Theor Appl Genet (2000) 100:614–620

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Host effect on genetic variation of *Marssonina brunnea* pathogenic to poplars

Received: 20 May 1999 / Accepted: 30 July 1999

Abstract A broad collection was made for 42 isolates of *Marssonina brunnea* affecting poplar trees from three different sections (*Leuce*, *Aigeiros* and *Tacamahaca*) within the same *Populus* genus in China. Genetic diversity among these isolates was analyzed for morphological traits, cultural features, pathogenicity, hyphal anastomosis and randomly amplified polymorphic DNA markers (RAPDs). No significant difference was found in conidial morphological features, such as size, shape and septum location. Yet, considerable differences occur in other characteristics, which leads to the classification of the 42 isolates into two distinct groups, *M. brunnea* f.sp. *monogermtubi* and *M. brunnea* f.sp. *multigermtubi*. Isolates of *M. brunnea* f.sp. *monogermtubi*, derived from section *Leuce*, germinate only one germ tube, grow fast, produce dark-reddish conidiosorus clusters on the PDA medium, and are highly pathogenic to *Populus tomentosa* of section *Leuce*. By contrast, isolates of *M. brunnea* f.sp. *multigermtubi*, derived from sections *Aigeiros* and *Tacamahaca*, germinate 1–5 germ tubes, grow slowly, produce yellow-greenish conidiosorus clusters on PDA medium, and are pathogenic to *Populus* ×*euramericana* cv I-45 and *Populus canadensis* of section *Aigeiros*. DNA amplification using 11 RAPD primers generate 78 polymorphic bands among isolates. Cluster analyses based on RAPD markers broadly support such a classification by phenotypes, but provide a new insight into the possible origins of *M. brunnea*. It is proposed that the pathogen co-evolves with the poplars of section *Leuce* and has been subsequently distributed to the poplars of sections *Aigeiros* and *Tacamahaca*. An isolate from *Populus adenopoda* of section *Leuce* is placed in the third group, which is most likely a transmission type from *M. brunnea* f.sp. *monogermtubi* to *M. brunnea* f.sp. *multigermtubi*.

Key words *Aigeiros* · *Leuce* · *Marssonina brunnea* · Poplar · RAPD · *Tacamahaca*

Communicated by P.M.A. Tigerstedt

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Introduction

A successful breeding program for disease resistance relies critically on an understanding of gene-gene interactions between hosts and pathogens. Quantitative and molecular genetic approaches have been used to study the genetic variation of resistance traits for host populations (Young 1996). With the aid of molecular markers, such as restricted fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP), many studies have been initiated to identify key genetic loci conferring disease resistance throughout the entire genome in forest trees (Devey et al. 1995; Cervera et al. 1996; Newcombe 1998; Newcombe and Bradshaw 1996; Newcombe et al. 1996; Wilcox et al. 1996; Lefevre et al. 1998). For example, a single dominant gene, which co-segregated with RAPD markers, is known to control resistance to white pine blister rust (*Cronartium ribicola* Fisch.) in sugar pine (*Pinus lambertiana* Dougl.) (Devey et al. 1995) and to *Cronartium quercuum* Berk., the casual agent of fusiform rust disease in loblolly pine (*Pinus taeda* L.) (Wilcox et al. 1996), respectively. However, these studies are based only on a limited number of types of pathogen. Without a detailed understanding of the genetic diversity of pathogens that infect forest trees, it is impossible to breed for resistant families or genotypes to a particular disease in a broad range of environments, across which different types of a pathogen are distributed (Bentley et al. 1998; Kinloch and Dupper 1999).

Populus, containing six different sections (*Abaso* Ecken., *Turanga* Bunge, *Leucoides* Spach, *Aigeiros* Duby, *Tacamahaca* Spach and *Leuce* Duby), is of increasing importance as a commercial source of fiber and fuel in many regions of the world (Stettler et al. 1996). One of the most significant threats to poplar wood production comes from Marssonina leaf spot of poplars, caused by the coelomycetous genus *Marssonina* Magnus (O'Riordain and Kavanagh 1965; Li 1984; Spiers 1990, 1998; Newcombe and Callan 1998). The fungus causes very small, dark brown spots on leaves and petioles and sometimes on young green twigs and capsules. Poplar trees attacked by *Marssonina* display premature leaf fall and reduced photosynthetic capacity and growth. *Marssonina* has been reported to have over ten species worldwide, but only four morphologically distinct species, *Marssonina castagnei* (Desm. & Mont.) Magn., *Marssonina populi* (Lib.) Magn., *Marssonina brunnea* (Ell. & Ev.) Magn. and *Marssonina balsamiferae* Hiratsuka, are recognized (Spiers 1984, 1988). The former three species have been observed in China (He and Wang 1991), of which the fungus *M. brunnea* has been considered the most important pathogen that occurs in most regions of China and leads to serious economic losses in poplar plantations.

