Hirsutella vermicola sp. nov., a new species parasitizing bacteria-feeding nematodes

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Xiang, M.C., Yang, E.C., Xiao, Q.M., Liu, X.Z. and Chen, S.Y. (2006). *Hirsutella vermicola* sp. nov., a new species parasitizing bacteria-feeding nematodes. Fungal Diversity 22: 255-266.

The phylogeny of nematode-endoparasitic *Hirsutella* species was studied based on morphology and DNA sequence analysis. *Hirsutella vermicola* is proposed as a new species that preys on bacteria-feeding nematodes and nonpathogenic or weakly pathogenic to plant-parasitic nematodes. It is distinguished from *H. rhossiliensis* by having shorter conidiogenous cells with a swollen base, helical necks and slightly wider conidia. Phylogenetic trees based on sequences of the ITS region, MAPK gene fragment, and the combined data revealed two clusters corresponding to these two species and thus supported the establishment of this new species.

Key words: biological control, *Hirsutella rhossiliensis*, *Hirsutella vermicola*, nematophagous, taxonomy

Introduction

There has been a surge in interest in nematode trapping fungi in recent years because their potential use in biological control. This has resulted in descriptions of several new species (Liu *et al*., 2005; Mo *et al*., 2005) and studies on nematicidal effects and virulence factors (Dong *et al*., 2004; Zhao *et al*., 2005). *Hirsutella rhossiliensis* Minter & Brady (= *Hirsutella heteroderae* Sturhan & Schneider) is endoparasite on vermiform nematodes (Sturhan and Schneider, 1980; Jaffee and Zehr, 1982). It parasitizes 80% of *Mesocriconema xenoplax* in California peach orchard soils (Jaffee *et al*., 1988) and 90% of *Heterodera schachtii* second-stage juveniles (J2s) in oil-radish fields in Germany (Müller, 1982). The fungus is widely distributed and has been isolated from *Heterodera humuli* (Sturhan and Schneider, 1980), *H. schachtii* (Müller, 1984), *H. avenae* (Stirling and Kerry, 1983), *H. glycines* (Chen,

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1997), *Meloidogyne javanica* (Cayrol *et al.*, 1986), *M. xenoplax* (Jaffee and Zehr, 1982), *Rotylenchus robustus* (Jaffee *et al*., 1991), *Xiphinema diversicacaudatum* (Ciancio *et al*., 1986), *Hoplolaimus galeatus*, bacterial-feeding nematodes, soil mites and soil in different areas of the world (Tedford *et al.*, 1994; Ma *et al.*, 2005). *Hirsutella rhossiliensis* has been extensively studied and has shown potential as a biological control agent (Jaffee and Zehr, 1982).

Although the fungus is generally isolated from only one species of nematodes in the field, at a time, at any site (Sturhan and Schneider, 1980; Jaffee and Zehr, 1985; Jaffee *et al.*, 1991; Timper and Brodie, 1993; Velvis and Kamp, 1995; Liu and Chen, 2000), Tedford *et al.* (1994) noted that variability among 25 isolates of the species occurred in morphology and their pathogenicity against *Heterodera schachtii*, *Meloidogyne javanica* and *Steinernema glaseri* on agar and in soil. Isolates from bacteria-feeding nematodes collected from soybean fields in Minnesota, USA, did not infect soybean-cyst nematode J2s within three days on agar plates (Liu and Chen, 2001). A recent study also showed that isolates from bacteria-feeding nematodes grew slower than those from plant-parasitic nematodes and were not or weakly parasitic to plant-parasitic nematodes (Xiang *et al*., unpublished). Preliminary observations indicated that the morphology of isolates from bacteria-feeding nematodes was different from plant-parasitic nematodes. A detailed study based on morphology and molecular data revealed that the isolates from bacteria-feeding nematodes represent a new species, described herein.

