Pak. J. Bot., 41(1): 185-193, 2009.

MOLECULAR PHYLOGENY OF *ERIOBOTRYA* LINDL. (LOQUAT) INFERRED FROM INTERNAL TRANSCRIBED SPACER SEQUENCES OF NUCLEAR RIBOSOME

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Abstract

Phylogenetic relationship of the genus *Eriobotyra* was investigated on the basis of the nuclear ribosomal DNA Internal Transcribed Spacer (ITS) sequence. A phylogenetic tree of 15 loquat accessions (species, varieties and types) was generated using *Photinieae serrulata* L., *Osteomeles anthyllidifolia* Lindl., *Sorbus scopulina* Hedl., *Malus prunifolia* (Willd.) Barkh, and *Pyrus pyrifolia* (Burm.) Nakai as outgroups, and *Rhaphiolepis indica* (L.) Lindl., as an ingroup. The study exhibited that loquat accessions formed a monophyletic group. In the consensus trees, loquat accessions were divided into six clusters, i.e., Cluster I: *Eriobotyra seguinii* Card. and *E. henryi* Nakai; Cluster II: *E. cavaleriei* Rehd and *E. fragrans* Champ; Cluster III: *E. malipoensis* Kuan, *E. prinoides* Rehd. & Wils.var. *dadunensis* H. Z. Zhang and *E. japonica* Lindl.; Cluster IV: *E.elliptica* Lindl., *E.bengalensis* Hook.f., *E.bengalensis* Hook. f. *forma angustifolia* Vidal; Cluster V: *E. salwinensis* Hand-Mazz and Cluster VI: *E. deflexa* Nakai *E. deflexa* Nakai var. *buisanensis* Nakai, *E. serrate* Vidal and *E. kwangsiensis* Chun. In addition, it was suggested that *E. cavaleriei* Rehd could be treated as a variety under *E. fragrans* Champ.

Introduction

Eriobotyra Lindl., belongs to the family Rosaceae, subfamily Maloideae (Lindley, 1822). The number of loquat species and their classification have been under dispute for several decades. In general, the species and varieties of *Eriobotyra* can be categorized into two groups, based on the presence or absence of pubescent on lower surface of old leaves (Yu *et al.*, 1974) and different flowering time (Zhang *et al*., 1990). A recent study has indicated that the genus *Eriobotyra* Lindl., comprises 32 species including some varieties and types all originating from China and Southeastern Asia (Lin *et al.*, 2004; Lin, 2007).

In the last twenty years, DNA molecular fingerprinting techniques are used to investigate genetic diversity of germplasm in fruit trees including loquat. Molecular markers, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and Inter Simple Sequence Repeat (ISSR), are applied to loquat for assessing the phylogenetic relationship. The genetic diversity of germplasm in loquat is reconstructed from molecular data based on the RAPD, AFLP and a part of morphological characters e.g., stamens, stigmas and leaves (Yang, 2005; Yang *et al.*, 2005; Yang & Lin, 2007). To our best knowledge, the phylogenetic relationship of the genus *Eriobotyra* Lindl., remains unclear. Moreover, the taxonomical status of some species, such as *E. cavaleriei* Rehd and *E. fragrans* Champ, needs to be further investigated.

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It is important that DNA data, especially DNA sequences should be applied for the classification and phylogeny of the plant (Jansen & Kim, 1996). Sequences of the internal transcribed spacers (ITS) of nuclear ribosome has proven to be of great significance in understanding the phylogeny of angiosperms at the specific and generic levels, and has been widely used for determining phylogenic relationships of plant groups at lower taxonomic level (Baldwin *et al.*, 1995; Tian & Li, 2002).

As a part of phylogenic study of loquat, ITS regions from 15 species of *Eriobotyra* Lindl., were determined and then analyzed in an attempt to construct a phylogeny of the family and then provide a new evidence for the phylogeny in *Eriobotyra* Lindl.

