Pakistan J. Zool., vol. 47(5), pp. 1405-1412, 2015.

Genetic Relationships Between *Tribolium castaneum* and *T. confusum* Based on Mitochondrial DNA Sequences

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Abstract.- The flour beetles *Tribolium castaneum* (Herbst) and *T. confusum* (Jacquelin du Val) are economically important, morphologically similar and sympatrically distributed stored-product pests throughout the world. To clarify their genetic relationships, two regions of the mitochondrial DNA, the cytochrome c oxidase subunit I (COI) and the cytochrome b (Cytb), were sequenced for 100 individuals. Three and four haplotypes were identified for the COI and Cytb sequences, respectively, and there were no shared haplotypes between the two species in either sequence. Haplotype sequences within species differed by small numbers of nucleotide substitutions in the COI and Cytb region, and the substitutions did not result in the change of the encoded amino acids in both regions. In contrast, there were many substitutions between haplotypes of *T. castaneum* and *T. confusum* in the COI and Cytb regions, and the substitutions resulted in some encoded amino acid changes. Phylogeny incorporating two other *Tribolium* flour beetle species revealed that, the two species pairs, *T. castaneum* and *T. freemani*, and *T. confusum* and *T. destructor*, were more closely related than *T. castaneum* and *T. confusum*, which was not completely consistent with the phylogenetic relationships inferred from morphological data. Therefore, these results demonstrate that *T. castaneum* and *T. confusum* are genetically distinct from each other even though their morphology and size are very similar, and the COI and Cytb sequences are very useful for quantifying the genetic differentiation and phylogenetic relationships between species, especially morphologically similar species.

Key words: *Tribolium*, flour beetles, COI, Cytb, genetic variability.

INTRODUCTION

The red flour beetle, *Tribolium castaneum* (Herbst), and the confused flour beetle, *T. confusum* (Jacquelin du Val) (Coleoptera: Tenebrionidae), are morphologically similar and sympatrically distributed stored-product pests. The latter is named because of confusion over its identity as it is so similar to the former (Walter, 1990). They are two cosmopolitan pests in flour mills and wherever cereal products and other dried foods are processed or stored and rank among the most important pests inhabiting grain processing plants and storage facilities (Campbell *et al.*, 2004). In laboratory, both the red and confused flour beetle males responded towards their conspecific females and completed the whole courtship sequence (contact, mount and copulation) in a mating area, whereas some males of both species mounted and attempted copulation to heterospecific females (Serrano *et al.*, 2000). Moreover, if confined in vials the two species can hybridize and produce offspring. Therefore, the genetic relationships between *T. castaneum* and *T. confusum* are of interest as both species show great morphological similarities and incomplete reproductive isolations.

Genetic relationships among *Tribolium* beetle species were studied by electrophoretic analysis of isozymes (Wool, 1982), and the results showed that *T. castaneum* and *T. confusum* were more closely related to the North American flour beetle *Tribolium brevicornis* than they were to each other, which was in disagreement with the morphological data. At the DNA level, relationships between some *Tribolium* species were inferred using characteristics of highly repetitive DNAs, their sequence homology and abundance (Ugarkovi *et al.*, 1996), in contrast to the chemotaxonomic phylogeny proposed by Howard (1987). Mestrovic *et al.* (2006) and Angelini and Jockusch (2008) inferred phylogenies of *Tribolium* species based on DNA sequence variations, and their results gave moderate support to the taxonomic phylogeny based on morphological traits. At the same time, their studies suggested that additional sequence data were needed to further resolve the genetic relationships between *Tribolium* species.

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0030-9923/2015/0005-1405 $ 8.00/0

Nucleotide sequences of several gene regions on mitochondrial DNA (mtDNA) have been used for evaluating genetic relationships among species or genetically heterogeneous populations of a single species because they show sufficiently high rates of nucleotide substitution (Havill *et al.*, 2007). In this study, partial regions of the cytochrome c oxidase subunit I (COI) and cytochrome b (Cytb) genes of *T. castaneum* and *T. confusum* were amplified and sequenced, and attributes of nucleotide sequence in each region as well as genetic differentiation between them were investigated. Also, the levels of sequence variation in the COI and Cytb regions of *T. castaneum* and *T. confusum* were compared with that found between other *Tribolium* flour beetle species to reveal their phylogenetic relationships.

MATERIALS AND METHODS

Beetles collection

Adult *T. castaneum* and *T. confusum* were each collected from wheat mills at five locations in China: Huhhot in northern China, Xi'an in central China, Chengdu in southern China, and Liaocheng and Xuzhou in eastern China. To minimize the possibility of collecting identical populations, the minimum distance between two sites was 500 km; the maximum distance was 1800 km. For each population, we randomly sampled 10 individuals and they were identified based on their morphological characteristics using the taxonomic key of Bousquet (1990).