Materials and methods

Morphological features

Seven isolates formerly identified as *Hirsutella rhossiliensis*, including three from bacteria-feeding nematodes and four from different plant-parasitic nematodes, and one isolate of *H. minnesotensis* Chen, Liu & Chen (Chen *et al.*, 2000) from *Heterodera glycines* were used in this study (Table 1). All fungi were maintained on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, Hampshire, England) slants at 4°C and cultured on PDA plates at room temperature. For morphological measurements of conidia and phialides, a PDA agar block (*ca*. 4 × 4 mm^2 and 4 mm thick) inoculated with fungus was placed on a sterile slide with a coverslip (Coetzee and Eicker, 1990). The slide culture was placed into a 90 mm Petri dish. After 7-10 days of incubation at room temperature, all of microscopic characteristics were measured from 30

Table 1. Details of of *Hirsutella* isolates used in this study.

Isolates	Taxon	Hosts	Isolated from	GeneBank No. (ITS/MAPK)
AS 3.7878	*H. vermicola*	Bacteria-feeding nematode	Watonwan, MN, USA	DQ345592 DQ452362
AS 3.7877	*H. vermicola*	Bacteria-feeding nematode	Jackson, MN, USA	DQ345589 DQ452358
AS 3.7879	*H. vermicola*	Bacteria-feeding nematode	Faribault, MN, USA	DQ345581 DQ452349
ARSEF 2006	*H. rhossiliensis*	*Criconemella xenoplax*	Erie, PA, USA	DQ345566 DQ452333
CBS 113353	*H. rhossiliensis*	*Heterodera glycines*	Beian, Heilongjiang, China	DQ345584 DQ452352
ARSEF 3755	*H. rhossiliensis*	*Rotylenchus robusta*	San Mateo, CA, USA	DQ345575 DQ452342
ARSEF 2894	*H. rhossiliensis*	*Heterodera humuli*	Hallertau, Germany	DQ345573 DQ452340
AS 3.7880	*H. minnesotensis*	*Heterodera glycines*	Redwood, MN, USA	DQ345591 DQ452361

individuals in water mounts at 1000× magnification. Observations, measurements, and photographs were taken with Nikon 80i microscope with differential interference contrast (DIC).

DNA extraction, amplification, and sequencing

Axenic mycelia (0.05-0.1 g) of all tested fungi were harvested from PDA plates and transferred into 1.5 ml Eppendorf tubes for genomic DNA extraction (Wu *et al.*, 2001). The ribosomal RNA gene ITS region was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990), and mitogen-activated protein kinase (MAPK) gene fragment amplified using the primers P1835 (5′-GAGGAGAATGCCGGTTACATGAC-3′) and P2370 (5′-CTCGTCAGATGCATCGTGCCA-3′). P1835 and P2370 were designed according to the highly conserved amino acid sequences at C-terminal of MAPK gene. PCR amplification was conducted as follows: 3 min at 95°C and 35 cycles of 95°C for 40 s, 53°C for 40 s (56°C for MAPK gene), 72°C for 60 s, followed by elongation at 72°C for 10 min. PCR products were purified using 3S PCR Products Purification Kit (Shenergy Biocolor BioScience & Technology Company, Shanghai, China) and sequenced.

Phylogenetic analysis

Nucleotide sequences were aligned using Clustal X 1.81 (Thompson *et al.*, 1997) and were manually realigned using BioEdit version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, Raleigh, NC 27695). Phylogenetic analysis was performed using PAUP* 4.0 beta 10 (Swofford, 2001) with gaps treated as missing data and all characters equally weighted. The robustness of branches was assessed by bootstrap analysis with 1000 replicates. *Hirsutella minnesotensis*, another nematode-endoparasitic, was selected as outgroup.

Results

Taxonomy

The isolates from bacteria-feeding nematodes were formerly identified as *Hirsutella rhossiliensis* based on colony and conidial morphologies. A detail examination however, revealed that the morphological and molecular characteristics of those isolates were different from those of isolates infecting plant-parasitic nematodes. Strains AS3.7877 and AS3.7878 of bacteria-feeding nematodes were selected for morphological study and compared with *H. rhossiliensis* isolates ARSEF2006 from *M. xenoplax* in California and CBS113353 from *Heterodera glycines* in Beian County, Heilongjiang, China. The features of AS3.7877 and AS3.7878 are not identical to any known species of *Hirsutella* and related genera. For reasons elaborated below, a new species of *Hirsutella* is proposed.

Hirsutella vermicola M.C. Xiang & X.Z. Liu, **sp. nov.** (Fig. 1)

MycoBank number: MB500924.