Strains of *M. brunnea* observed in eastern China have been classified into two specialized forms based on its host range in the field (Li 1984; He and Wang 1991). One form, named *M. brunnea* f.sp. *monogermtubi*, attacks poplars of the section *Leuce*, such as *Populus adenopoda* and *Populus tomentosa*, and their hybrids, in both the field and by artificial inoculation of detached leaves in vitro. The other form, named *M. brunnea* f.sp. *multigermtubi*, is hosted on many species of section *Aigeiros* and their hybrids, as well as hybrids of sections *Aigeiros* and *Tacamahaca*. These two forms can also be separated by conidial morphology. In *M. brunnea* f.sp. *monogermtubi*, conidia germinate one germ tube, whereas in *M. brunnea* f.sp. *multigermtubi* conidia germinate 1–5 (mostly 2–3) germ tubes. The specialized forms of *M. brunnea* are not defined genetically, but are groups of isolates attacking different hosts in the field. This variation in conidial feature can be induced by environmental, host and cultural factors, which often obscures taxonomic differences between isolates, even between species (Boyer 1961).

M. brunnea is considered to be a highly complex pathogen with a broad host range (Spiers 1990). Its genetic variation in morphology and pathogenicity has been studied extensively (Thompson 1937; Spiers and Hopcroft 1983, 1986; O'Donnell et al. 1998; Spiers 1988, 1998). Unaffected by environmental factors, various genetic marker systems that have been extensively developed in recent years provide a unique means of determining genetic diversity among different isolates within a fungus species (Bentley et al. 1995). Since *M. brunnea* is a haploid asexual pathogen, arbitrary primer techniques such as RAPD can be used efficiently. The aims of the present study were to: (1) assess the genetic variation of morphological features, pathogenicity and hyphal anastomosis among isolates of *M. brunnea* collected from different poplar species in different regions of China; (2) determine the association of the genetic structure of *M. brunnea* with *Populus* sections at the molecular level; and (3) delineate the genetic relatedness of different origins within *M. brunnea*. The racial structure and possible origins of the pathogen are also discussed.

Materials and methods

Fungal isolates

The fungal materials studied include 42 isolates of *M. brunnea* collected from different species or hybrids of poplars that belong to three poplar sections (*Leuce*, *Aigeiros* and *Tacamahaca*) distributed in nine different geographic origins within China (Table 1). The leaf pieces infected by *M. brunnea* were sterilized by dipping them in 70% alcohol for 20 s followed by a 3.5-min immersion in 0.1% mercuric chloride. The leaf pieces were rinsed three times in sterile distilled water and the isolates were then established by streaking conidia onto potato-dextrose agar (PDA). The isolates were maintained as single-spore cultures.

Phenotypic and molecular characterization

Conidial suspensions were prepared with the isolates derived both from 15-day old PDA cultures and fresh infected leaves. In each case, 50 arbitrarily chosen conidia per isolate were measured using an eyepiece micrometer. The morphological traits recorded were conidium length (L), breadth (B) and the distance from the conidium base to the septum (LS). From these the length to breadth ratio (L : B) and septum location, expressed as a percentage of total conidium length (%Sept = LS/L × 100), were calculated. A further study was conducted to compare the growth rate of different isolates cultured on PDA Petri plates. The diameters of 4–6 colonies per isolate were measured at days 5, 15, 25 and 35 after culture. Colony characteristics and germination types were also observed.