Materials and Methods

Plant samples of the genus *Eriobotrya* were ex situ preserved in the Loquat Germplasm Center at Horticultural College, South China Agricultural University, China and 17 species were selected as operational taxonomic units (OUTs) or terminal taxa, including 15 *Eriobotyra* Lindl., and 2 relatives (Table 1). In the ITS separate data set, the sequences of 4 species of *Osteomeles anthyllidifolia* Lindl., *Sorbus scopulina* Hedl., *Malus prunifolia* (Willd.) Barkh and *Pyrus pyrifolia* (Burm.) Nakai of subfamily Maloideae were obtained from the GeneBank. Table 1 gives the taxa, vouchers and GeneBank accession numbers.

The previous study indicated that *Eriobotyra* Lindl., was closely related to *Rhaphiolepis indica* (L.) Lindl., *Osteomeles anthyllidifolia* Lindl., *Sorbus scopulina* Hedl., *Malus prunifolia* (Willd.) Barkh and *Pyrus pyrifolia* (Burm.) Nakai, especially to *Rhaphiolepis indica* (L.) Lindl., (Campbell *et al.*, 1995; 2007). In this study, *Osteomeles anthyllidifolia* Lindl., *Sorbus scopulina* Hedl., *Malus prunifolia* (Willd.) Barkh and *Pyrus pyrifolia* (Burm.) Nakai were designated as multiple outgroups while *Rhaphiolepis indica* (L.) Lindl., was designated as an ingroup.

Total DNA was extracted from fresh leaf, by following the method of Liu *et al.* (2005). Double strands were directly amplified by the symmetric PCR with the ITS1 and ITS4 primers (White *et al.*, 1990). PCR amplification consisted of the initial denaturation for 7 min at 94°C, followed by 30 cycles of a 94°C denaturation for 1 min at 94°C, an 58°C annealing for 1 min at 58°C, and an 72°C extension for 1 min at 72°C, and with a final extension for 10 min at 72°C. PCR products were separated with 1% agarose TAE gel and purified using Watson's PCR minicolumns. Sequencing reactions were performed using the dye-terminator cycle-sequencing ready-reaction kit following the manufacture's protocol, and then analyzed on an ABI 377 Automated DNA Sequencer (Applied Biosystems).

We obtained the DNA sequence of *E. deflexa* Nakai, and then designed the specific primer "ITSF" 5'-AAAAGTCGTAACAAGGTTTCC-3' and "ITSR" 5'-GCTTAAATT CAGCGGGTAA-3' based on the DNA sequence of *E. deflexa* Nakai. ITS sequences of the other species were amplified using the primers "ITSF" and "ITSR". Reaction was conducted in thin-walled microcentrifuge tubes (0.2mL), which contained 10 × PCR buffer without Mg^{2+}, 0.5 μL of 5 U/μL Ampli*Taq* DNA polymerase, 4.0 μL of 25 mmol/L $MgCl_2$, 1.0 μL of 25 mmol/L dNTPs, 1.0 μL of ITSF (10 μmol/L), 1.0 μL of ITSR (10 μmol/L), and 20−50 ng sample DNA.The procedures of PCR thermal cycle and DNA sequencing were also conducted as mentioned above.

DNA sequences were edited, aligned with ClustalX software (Thompson *et al.*, 1997) and then adjusted manually where necessary. Phylogenic analyses by the maximum-parsimony method were performed with PAUP4.0b10 (Swofford, 2003). In phylogenic analysis, ambiguous sites were excluded from the matrix. Gaps were treated as missing while the inferred index of unambiguous alignment were recorded as unordered separated characters. All unambiguous characters and character-state transformations were given an equal weight. A heuristic search was performed for each data set, with RANDOM stepwise data addition (1000 replications with a start seed of 1) and TBR branch-swapping algorithm options. To assess the relative support for each clade, bootstrap values were calculated from 1000 replicate analyses with the heuristic search strategy and simple addition sequence of the taxa. The amount of phylogenic information in the MP analysis was constructed with the consistency index (CI) and retention index (RI). Maximum parsimony trees were constructed using PAUP4.0b10 program (Swofford, 2003). Cladistic analysis of the phylogenic relationship was conducted by using Wagner parsimony and applying heuristic search with tree bisection reconnection (TBS) branch-swapping and simple stepwise taxon application of 1000 replications. At the same time, Kimura 2-parameter distance of pairwise divergence of ITS sequences was calculated using Mega 3.1 Software (Kumar *et al.*, 2004).