Etymology: Species epithet in reference to the eelworm host.

Coloniae in PDA agaro lente crescentes, albae, 12-17 mm diam post 21 dies. *Mycelium* superficiale, hyalinum, septatum, laeve. *Conidiophoris* ad cellulas conidiogenas sessilibus, reductis, singulatim producentis ex hyphis vegetativis. *Cellulis conidiogenis* monophialidicis, rarius polyphialidicis, 14-20-26 µm longis, basi parte inflatis, 7-10.5-15 × 3-4.5-5 µm, apice 1-2 µm latis, attenuatis. *Conidiis* aseptatis, levibus, singulatim vel 2-3 aggregate ad colli apicem facientibus, plus minusve ellipsoideis, 6-6.5-8 µm longis, 3-4-5 µm latis, in muco involutis.

Holytypus: HMAS B4501 (cultura exsiccata) isolatus ex nematodo bacteriivoro, X.Z. Liu, in Herbario Mycologico Academiae Sinicae (HMAS) in Beijing conservatus.

Colonies on PDA growing much slowly, attaining a diam. of 12-17 mm within 3 weeks. *Mycelium* moderate, superficial, hyaline, septate, smooth. *Conidiogenous cells* arising singly, or occasional in pairs oppositely, more or

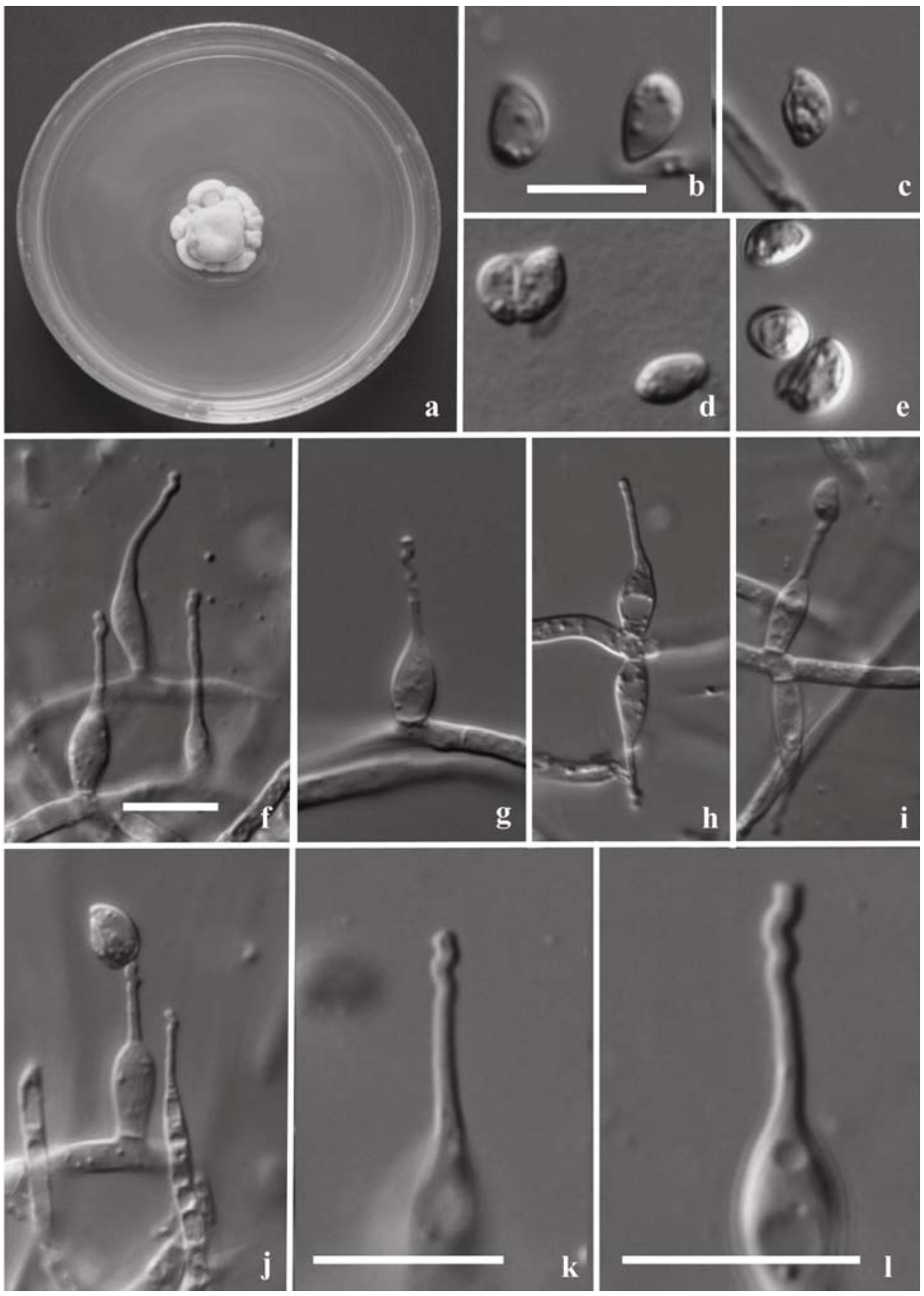

Fig. 1. *Hirsutella vermicola*. **a.** Colony on PDA for 3 wks. **b-e.** Conidia and one or two in a mucous sheath. **f-j.** Monophialides of conidiogenous cells with or without conidia. **k-l.** Twisted neck of conidiogenous cell apex. Bars are the same in b-e, f-j, k and l respectively and represent 10 µm.