Pathogenicity was compared based on the infection severity of each isolate that was inoculated to poplar leaves. All the isolates were grown on PDA tube-slants at 25°C for 15 days. The spores were flooded with sterilized water (10 mlper tube) and were centrifuged (2000 g) twice each for 5 min to remove the substance which influences spore germination. The spore suspension of each isolate was adjusted to approximately 3×10^6 spores per ml. The poplar species used for inoculations include *Populus alba* and *Populus tomentosa* (*Leuce*), *Populus canadensis* and *Populus ×euramericana* cv I-45 (*Aigeiros*) and *Populus cathayana* and *Populus simonii* (*Tacamahaca*). From these species, at least three nearly mature leaves (the second or third leaves from the terminal bud) were collected from the shoots cultured with water in the greenhouse in early spring or from trees in field trials in late spring or early summer. Leaf surfaces were atomized with suspensions of the conidia at approximately 0.05 ml/cm^2. Atomized leaves were transferred in culture dishes that were spread with wet filter papers at the bottom to maintain high humidity. Infection severity was determined after 10–12 days following leaf inoculation and expressed as the number of spots per unit leaf area (cm^2), with the following scales: 0 = no spots, 1=1–2 spots, 2=3–5 spots, 3=6–9 spots, and 4>10 spots or spots cluster.

To observe anastomosis between different isolates of *M. brunnea*, 5-cm long cotton threads were completely submerged under 50-ml of potato-dextrose liquid medium in 150-ml Erlenmeyer flasks. The medium was inoculated with a 2-ml spore suspension containing 10^5–10^6 spores per ml. The flasks were placed on a shaker (100 rpm) at 25°C for 5 days. Two threads adhered with different isolates were paired on the slides with 3–4 drops of potato-dextrose liquid medium, at a distance of 0.5–1.0 cm. The

Table 1 Geographic origin, poplar host, and original collection date of isolates of *M. brunnea* used in this study

Poplar host			
Section	Species	Isolate	Year
(1) **Nanjing, Jiangsu, 32°N, 118°E**			
Leuce	*P. adenopoda*	LAJ	1994
	P. tomentosa	LTJ1	1994
Aigeiros	*P. ×euramericana* cv I-45	AEJ1	1994
	P. ×euramericana cv 'Robusta'	AEJ2	1994
	P. ×euramericana cv I-214	AEJ3	1994
	P. nigra cv A86	ANJ1	1994
	P. nigra	ANJ2	1994
	P. canadensis	ACJ	1994
	P. illiensis	AIJ	1994
Tacamahaca	*P. ussuriensis*	TUJ	1994
	P. laurifolia	TLJ	1994
	P. talassica	TTJ	1994
	P. simonii	TSJ1	1994
	P. szechuanica	TSJ2	1994
	P. cathayana	TCJ	1994
(2) **Xinyang, Henan, 33°N, 114°E**			
Tacamahaca	*P. cathayana*	TCH	1995
(3) **Nanyang, Henan, 33.5°N, 112.5°E**			
Tacamahaca	*P. simonii*	TSH	1995
(4) **Xuzhou, Jiangsu, 34°N, 117°E**			
Leuce	*P. tomentosa*	LTJ2	1995
Aigeiros	*P. ×euramericana* cv I-214	AEX1	1995
	P. ×euramericana cv I-45	AEX2	1995
Tacamahaca	*P. cathayana*	TCX	1995
(5) **Zhenzhou, Henan, 34.5°N, 114°E**			
Leuce	*P. tomentosa*	LTH	1995
Aigeiros	*P. ×euramericana* cv 'Sacrau79'	AEH1	1994
	P. ×euramericana cv I-45	AEH2	1995
(6) **Tai-an, Shandong, 36°N, 117°E**			
Aigeiros	*P. deltoides* cv 'Harvard'	ADS1	1995
	P. deltoides cv I-69	ADS2	1995
	P. ×euramericana cv I-45	AES1	1995
	P. ×euramericana cv 'Robusta'	AES2	1995
	P. ×euramericana cv 'Sacrau79'	AES3	1995
	P. ×euramericana cv I-214	AES4	1995
	P. ×euramericana cv 158	AES5	1995
	P. canadensis	ACS	1995
	P. beijingensis	ABS	1995
	P. ×xiaozhuanica cv 'Dekuar'	AXS	1995
Tacamahaca	*P. simonii × P. pyramidalis*	TSS	1995
(7) **Beijing, 40°N, 116.3°E**			
Leuce	*P. tomentosa*	LTB	1996
Aigeiros	*P. deltoides*	ADB	1996
Tacamahaca	*P. cathayana*	TCB	1996
(8) **Yangling, Shanxi 34°N, 108°E**			
Aigeiros	*P.×euramericana* cv 'Sacrau79'	AESX	1995
	P. nigra var. thevestina	ANSX	1995
(9) **Baicheng, Jilin, 46°N, 123°E**			
Tacamahaca	*P. simonii*	TSJL	1996