Results

Specific primer polymerase chain reaction on ITS sequence of *Eriobotyra* Lindl: Seventeen plant samples were amplified using the primer "ITSF" and "ITSR". The cloned full-length DNA of all samples was 650-700 bp. Fig. 1 gives the result of PCR amplification.

Phylogenetic analysis

MP tree of *Eriobotyra* Lindl: The aligned ITS data matrix consisted of 629 alignment positions, with 154 variable sites and 132 informative sites. The percentage of phylogenic informative sites was 20.9% while the percentage of GC was 63.5%. A total of 841 most parsimonious trees were obtained in the maximum parsimony analysis of the ITS sequences when gaps were treated as missing. Each of the trees had a minimal length of 657 steps, with a consistency index (CI) of 0.8128 and a retention index (RI) of 0.7953. Fig. 2 shows the strict consensus tree. The study indicated that the outgroups of *Osteomeles anthyllidifolia* Lindl., *Sorbus scopulina* Hedl., *Malus prunifolia* (Willd.) Barkh, *Pyrus pyrifolia* (Burm.) Nakai and *Photinieae serrulata* L., formed a strongly supported monophyletic group (Bootstrap value= 88%). *Photinieae serrulata* L., and *Malus prunifolia* (Willd.) Barkh were each other related to form a group, but the internal support was relatively low (bootstrap = 50%). While *Rhaphiolepis indica* Lindl., formed a monophyletic group as the ingroup, the genus of *Eriobotyra* Lindl., was resolved as a monophyletic group with a high bootstrap support (Bootstrap value = 73%). It was divided into six clades. Clade I included *E. seguinii* Card and *E. henryi* Nakai (Bootstrap value = 56%), which may be a primitive group of *Eriobotyra* Lindl., Clade II contained *E. cavaleriei* Rehd and *E. fragrans* Champ, which were supported by bootstrap value (93%). *E. malipoensis* Kuan, *E. prinoides* Rehd. & Wils. var. *dadunensis* H.Z. Zhang and *E. japonica* Lindl., formed Clade III, with a high bootstrap support of 98%. Clade IV comprised *E.elliptica* Lindl., *E. bengalensis* Hook. f. and forma *angustifolia* Vidal

(Bootstrap value = 55%). *E. salwinensis* Hand-Mazz formed a monophyletic Clade V. Clade VI comprised *E. deflexa* Nakai, *E. deflexa* Nakai var. *buisanensis* Nakai, *E. kwangsiensis* Chun and *E. serrate* Vidal (Bootstrap value = 58%). The relationship between *E. deflexa* Nakai, and *E. deflexa* Nakai var. *buisanensis* Nakai was very close while *E. deflexa* Nakai, and *E. deflexa* Nakai var.*buisanensis* Nakai were also interrelated closely with *E. kwangsiensis* Chun.

Genetic relationship between *E. cavaleriei* Rehd and *E. fragrans* Champ: The pairwise divergence of ITS sequences using Kimura 2-parameter distance between *E. cavaleriei* Rehd and *E. fragrans* Champ was determined to be 0.003 ± 0.002 (Table 2). While the pairwise divergence of ITS sequences ranged from 0.016 to 1.154 within the other loquat accessions (data not shown). Because of a higher evolutionary rate of ITS sequence and the average substitution rate of ITS fragment of 4.5×10 base substitutions /sites in high plant (Suh *et al.*, 1993), it could be concluded that the two species of *E. cavaleriei* Rehd and *E. fragrans* Champ might be the same species. The study proved the ITS sequences between the two species were hardly different and the microscopic difference of the ITS sequence might be only lie in the same sample among different plant varieties or sequence copies. The analysis of ITS sequences using Kimura 2-parameter distance also supported the close molecular evolution time and relationships between *E. cavaleriei* Rehd and *E. fragrans* Champ.

Discussion

Phylogeny of the *Eriobotyra* Lindl., based on ITS sequence: The entire ITS region length of angiosperms ranged from 565−700 bp, which means that nrDNA ITS sequences of angiosperms were very conserved in length because of its faster concerted evolution rate (Baldwin *et al.*, 1995; Wendel *et al.*, 1995). For the relatively simple nuclear genomes, the sequence of the ITS is of great significance in the phylogenetic and evolutionary studies of many angiosperm taxa at specific sectional and generic levels (Hsiao *et al.*, 1994). Based on ITS sequences data, the relationship was analyzed in *Populus* sections (Shi *et al.*, 2001), *Rhododendron* sections *Azaleastrum* (Ericaceae) (Gao *et al.*, 2003) and *Aconitum delavayi* complex (Ranunculaceae) (Zhang *et al.*, 2003).