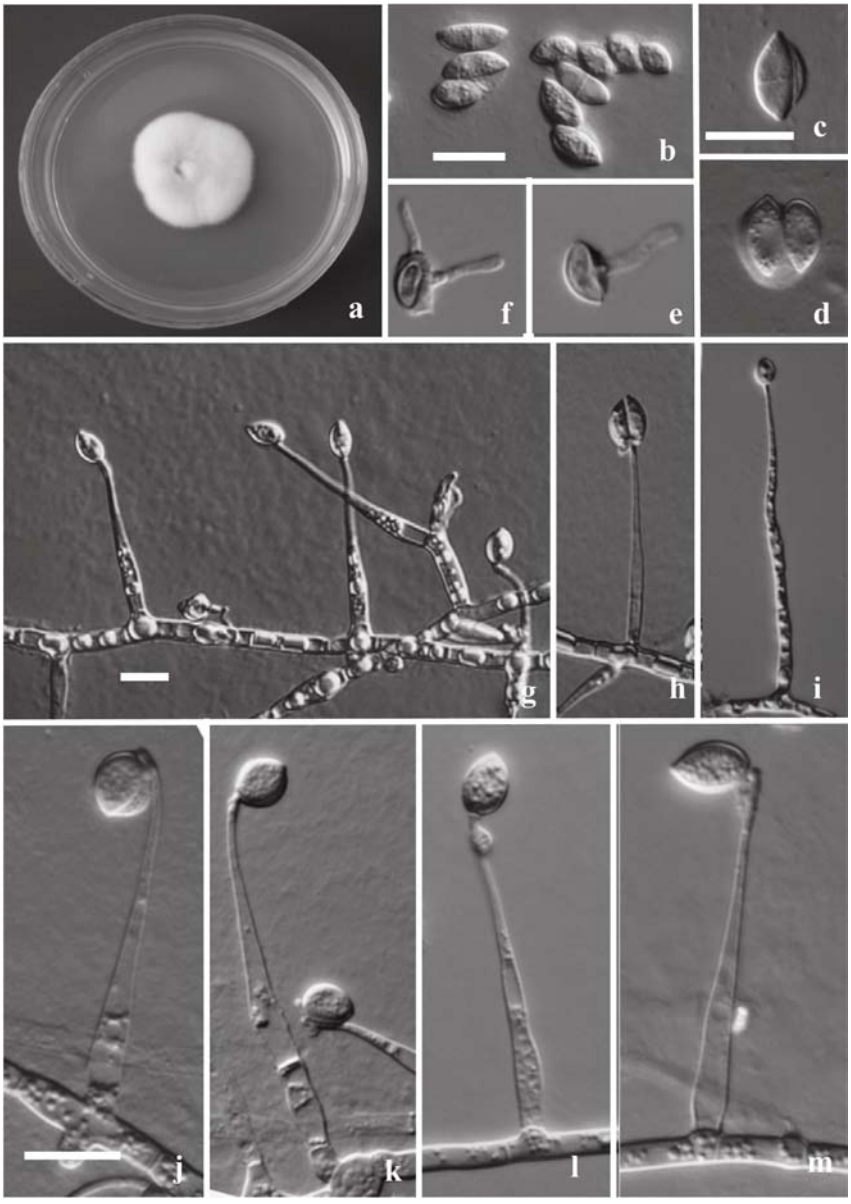

Fig. 2. *Hirsutella rhossiliensis*. **a.** Colony on PDA for 3 wks. **b-d.** Conidia with 0-1 septum, one or two in a mucous sheath. **e-f.** Germinating conidia. **g-i.** Monophialides or polyphialides of conidiogenous cells with conidia. **j-m**. Magnification of conidiogenous cells. Bars are the same in c-d, b and e-f, g-i, and j-m respectively and represent 10 µm.

less right angles from vegetative hyphae, monophialidic or polyphialidic, hyaline, smooth, 14-20 (average)-26 µm long, significantly swollen to 3-4.5-5 µm wide towards the base, tapering to 1-2 µm wide and 5-9.5-15 µm long neck that twists in a helix towards the apex. *Conidia* hyaline, aseptate, smooth, more or less ellipsoid (often the shape of an orange segment), arising from the apex of the neck singly or in a groups of 2-3, 6-6.5-8 µm long, 3-4-5 µm wide, enveloped in a hyaline mucous sheath.

Habitat: Parasitizing bacteria-feeding nematodes.

Known distribution: Minnesota, United States

Material examined: Strains AS3.7877 and AS3.7878 isolated from bacteria-feeding nematodes by X.Z. Liu in USA, Minnosota, Jackson and Watonwan Counties in 1998. The living cultures were deposited in China General Microbiological Culture Collection Center.

Notes: Due to the similarity between *H. vermicola* and *H. rhossiliensis* in morphology, a comparative description of *H. rhossiliensis* is provided as follows.

Hirsutella rhossiliensis Minter & Brady (Fig. 2)