Table 2 Nucleotide sequences of 11 RAPD primers and number of polymorphic fragments generated among 42 isolates of *M. brunnea*

Primer	Sequence 5′ to 3′	Total number of fragments scored	Number of polymorphic fragments
A03	AGT CAG CCA C	6	6
A07	GAA ACG GGT G	4	3
A11	CAA TCG CCG T	4	4
A18	AGG TGA CCG T	4	4
A19	CAA ACG TCG G	12	12
A20	GTT GCG ATC C	8	8
J19	GGA CAC CAC T	11	11
J20	AAG CGG CCT C	9	6
K01	CAT TCG AGC C	11	10
X02	TTC CGC CAC C	7	7
X08	CAG GGG TGG A	7	7
Total		83	78

slides were then put in culture dishes that contained 10-ml of 2% water agar. Mycelia from the threads were anastomosed after incubation at 25°C for 30–35 days. The degree of anastomosis between different isolates was examined under the microscope.

Randomly amplified polymorphic DNA (RAPD) markers were used to detect genetic polymorphisms among the isolates of *M. brunnea* from different regions. Erlenmeyer flasks containing 100 ml of potato-dextrose liquid medium were inoculated with conidial suspensions (approximately 10^6 spores per flask) of *M. brunnea* isolates. The flasks were shaken (100 rpm) at 25°C for 12 days. Mycelia growing from the isolates were harvested by filtering the cultures through Miracloth and washed three times in distilled water. Genomic DNA was isolated from mycelia of *M. brunnea* using a modified procedure described by Fan and Wood (1991). Through the screening of 80 RAPD primers ordered from Operon Technologies (Alameda, Calif.), 11, with the DNA sequences listed in Table 2, were detected to generate 3–11 polymorphic bands among *M. brunnea* isolates. Each RAPD reaction (12 µl) contains 5 ng of DNA templates, 5 pmol of primer, 200 µmol/l of dNTPs, 1.5 µl of 10× reaction buffer (100 µmol/l Tris-HCl, pH 8.3, 500 µmol/l KCl, 20 mmol/l $MgCl_2$, 5.0 g/l BSA) and 1 U of *Taq* DNA polymerase (28). Reactions were performed on a programmable thermal controller 1605 (Idaho Company). The amplified products were then separated on a 1.5% agarose gel using Tris-boric acid-EDTA buffer (TBE) and detected by staining with ethidium bromide.

Data analysis

Differences among the isolates of *M. brunnea* derived from PDA cultures or fresh infected leaves were analyzed using a simple statistical model expressed as:

$$y_{ij} = \mu + \alpha_i + \varepsilon_{ij}, \quad (1a)$$

where y_{ij} is the observation value of the *j*th conidium of the *i*th isolate, μ is the overall mean, α_i is the effect of the *i*th isolate and ε_{ij} is the error term. In this study, the effect of the *i*th isolate was partitioned into two components based on the geographic origins and taxonomic sections of the host species, i.e.,

$$\alpha_i = \beta_j + (\gamma/\beta)_{k/j}, \quad (1b)$$

where β_j is the effect of the *j*th origin, $(\gamma/\beta)_{k/j}$ is the effect of the *k*th section from the *j*th origin. A two-way factorial analysis of

variance method was used to analyze variation in the pathogenicity of isolates from different poplar sections (*Leuce*, *Aigeiros* and *Tacamahaca*) inoculated on various hosts in the laboratory. The underlying model is expressed as:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ijk}, \quad (2)$$

where y_{ijk} is the infection severity of the *k*th leaf from the *j*th poplar host affected by the isolate from the *i*th section, μ is the overall mean, α_i is the effect of the isolates from the *i*th section, regardless of its geographic origin, β_j is the effect of the *j*th host section inoculated in the laboratory, $(\alpha \times \beta)_{ij}$ is the interaction effect between the isolates from the *i*th section and *j*th host section and ε_{ijk} is the error term.