Some researchers discussed the close relationships between *Eriobotyra* Lindl., and *Rhaphiolepis indica* (L.) Lindl., (Campbell *et al.*, 2007). However, the phylogenetic relationship between the genus *Eriobotyra* Lindl., and the related genera was not clarified because the genus *Eriobotyra* Lindl., contains only one sample. In this study, we reconstruct phylogeny of the genus *Eriobotyra* Lindl., based on nr DNA ITS sequence (Fig. 2) and found that the genus *Eriobotyra* Lindl., formed a monophyletic group, and *E. serrate* Vidal and *E. kwangsiensis* Chun were basal to the clade of *E. deflexa* Nakai and *E. deflexa* Nakai var.*buisanensis* Nakai, which implied that *E. serrate* Vidal and *E. kwangsiensis* Chun should be primitive relative to Clade VI. The primitive group of *Eriobotyra* Lindl., could be *E. seguinii* Card, *E. henryi* Nakai, *E. malipoensis* Kuan or *E. japonica* Lindl. In brief, in the parsimonious tree based on nr DNA ITS sequences, *Rhaphiolepis indica* (L.) Lindl., formed a robust monophyletic group. The loquat accessions formed a monophyletic group. The strict consensus tree was divided into six lineages. Therefore, the relationship among these species and the systematic positions of the section *Eriobotyra* Lindl., needs to be further investigated.

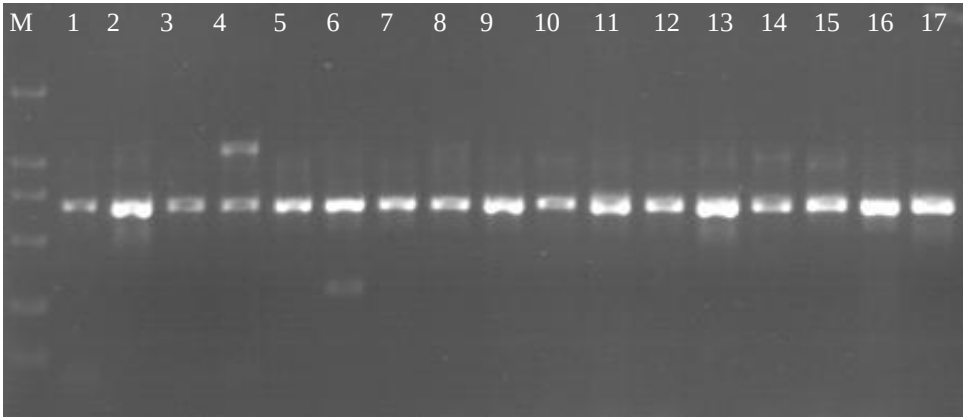

Fig. 1. Products of PCR amplified on ITS sequences of samples on 15 *Eriobotrya* and *Rhaphiolepis indica* (L.*)* Lindl., and *Photinieae serrulaia* L.
From the left to the right: M: DL2000 1. *E.elliptica* Lindl. 2. *E.serrate* Vidal 3. *E. salwinensis* Hand-Mazz 4. *E. deflexa* Nakai var. *buisanensis* Nakai 5. *E. deflexa* Nakai 6. *E. malipoensis* Kuan 7. *E. japonica* Lindl. 8. *E.kwangsiensis* Chun 9. *E. prinoides* Rehd. & Wils. var. *dadunensis* H.Z.Zhang 10. *E.bengalensis* Hook.f. 11. *E.bengalensis* Hook.f. *forma angustifolia* Vidal 12. *E. fragrans* Champ 13. *E. cavaleriei* Rehd 14. *E. seguinii* Card 15. *E. henryi* Nakai 16. *Photinieae serrulaia* L., 17. *Rhaphiolepis indica* (L.) Lindl.