Colonies on PDA growing slowly, attaining a diam. of 16-24 mm within 3 weeks. *Mycelium* abundant, superficial, hyaline, septate, smooth. *Conidiogenous cell*s arising singly, more or less right angles from vegetative hyphae, monophialidic, occasional polyphialidic, hyaline, smooth, 22-32.5-42 µm long, 2-4-5 µm wide at the base, tapering to a straight neck, 1 µm wide at the apex. *Conidia* hyaline, aseptate or occasionally with 1 septum, smooth, more or less ellipsoid (often the shape of an orange segment), arising from the apex of the neck singly or 2 in a group, 6-7-10 µm long, 3-4.5-6 µm wide, enveloped in a hyaline or pigmented mucous sheath.

Strains examined: ARSEF2006 isolated from *M. xenoplax* by B.A. Jaffee in USA, PA, Erie County in 1983. CBS113353 isolated from *H. glycines* by R. Ma, in China, Heilongjiang, Beian County in 1999.

DNA sequence analysis

A neighbor-joining tree was constructed based on sequences from the ITS region, MAPK gene fragment and the combined data of seven isolates of *H. rhossiliensis* and *H. vermicola* with *H. minnesotensis* as the outgroup. The resultant phylogenetic tree was mainly divided by two clades with 100% bootstrap values, one of which encompassed the isolates from plant-parasitic nematodes and the other included isolates from bacteria-feeding nematodes (Fig. 3). The molecular data were consistent with the morphological characteristics and supported the establishment a new species.