The RAPD bands scored were used to calculate Nei and Li's (1979) distance index using formula:

$$D_{xy} = 1–2N_{xy}/(N_x + N_y), \quad (3)$$

where N_{xy} is the number of bands shared between a pair of isolates and N_x and N_y are the number of bands present in isolates *x* and *y*, respectively. A distance matrix among the 42 isolates was clustered by the unweighted pair group method with arithmetic means (UPGMA) using the RAPDistance software, v. 1.04.

Results

Morphological features and germination type

Based on statistical tests using model (1a), conidia both from fresh infected leaves and PDA cultures displayed significant variation in size and shape among the 42 isolates of *M. brunnea* collected from a wide range of environments in China (data not shown). Conidia from infected leaves were 15.5 μm long and 6.3 μm broad, with septa located at 31.8% of the total conidium length from the bases (%Sept), whereas those cultured on PDA for 15 days were 5–10% larger and had septa 10% farther from the conidial bases. The conidia from leaves and cultures had similar shape, with a length:breadth (L : B) ratio of 0.3–0.4. Results from the analysis of variance using model (1b) indicated that the effects of the geographic origin and section of the host poplar on conidial size and shape were statistically not significant for *M. brunnea* isolates from the two sources (data not shown). The conidia were hyaline, narrowly obovoid to obovoid, straight to slightly curved, and divided unequally by septa into a smaller basal cell and a longer rounded cell. The size of conidia was highly variable within each isolate, especially in the PDA culture.

Although there was no difference in conidial size and shape before day 15 of the culture, the growth rate of the *M. brunnea* isolate varied dramatically among the isolates from different sections cultured on PDA Petri plates after day 15 (Fig. 1). Colonies of five isolates from section *Leuce* (L-isolate) grew faster than those of the other 37 isolates from sections *Aigeiros* (A-isolate) and *Tacamahaca* (T-isolate). The colony diameter of the L-isolate at 25-days old averaged 1.35 cm, ranging from 1.12 to 1.50 cm, whereas those of the A- and T-isolates averaged 0.73 cm, ranging from 0.56 to 0.90 cm. Colony characteristics and germination type also differed among isolates of the three sections. Colonies of L-isolates were coarse with aerial mycelia, dark brown, and produced dark-reddish conidiosorus clusters after 12 days. Colonies of A- and T-isolates, however, were fine in texture, lightly colored, and produced yellow-greenish conidiosorus clusters. Germination types of conidia were compared among isolates of the three sections cultured with concave slits. After a 2-day culture, conidia from the L-isolate germinated only one germ tube, whereas those from the A- and T-isolates germinated 1–5 germ tubes, and mostly 2–3.

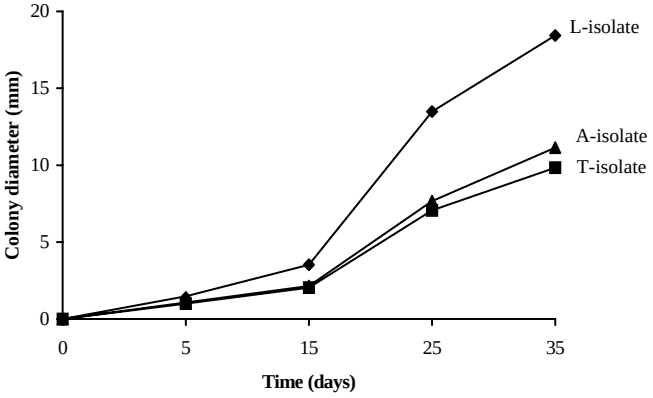

Fig. 1 Comparison of the colony growth rate of *M. brunnea* isolates from different poplar sections

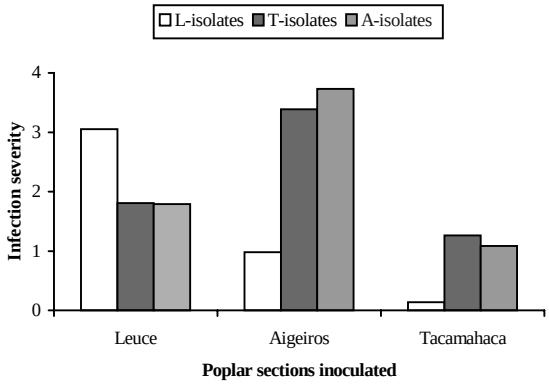

Fig. 2 Differences of infection severity on inoculated poplars of three sections by *M. brunnea* isolates of different hosts