DNA extraction

Adults of both beetle species were individually ground into fine power with a mortar and pestle, and total DNA was extracted from single beetle according to the procedure of Ming *et al.* (2014). After air-drying DNA, it was re-suspended in 20 µl ultrapure water and then stored at -20°C.

PCR amplification and sequencing

The primers used in both amplification and sequencing were COIF (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') and COIR (5'-ATC TCC CAC ATT ATT AGA CAA G-3') (Simon *et al.*, 1994) for the COI gene, and CytbF (5'-TAT GTA CTA CCA TGA GGA CAA ATA TC-3') and CytbR (5'-ATT ACA CCT CCT AAT TTA TTA GGA AT-3') (Jermiin and Crozier, 1994) for the Cytb gene, and they were synthesized by Generay Biotech Co. Ltd. (Shanghai, China). PCR amplification was carried out in a 30 µl reaction volume containing 2× PCR Reaction Mix (TIANGEN, Beijing, China), 0.4 µM of each primer, 0.6 unit Taq DNA polymerase (TIANGEN, Beijing, China), 1.3 µl of DNA extraction, and 11 µl of ultrapure water in a little genius thermal cycler (BIOER, Hangzhou, China). The thermal cycling profile was as follows: 94°C for 5 min, followed by 33 cycles of denaturation at 94°C for 50 s, annealing at 55°C (COI) and 51°C (Cytb) for 45 s and extension at 72°C for 1 min, with a final extension for 10 min at 72°C. The PCR products were electrophoresed on 0.8% agarose gel, stained with ethidium bromide (0.5 µg/ml), and visualized under a UV transilluminator in a LG2020 Gel Documentation System (LongGene, Hangzhou, China). Fifty PCR product samples of each species on either gene were sent to the Invitrogen Trading DNA Sequencing Facility (Shanghai, China) for direct sequencing.

Sequence analysis

COI and Cytb sequences of *T. castaneum* and *T. confusum* were aligned using Clustal X version 2.0 (Larkin *et al.*, 2007), and the same sequences were grouped under one haplotype. Haplotype sequences were further edited and checked according to the derived amino acid sequences, and nucleotide composition, nucleotide substitutions, inter- and intra-specific genetic variations and genetic distances for *T. castaneum* and *T. confusum* were calculated based on them by using MEGA 5.05 (Tamura *et al.*, 2011). The phylogenetic analyses based on the neighbor-joining (NJ) and maximum likelihood (ML) methods were performed separately for the COI and Cytb regions using MEGA 5.05 (Tamura *et al.*, 2011). In the phylogenetic analyses, two other flour beetle species *Tribolium destructor* and *Tribolium freemani* and one beetle species *Zophosis punctata* as an outgroup species were also used, and their sequences were published in GenBank under accession number FJ743723, FJ743724 and FM876573, respectively.

Table I.- Summary of nucleotide composition for the mitochondrial gene sequences of *T. castaneum* (CS) and *T. confusum* (CF).

Gene sequences	Species*	Base pairs	N ucleotide %				
			A	T	C	G	A+T
COI	CS h1	738	29.0	32.7	22.8	15.6	61.7
	CS h2	738	29.0	32.8	22.6	15.6	61.8
	CF h1	738	31.8	32.9	21.0	14.2	64.7
Cytb	CS h1	432	30.8	34.0	24.3	10.9	64.8
	CS h2	432	30.6	33.8	24.5	11.1	64.4
	CF h1	432	30.3	37.3	20.6	11.8	67.6
	CF h2	432	30.6	37.3	20.6	11.6	67.9

* Acronym "h" in the names of species denotes haplotype numbers.

Table II.- Statistics for nucleotide substitutions between *T. castaneum* and *T. confusum* in the mitochondrial gene regions.

Gene sequences	Position	Variable sites	Transitions (ti)		Transversions (tv)				ti/tv ratio
			A-G	C-T	A-C	A-T	C-G	G-T	
COI	all	144	25	46	27	39	4	3	0.97
	1st pos	30	5	12	6	6	0	1	1.31
	2nd pos	9	2	4	0	1	2	0	2.00
	3rd pos	105	18	30	21	32	2	2	0.84
Cytb	all	92	11	47	8	23	0	3	1.71
	1st pos	21	3	10	3	4	0	1	1.63
	2nd pos	7	0	4	0	3	0	0	1.33
	3rd pos	64	8	33	5	16	0	2	1.78

RESULTS

The aligned sequence lengths of the partial COI and Cytb regions analyzed were 738 and 432 bp, respectively. No insertions or deletions were found in either region. Three haplotypes in the COI region and four in Cytb were detected among 100 individuals of *T. castaneum* and *T. confusum*, and there were one to two haplotypes per species and no shared haplotypes between the two species. All seven sequences had been deposited in GenBank DNA databases (Accession numbers: KC139705-KC139711).

