGMA tree, ISSR analysis was successful in differentiating the *Hevea* rubber isolates and other host isolates in this study. All 11 *Hevea* rubber isolates clustered together (C). Four papaya isolates clustered together with one cucumber isolate (CC-003), the eggplant isolate (C-007), the bean isolate (CC-043), the vigna isolate (CC-044) and the sesame isolate (CC-045) (D), while one cucumber isolate (CC-002) and the tomato isolate (CC-042) clustered together (E), but they are genetically distinct from *Hevea* rubber isolates. This suggests that ISSR clusters have an obvious correlation to their host origins.

According to the data made available here, it is the first report on morphological and ISSR analysis of genetic variability among *C. cassiicola* isolates from different host origins in China. The high degree of morphological and molecular variability found among isolates was unexpected. We can confirm that there was a link between ISSR clusters and their host origins, and ISSR markers can be used for intra-species population studies.

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