Pathogencity test

The pathogenicity of *M. brunnea* was tested by inoculating isolates on poplar leaves of three different sections, *Leuce* (*P. tomentosa*), *Aigeiros* (*P. canadensis* and *P. ×euramericana* cv I-45) and *Tacamahaca* (*P. cathayana and P. simonii*). A relatively high percentage of conidial germination (30–40%) was obtained with each inoculation. The leaves developed necrotic local lesions at days 7–8 after inoculation. Results from the analysis of variance based on model (2) indicated that isolates from different sections, inoculated host poplar sections, and their interactions all had significant effects on the infection severity of

Table 3 Hyphal anastomoses among different isolates of *M. brunnea*. Symbols: "+" = the hyphae of two different isolates were anastomosed; "–" = no hyphal anastomosis occurred; "/" = two hyphae of different isolates did not meet

	LAJ											
LAJ	+	LTJ1										
LTJ1	+	+	LTJ2									
LTJ2	+	+	+	LTH								
LTH	+	+	+	+	AEJ3							
AEJ3	—	–	–	+	ACJ							
ACJ	—	–	–	+	+	AXS						
AXS	—	–	–	+	+	+	ACS					
ACS	—	–	–	+	+	+	+	ANSX				
ANSX	—	–	–	+	+	/	+	+	TSJ1			
TSJ1	—	–	–	+	+	+	+	+	+	TSJ2		
TSJ2	—	–	–	/	+	+	/	+	+	+	TTJ	
TTJ	—	–	–	+	/	/	+	+	/	+	+	TCX
TCX	—	–	–	+	+	+	+	/	+	/	+	+
TSS	—	/	–	+	/	+	+	+	/	/	/	+

poplar leaves at day 10 after inoculation (data not shown). Five L-isolates were mostly pathogenic to *P. tomentosa*, but moderately to *P. canadensis* and *P. ×euramericana* cv I-45 (Fig. 2). Species *P. cathayana* and *P. simonii* from section *Tacamahaca* were only slightly susceptible or else not infected by these isolates. Both A- and T-isolates displayed a similar trend, being mostly pathogenic to *P. canadensis* and *P. ×euramericana* cv I-45, moderately pathogenic to *P. tomentosa*, and slightly to *P. cathayana* and *P. simonii*. Poplars from section *Tacamahaca* were least susceptible to *M. brunnea* pathogens among the three sections. Surprisingly, all the isolates did not infect *P. alba*, which is a species of section *Leuce* (Han et al. 1998). Cluster analyses based on pathogenic index classified the 42 isolates into two groups: L-isolate and A- and T-isolates, analogous to the classification based on cultural characterization and germination type of the conidia.

Hyphal anastomosis

Four L-isolates, five A-isolates and five T-isolates were randomly chosen to examine hyphal anastomosis by making all possible pairs each with two replications. The degree of hyphal fusion varied depending on host sections from which isolates for pairing were derived (Table 3). Hyphae from the same isolates or host sections could be well fused. L-isolates could not be fused with A- or T-isolates, yet haphal fusion occurred between T- and A-isolates. After hyphae from two compatible isolates met, their cell walls were dissolved, which was immediately followed by plasma fusion and the formation of double-nucleus cells. By contrast, when hyphae from two incompatible isolates were paired on the slide, one hypha crossed over another and no anastomosis occurred even if the two hyphae were contracted.

RAPD polymorphism

DNA amplification using 11 RAPD primers produced a total of 78 polymorphic bands among the isolates of *M. brunnea* (Table 2). Nei and Li's distance analysis was used to estimate the distance index between each pair of the 42 isolates from which a 42-dimensional distance matrix was generated. Cluster analysis based on the distance matrix using the UPGMA method indicated that