Attributes of nucleotide sequences

In the COI region, the average nucleotide composition (A, T, C, G) of *T. castaneum* and *T. confusum* was 29.9%, 32.8%, 22.1%, 15.1%, and in Cytb it was 30.6%, 35.6%, 22.5%, 11.3% (Table I). The average A+T contents were 62.7% in COI and 66.2% in Cytb, and both sequence regions showed AT-rich nucleotide composition. In Cytb, the transitions (ti = 58) were more frequent than transversions (tv = 34), and in COI, the transitions (ti = 71) and transversions (tv = 73) were approximately equal, which showed a higher average transition/transversion (ti/tv) ratio in Cytb than in COI (Table II). Of the 144 variable positions in COI, 73.9% were at third codon positions, 20.8% at first, and 6.3% at second, and of the 92 variable positions in Cytb, 69.6% were at third codon positions, 22.8% at first, and 7.6% at second, which demonstrated that in both regions the third codon positions were most variable while the second codon positions were most conserved (Table II).

Of the 738 sites in the COI region and 432 in Cytb, 594, 144, 144, 0, and 340, 92, 92, 0 were conserved, variable, parsimony informative, and singleton, respectively, and there were a higher proportion of variable sites in Cytb (21.3%) than in

A

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[                    1111 1111111122 2222222222 2222223333 3333333333 ]
[       2344445 6677991223 3444789900 0011134455 6667780011 2334445567 ]
[    3674902381 3658064035 8147430214 7836956925 1573650925 1092581763 ]
 CS1 AACAGCCTTT CTTCCTTCAT CACGTCATCT TAAGACGGAA AATATTCCCA TCTTAGCCGT
 CS2 .......... .......... ........T. .......... .......... ..........
 CF  CCTCATACGC ACCTTCAATA TCTACATAAC ACTATTAATT TGACCCTTTT CTACGATTAC

[    3344444444 4444444444 4555555555 5555555555 5555566666 6666666666 ]
[    8800000022 3334445689 9002233344 4445566668 8999900111 2222234555 ]
[    1823456919 0122343502 3475846701 2393812470 8127869028 0245702124 ]
 CS1 TTCTCTACTG GCGACCGAGC CGACAGATCC TACAGAATGT TTATTTCCCG GTCACCCCCT
 CS2 .......... .......... .......... .......... .......... ..........
 CF  CCAGTAGACA ATCGTAAGAA TATTTTTCAA CTTTCTGAAA AACCCCAAAA CAATATTATA

[    6666666666 6777777777 7777]
[    6667788899 9000112222 3333]
[    3692967803 9678173679 2578]
 CS1 ACATCGACTT TTCTCGAACT ATAG
 CS2 .......... .......... ....
 CF  TACCTATAAA AAGATATGTA TAGA
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B

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[      14444556 6677888990 0122333355 5556888999 001122]
[    3630258570 3625478692 8736235803 4675369056 173958]
 CS1 CCTTATTATA GGACTTCACC CTATTTACCC CCTTCTACCT TCCTTC
 CS2 .....C.... .......... .......... .......... ......
 CF1 TACAT.GGAG ATTTAATGTT TCTCAGTTTT TACCTCTTAA CTTCCT
 CF2 TACAT.GGAG ATTTAATGTT TCTCAGTTTT TACCTCTTAA CTTCCT

[    2222222222 2222222222 2222333333 3333333333 334444]
[    3444566777 7777888888 8999011222 3333344556 890013]
[    7023401123 6789035678 9478103147 0356989176 802812]
 CS1 TAATCACTTA AATTTACAAC TCTCGACCGC CCCTTACAAC CCCTGA
 CS2 .......... .......... .......... .......... .....G
 CF1 CTTCTTTACG TTCACGTCTT CTAAACTTAT TTTCATTGTT TAACAG
 CF2 CTTCTTTACG TTCACGTCTT CTAAACTTAT TTTCATTGTT TAACA.
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Fig. 1. Variable sites of two mitochondrial gene sequences of *Tribolium castaneum* (CS) and *Tribolium confusum* (CF). **A**, COI, and **B**, Cytb. The top numbers in square brackets denote base sites that are relative to the consensus sequence established here. Arabic numerals in the names of species denote haplotype numbers.