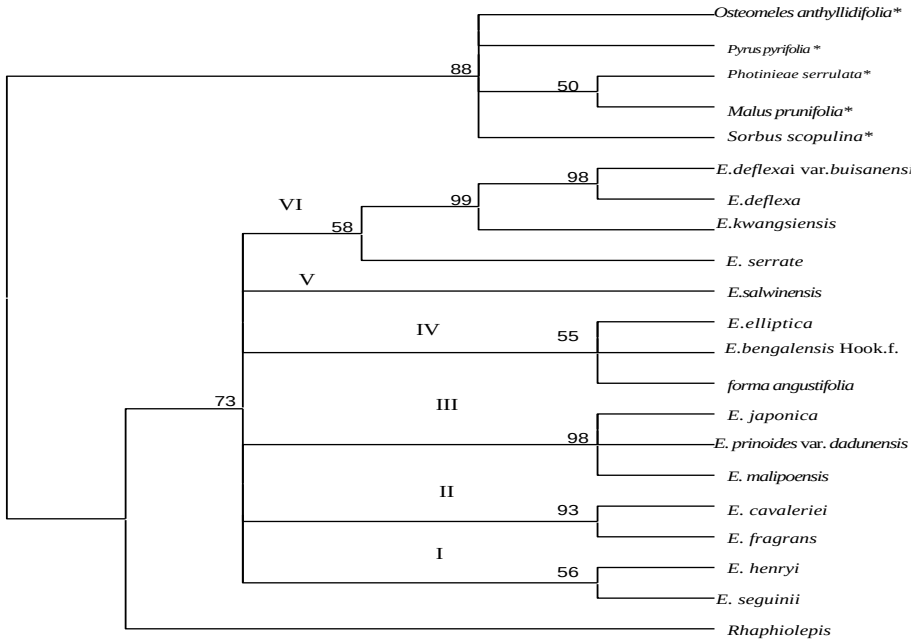

Fig. 2. Majority-rule consensus trees (a bootstrap percentage of >50%) of equally most parsimonious trees based on ITS sequences, with all characters equally weighted (Length=657, *CI*=0.8128, *RI*=0.7953). Numbers in the branches were the bootstrap percentages of 1000 replications. *indicated the outgroup.

Table 2. Pair wise divergence of ITS sequences using Kimura 2-parameter distance.

	E. fragrans Champ	*E. cavaleriei* Rehd
E. fragrans Champ		0.002
E. cavaleriei Rehd	0.003	

Distance values were in the lower left matrix while standard errors were in the upper right matrix.

The genus *Eriobotyra* Lindl., was divided into three large groups, which were mainly characterized by the morphological traits such as pistil, carpel and the size of leaf, as well as the results of RAPD and AFLP (Yang & Lin, 2007). In this system, the genus *Eriobotyra* Lindl., was divided into three groups: Group I, few pistil and small leaf (including *E. seguinii* Card, *E. henryi* Nakai); Group II, more pistil and carpel and big leaf (including *E. japonica* Lindl., *E. malipoensis* Kuan and *E.elliptica* Lindl.) and Group III, more pistil, few carpel and middle leaf, which included 4 subgroups: Yunnan-Sichuan subgroup (*E. prinoides* Rehd. & Wils. var. *dadunensis* H.Z. Zhang and *E. prinoides* Rehd. & Wils. *var. dadunesis*), widely distributed subgroup (*E. cavaleriei* Rehd and *E. fragrans* Champ), pearl river subgroup (*E. kwangsiensis* Chun and *E. deflexa* Nakai), and Western Yunnan subgroup (*E. bengalensis* Hook.f., *Forma angustifolia* Vidal, *E. salwinensis* Hand-Mazz, *E. tengyuehensis* W.W.Smith). *E. seguinii* Card and *E. henryi* Nakai which exhibited few pistils and small leaves were treated as an individual group. With the exception of two species *E. prinoides* Rehd. & Wils. var. *dadunensis* H. Z. Zhang and *E.elliptica* Lindl., whose relationship among the genus *Eriobotyra* Lindl., was supported in the ITS phylogeny in agreement with the report of Yang & Lin (2007).