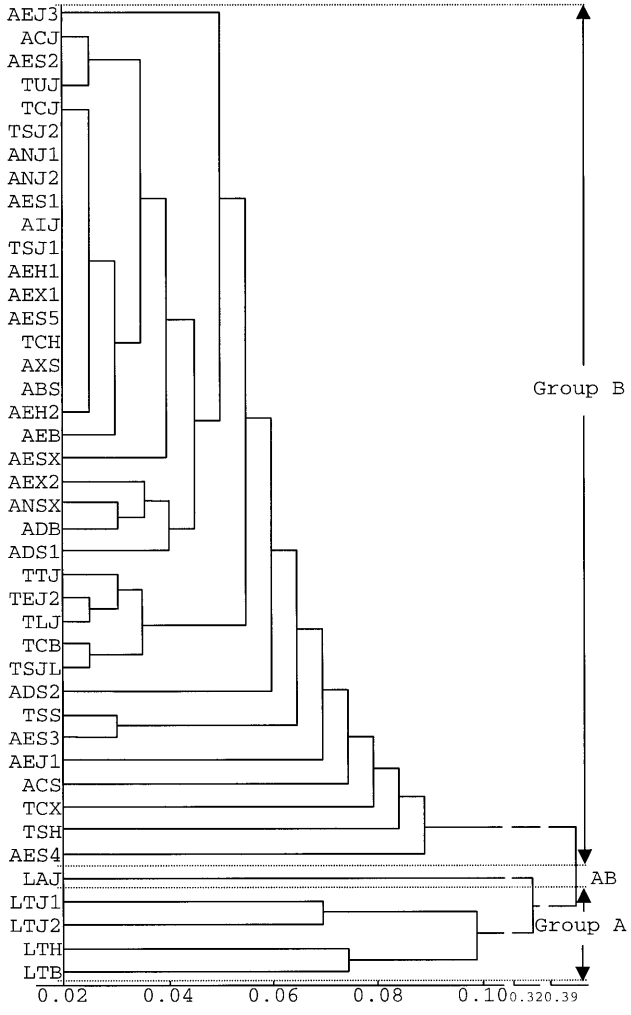

Fig. 3 UPGMA dendrogram based on RAPD polymorphism among 42 isolates of *M. brunnea*

the *M. brunnea* isolates studied were separated into three groups using 80% similarity (1 – distance index) as a cut-off point (Fig. 3). The three groups consisted of four isolates from *P. tomentosa* (group A), 37 isolates from the poplars of sections *Aigeiros* and *Tacamahaca* (group B), and one isolate, LAJ, from *P. adenopoda* (group C), respectively. Despite their broader geographic range, isolates within group B displayed a greater similarity index (88%) than isolates within group B collected from a more limited range (83%) (see Table 1). Isolate LAJ within group C formed an independent group, with a similarity index of 61% with isolates of group A and 32% with isolates of group B. Partially shared by groups A and B, isolate LAJ most likely provides a connection type between these groups, and is therefore named group AB.

Discussion

Conidial morphological features of the 42 isolates of *M. brunnea* collected from a wide range in China display considerable variation. However, such variation is statistically independent of host species and their geographic origins. The values for dimensions and septum locations of *M. brunnea* isolates observed in this study are broadly in agreement with those reported by Spiers (1988) who collected specimens of this species from Europe and North America. These results suggest that conidial size and shape cannot be used as a criterion to classify types of isolates within *M. brunnea*. However, based on host specificity, previous studies of diversity within *M. brunnea* have divided isolates collected in eastern China into two specialized forms, *M. brunnea* f.sp. *monogermtubi* and *M. brunnea* f.sp. *multigermtubi* (Li 1984). The results from the present study are consistent with this classification but are based on expanded collections into a wider region and molecular genetic analysis. Of the 42 *M. brunnea* isolates studied, five from section *Leuce* and 37 from *Aigeiros* and *Tacamahaca* can be well separated. The isolates from section *Leuce*, named *M. brunnea* f.sp. *monogermtubi*, germinate only one germ tube, grow rapidly and produce dark-reddish conidiosorus clusters on the PDA culture, whereas the isolates from sections *Aigeiros* and *Tacamahaca*, named *M. brunnea* f.sp. *multigermtubi*, germinate 1–5 germ tubes, grow slowly and produce yellow-greenish conidiosorus clusters. Anastomosis tests showed that mycelia could be fused from the isolates of the same group but not from different groups. Thus, culture characteristics, growth rate, germination type and the host range of isolates can be used as morphological markers for classifying *M. brunnea* isolates into different groups.