COI (19.5%). The average genetic distances between *T. castaneum* and *T. confusum* in the COI and Cytb regions were 0.227 and 0.253 respectively. By contrast, the intraspecific genetic distances within *T. castaneum* and *T. confusum* were 0.001 and 0 in the COI region, 0.003 and 0.001 in Cytb. Therefore, when variability and genetic distance of two sequences from *T. castaneum* and *T. confusum* were compared, the evolutionary rate in the Cytb region appeared to be slightly higher than in COI.

Genetic variation within and between species

Haplotype sequences within species differed from each other by no more than two nucleotide substitutions in the COI and Cytb regions (Fig. 1), and the substitutions did not result in the change of encoded amino acids (Fig. 2). In contrast, the numbers of nucleotide substitutions between haplotypes *T. castaneum* and *T. confusum* were very large, 144 nucleotide substitutions for the 738-bp COI fragment (Fig. 1A) and 90 to 92 for the 432-bp

A

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CS1 GTLGMIYAMM AIGLLGFVVW AHHMFTVGMD VDTRAYFTSA TMIIAVPTGI  [ 50]
CS2 .......... .......... .......... .......... ..........  [ 50]
CF  .......... .......... .......... .......... ..........  [ 50]

CS1 KIFSWLATLH GTQINYSPSM MWALGFVFLF TVGGLTGVIL ANSSIDIMLH  [100]
CS2 .......... .......... .......... .......... ..........  [100]
CF  .......... ...L.....L I......... ........V. ..........  [100]

CS1 DTYYVVAHFH YVLSMGAVFA IMAGLVHWFS LFTGSTLNPK LCKAQFLTMF  [150]
CS2 .......... .......... .......... .......... ..........  [150]
CF  .......... .......... .........P ....VAM... ...I...V..  [150]

CS1 MGVNLTFFPQ HFLGLSGMPR RYSDYPDAYT LWNIISSIGS IISLIGVMFF  [200]
CS2 .......... .......... .......... .......... ..........  [200]
CF  .......... .......... ........F. T...F..V.. ...M...L.L  [200]

CS1 IFILWEGFTS SRKTIIPLNM TSSIEWLQSL PPAEHSYSEL PMLSSK [246]
CS2 .......... .......... .......... .......... ......  [246]
CF  ..MM..AMS. .......... ........NM .......... .....S [246]
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B

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CS1 ITNLLSAIPY LGTSIVQWIW GGFAVDNATL TRFFSFHFLL PFIVSAMVMI  [ 50]
CS2 .......... .......... .......... .......... ..........  [ 50]
CF1 .......... ...T....V. .......... .......... ....A.....  [ 50]
CF2 .......... ...T....V. .......... .......... ....A.....  [ 50]

CS1 HLLFLHQTGS NNPLGLNSNI DKIPFHPYFS YKDIAGYLIM LMILINLSLL  [100]
CS2 .......... .......... .......... .......... ..........  [100]
CF1 .......... .....M.... .......... F...V.F... TIS.VL...M  [100]
CF2 .......... .....M.... .......... F...V.F... TIS.VL...M  [100]

CS1 DPYMLGDPDN FTPANPLVTP VHIQPEWYFL FAYAILRSIP NKLG [144]
CS2 .......... .......... .......... .......... ....  [144]
CF1 N..L...... .I........ .......... .......... ....  [144]
CF2 N..L...... .I........ .......... .......... ....  [144]
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Fig. 2. Amino acid sequence alignment of two mitochondrial genes of *Tribolium castaneum* (CS) and *Tribolium confusum* (CF). **A**, COI, and **B**, Cytb. Symbols '.' indicate conserved amino acid residues. Numbers in square brackets indicate the number of amino acid residues, and the amino acid numbering is relative to the consensus sequence established here. Arabic numerals in the names of species denote haplotype numbers.

Cytb fragment (Fig. 1B). Moreover, the nucleotide substitutions between species resulted in 25 encoded amino acid changes in the COI region (Fig. 2A) and 16 in Cytb (Fig. 2B).

Phylogenetic analysis

Since tree topologies obtained from COI and Cytb data and those by neighbor-joining (NJ) and maximum likelihood (ML) methods on the Kimura two-parameter distances for phylogenetic analysis were almost consensus, only the result of NJ method using the COI data was shown in Figure 3. In the genus *Tribolium*, two groups were formed with bootstrap confidence (<50%): one consisted of *T. castaneum* and *T. freemani*, and the other consisted of *T. confusum* and *T. destructor*. Within the two groups, the relationships between the species (*T. castaneum – T. freemani* and *T. confusum –T. destructor*) were clearly resolved.