This study also showed that the distribution pattern of nr DNA ITS sequences was very useful in tracking the evolutionary history of *Eriobotyra* Lindl. In the ITS phylogeny, some species, such as *Rhaphiolepis indica* Lindl., and *E. salwinensis* Hand-Mazz, formed a respective monophyletic clade in the strict consensus tree. This study indicated that *E. salwinensis* Hand-Mazz could play important roles in the evolution of pearl river subgroup and Western Yunnan subgroup. In contrast, *E. deflexa* Nakai, *E. deflexa* Nakai var. *buisanensis* Nakai, *E. kwangsiensis* Chun and *E. serrate* Vidal., formed a clade, which also showed that the species diversification of pearl river subgroup might result from radiation on the river and its vicinity considering extensive nr DNA exchange in this group.

Phylogenetic relationships between *E. cavaleriei* Rehd and *E. fragrans* Champ: In the strict consensus tree, *E. cavaleriei* Rehd and *E. fragrans* Champ formed a robust group with high bootstrap values (93%). This result showed a close phylogenetic relationship between *E. cavaleriei* Rehd and *E. fragrans* Champ. Because of the similar bionomics to *E. cavaleriei* Rehd and *E. fragrans* Champ, the two species could not be classified based only on the morphological data, i.e., the size of leaf under some ecological condition (Yang, 2005). Obviously, it was a tricky matter concerning the genetic distance as the classified level of axonomic treatment and lack of the strict standards that distinguished two accessions with species or varieties based on the difference of DNA sequence. The evolutional interval between *E. cavaleriei* Rehd and *E. fragrans* Champ had little difference in the pairwise divergence of ITS sequences. To clarify this, a further study is needed as the relative fast morphological divergence could result in "reproductive isolation", i.e. the forming of the species.

Because *E. cavaleriei* Rehd was very close to *E. fragrans* Champ (similarity in the level of intraspecific), Yang (2005) suggested that one of them (*E. cavaleriei* and *E.*

fragrans) should be descended to a variety of the other based on molecular analysis in future. This study supported this suggestion based on the data of ITS sequences i.e., the taxonomic treatment of *E. cavaleriei* Rehd as a variety of *E. fragrans* Champ for the following reasons. 1, Traits of species: With the exception of the distinctive difference of the number of carpels, the traits of *E. cavaleriei* Rehd was similar that of *E. fragrans* Champ. The original description of the number of carpels on *E. fragrans* Champ was 4–5, while in *E. cavaleriei* Rehd 2–3. However, sometimes there were 3 carpels on the living plants of *E. fragrans* Champ according to our investigation. Therefore, if the description of the number of carpels on *E. fragrans* Champ was designated to 3–5, *E. cavaleriei* Rehd was then considered to be a variety of *E. fragrans* Champ. 2, Geographical distribution: In general, the distribution regions of *E. cavaleriei* Rehd might be wider than that of *E. fragrans* Champ. While the distribution regions of *E. fragrans* Champ might be much wider than that of *E. cavaleriei* Rehd due to different geographical distribution and ecological characteristics. *Eriobotyra* Lindl., was a genus with a East Asia distribution (70 genera like *Eriobotyra* Lindl., among 278 genera of angiosperms in East Asia). *Eriobotyra* Lindl., is distributed not only in region I (from Japan to Hengduan Mountains Region), but also in region II (from Hengduan Mountains Region to Kashmir). In fact, it is only *E. elliptica* Lindl., and *E. fragrans* Champ that are found to be distributed like this in genus *Eriobotyra* Lindl. Therefore, the geographical distribution pattern of *E. fragrans* Champ., was more complicated than that of *E. cavaleriei* Rehd. 3, *E. fragrans* Champ., was found and named by the taxonomists in 19th century just after *E. japonica*, while *E. cavaleriei* Rehd was found and named in 20th century like most of loquat accessions (Yu, 1974), so *E. cavaleriei* Rehd might not have been named as a species, but a variety or variant, if the results on morphology, geographical distribution and molecular data from DNA had been applied at that time. As mentioned above, it was suggested the taxonomic treatment of *E. cavaleriei* Rehd as a variety of *E. fragrans* Champ.

Acknowledgements

We were grateful to Prof. S.H. Shi and Dr. R.C. Zhou of Zhongshan University, China, and Prof. M. Ashraf of University of Agriculture, Faisalabad, Pakistan. for their valuable comments on the manuscript. This work was supported by Guangdong Provincial Natural Science Foundation (No. 07118121).

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(Received for publication 6 October 2008)