In nature, host ranges for each of the two specialized forms are stable, which is viewed as a characteristic of *Marssonina* different from *Melampsora* (Spiers 1998). For example, in a wide geographic region, *M. brunnea* f.sp. *multigermtubi* has not been found from poplars of section *Leuce* whilst *M. brunnea* f.sp. *monogermtubi* has not been found from poplars of sections *Aigeiros* and *Tacamahaca* (Z.M. Han, unpublished data). However, such host specialisation does not exist under inoculation conditions (see Fig. 2) or when multiple poplar species are grown together. In fact, host ranges of the specialized forms are not absolutely associated with poplar sections. Even under inoculation conditions, for example, five isolates derived from section *Leuce* do not infect *P. able* of the same section, whereas 37 isolates from sections *Aigeiros* and *Tacamahaca* do not infect *P. deltoides* cv I-69 and *P. deltoides* cv I-63, both of which belong to section *Aigeiros* (Li 1984).

Although morphological markers or host ranges are a useful means of grouping similar isolates, they do not provide any indication of the genetic relatedness between isolates derived from different poplar hosts. Using DNA-based marker analysis, we can determine the genetic variation among isolates on different hosts from different regions. Based on the cluster analysis of 78 polymorphic RAPD markers, the 42 isolates studied can be divided into three groups: group A including four isolates from *P. tomentosa* of section *Leuce*, group B including 37 isolates from both sections *Aigeiros* and *Tacamahaca*, and group AB including a single isolate from *P. adenopoda* of section *Leuce*. Results from DNA analysis basically support the classification of *M. brunnea* by the phenotype, but gain new insights into the origin of this pathogen. We propose that *M. brunnea* co-evolves with poplars of section *Leuce* and has been subsequently distributed to poplars of sections *Aigeiros* and *Tacamahaca*. It is less likely that the pathogen evolves independently from poplars of different sections. Our hypothesis for the origin of *M. brunnea* is based on the fact that there is greater diversity within group A than group B. Although group A contains only four isolates collected from a limited number of geographic locations (Table 1), the genotypes within this group were highly diverged. By contrast, group B contains 37 isolates from a variety of host species (two sections) and geographic locations whose genotypes are closely related (Fig. 3). Similar analysis has been carried out by Bentley et al. (1998) and O'Donnell et al. (1998) who based their inferences of fungal origins on the variation of isolates at the DNA level.

Further evidence for the hypothesis of co-evolution with section *Leuce* and subsequent distribution to other sections is that a transition type, group AB, exists between two different groups. Isolate LAJ derived from *P. adenospoda* of section *Leuce* is the only representative of this group. Derived from section *Leuce*, this isolate has a similarity of 61% with group A, larger than its similarity with group B (32%). There is no evidence that group AB results from genetic recombination between groups A and B, because no sexual propagation has been detected for *M. brunnea* in China. Also, there is a low possibility that group AB derives from somatic hybridization between groups A and B. Hyphal anastomosis tests indicate that anastomosis cannot occur between the hyphae of the isolates from the poplars of section *Leuce* and from sections

Aigeiros or *Tacamahaca* (Li 1984). The co-evolution hypothesis has important implications for the selection of poplar cultivars with resistance to Marssonina leaf spot, as resistant cultivars are most likely to be present in regions where there is greatest diversity within the host and the pathogen (Vavilov 1922). It is apparent that further collection and genetic analysis of *M. brunnea* strains from poplars of section *Leuce* is necessary.

In an early study, Spiers (1988) divided *M. brunnea* collected in Europe into two *formae speciales*: (1) *M. brunnea* f.sp. *trepida*e, which is pathogenic to *P. tremula* and *P. tremuloides* in subsection *Trepidae* of section *Leuce* but not pathogenic to *P. deltoides* and its hybrids with *P. nigra*, and (2) *M. brunnea* f.sp. *brunnea*, which is pathogenic to P. deltoides and its hybrids with *P. nigra* but not pathogenic to *P. tremula* and *P. tremuloides*. These two forms may represent *M. brunnea* f.sp. *monogermtubi* and *M. brunnea* f.sp. *multigermtubi*, respectively, except for a difference of host range. However, further molecular genetic analysis is needed to accurately compare these two Europe-collected types with the two specialized forms native to China. With such a comparison, our understanding of the overall genetic diversity of *M. brunnea* will well be enhanced.

Acknowledgements We thank K.M. Yang and M. Li for technical assistance in the laboratory and Dr. B.K. Shen for helpful discussion regarding this study, which was partially supported by a grant from the National Natural Science Foundation of China (39870628).

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