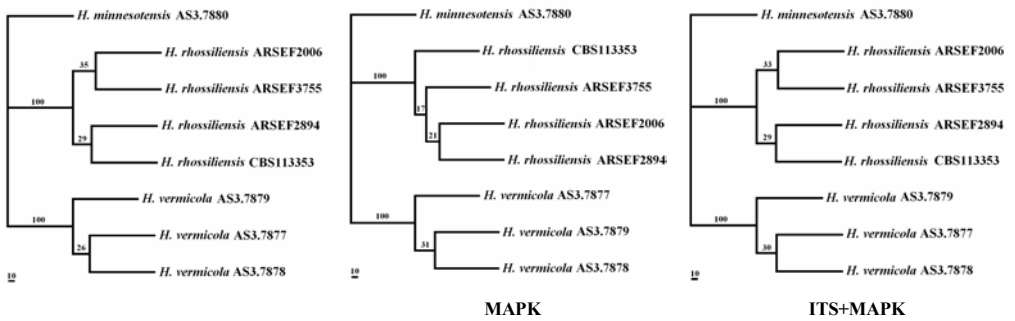

Fig. 3. Dendrogram constructed from neighbor-joining analyses based on sequences of rDNA ITS region and MAPK gene fragment and their combination of *Hirsutella rhossiliensis*, *H. vermicola* and *H. minnesotensis*. Bootstrap values are placed on the tree nodes.

Discussion

The new species is distinguished from *Hirsutella rhossiliensis* by the strongly swollen base and a conspicuous helical twist at the apex of conidiogenous cell. *Hirsutella nodulosa* and *H. brownorum* are the only species of *Hirsutella* with conidiogenous cells with a twisted neck (Minter and Brady, 1980). The new species resembles these two species in the helical neck and size of conidiogenous cell, but its smooth conidiogenous cells differ from *H. nodulosa* (with tiny warts) and the polyphialidic conidiogenous cell from *H. brownorum* (versus only monophialidic in *H. vermicola*) (Minter and Brady, 1980). Both *H. nodulosa* and *H. brownorum* are parasites of insects, while the new species is a parasite of bacteria-feeding nematodes.

Hirsutella rhossiliensis was described by Minter and Brady (1980) based on the fungus isolated from soil in Glamorgan, Wales in 1953. In the same year, *H. heteroderae* was described by Sturhan and Schneider (1980) based on a fungus isolated from *H. humuli* juveniles in Hallertau, Germany in 1976. Due to the morphological similarity between those two species, *H. heteroderae* was considered to be a synonym of *H. rhossiliensis* (Jaffee and Zehr, 1985). *Hirsutella rhossiliensis* has been isolated from various nematodes, soil mites, springtails and soils from a wide geographic range (Tedford *et al*., 1994; Liu and Chen, 2000). Although Tedford *et al*. (1994) found that the isolates from *Hoplolaimus galeatus* and *Rotylenchus robustus* grew slower in culture, produced larger conidia and were weakly parasitic to *Heterodera schachtii, Meloidogyne javanica* and *Steinernema glaseri* on agar than the other nematodes, there were no significant divergence of sequences of ITS and MAPK gene among isolates from various hosts except bacteria-feeding

nematodes (Xiang *et al*., unpublished). The isolates of the new species did not or only weakly infected these six nematodes, e.g. *Heterodera glycines*, *H. avenae*, *Meloidogyne hapla*, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, *Heterorhabditis bacteriophora* Poinar and *Steinernema* sp.), which further supports the establishment of the new species.

Hirsutella rhossiliensis has a broad host range and wide distribution (Sturhan and Schneider, 1980; Jaffee and Zehr, 1985; Timper and Brodie, 1993; Velvis and Kamp, 1995; Liu and Chen, 2000), probably is an obligate parasite in nature (Jaffee *et al*., 1991), can parasitize a high percentage of nematode population and may be responsible for nematode natural suppression in certain locations (Müller, 1982; Jaffee and Zehr, 1985; Jaffee *et al*., 1991; Chen, 1997; Ma *et al*., 2005). The isolate OWVT-1 was highly effective in suppressing soybean cyst nematode population density in the soil under greenhouse conditions, and reduced the nematode egg density by 95% and J2 population density by 98% when compared with the control (Liu and Chen, 2001). These characteristics make the fungus an attractive candidate in nematode biological control. The investigation on the differentiation of the fungus may result in discovery of effective biological control agents against plant-parasitic nematodes.

Acknowledgements

We thank R.A. Humber for providing cultures of *Hirsutella* spp., J.Y. Zhuang for his help in Latin diagnosis, R.A. Humber, W.Y. Zhuang and anonymous reviewers for useful suggestions. This research was supported by National Natural Scientific Foundation of China (No. 30370953).

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(Received 11 January 2006; accepted 15 March 2006)