DISCUSSION

Three specific questions were addressed to explore the genetic relationships between *T.*

castaneum and *T. confusum*: 1) the attributes of nucleotide sequence from COI and Cytb region; 2) the levels of genetic variation; and 3) the consistency of phylogenies from DNA sequences and morphological data.

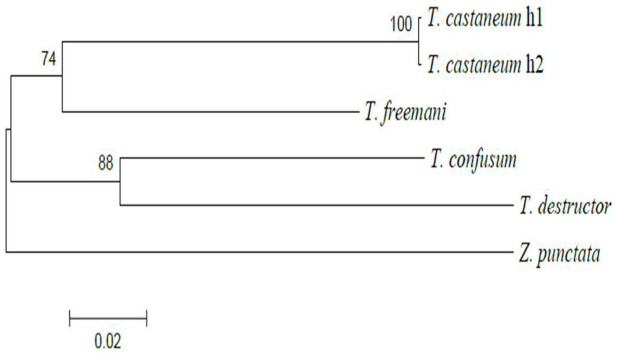

Fig. 3. Neighbour Joining (NJ) phylogenetic tree of four *Tribolium* flour beetle species and one beetle species *Zophosis punctata* as an outgroup inferred from DNA sequences of mitochondrial COI gene. Acronym "h" in the names of *Tribolium castaneum* denotes haplotype numbers. Numbers on the branches represent bootstrap values obtained from 1000 replications (only values greater than 50% are shown).

First, the attributes of nucleotide sequences were summarized in Tables I and II. The AT contents of the Cytb and COI regions were relatively high in both *Tribolium* species, which was typical of insect mitochondrial genomes (Boore, 1999). Crozier and Crozier (1993) reported an overall AT content (84.9%) in the honeybee *Apis mellifera* based on complete mtDNA genome, and it has been cited to be the most AT biased insect mtDNA genome sequenced (Simon *et al.*, 1994). In fact, Silvestre *et al.* (2008) reported an overall higher AT bias (86.7%) in the stingless bee *Melipona bicolor* mtDNA. One hypothesis that attempted to explain this bias was that the DNA polymerase could use those bases in a more efficient way during mtDNA replication (Clary and Wolstenholme, 1985), and the lower energetic cost to break the A-T links during mtDNA replication and transcription would generate AT bias on organisms that rely on mitochondrial efficiency to keep a high metabolic rate (Xia, 1996).

Although sequence analysis revealed a higher average ti/tv ratio in Cytb (1.71) than in COI (0.97), the ti/tv bias had been noted to occur in both regions, which was in accordance with the previous reports on mtDNA in insect (Brown *et al.*, 1994; Sperling and Hickey, 1994). The ratio becomes 0.5 when there is no bias towards either transitional or transversional substitution because, when the two kinds of substitution are equally probable, there are twice as many possible transversions as transitions.

Second, emphasis was given to the genetic differentiation between *T. castaneum* and *T. confusum*. This study revealed that, based on the partial sequence information of both COI and Cytb genes, both *Tribolium* species were genetically distinct from each other at the nucleotide and amino acid sequence levels even though they show great morphological similarities. This suggested that the two regions are highly differentiated and might be used diagnostically to distinguish the two species.

Fumigants and other chemical pesticides are widely used to protect stored commodities from their infestations and contamination, and studies have shown that, not only are immature stages (eggs, larvae and pupae) of *T. castaneum* and *T. confusum* more susceptible to chemical pesticides than their adults, but *T. castaneum* is more susceptible to chemical pesticides than *T. confusum* (Arthur *et al.*, 2009). To reduce pesticide use and improve the level of pest management programs, it is important for a pest manager to identify and control them in immature stages. However, for the mixed populations of *T. castaneum* and *T. confusum*, eggs, larvae and pupae are virtually indistinguishable according to their morphological characteristics. As shown here, either sequence of COI and Cytb genes successfully differentiated adult individuals of *T. castaneum* and *T. confusum* but the assay could be used with the larvae and pupae ones because genome DNA did not vary between adults and immature forms.

Third, this study focused on phylogenetic analysis of *T. castaneum* and *T. confusum* incorporating two other *Tribolium* species. The molecular phylogenetic analysis confirmed, in agreement with the molecular analysis of Mestrovic *et al.* (2006) and Angelini and Jockusch (2008), the two species pairs, *T. castaneum* and *T. freemani*,

and *T. confusum* and *T. destructor*, were more closely related than *T. castaneum* and *T. confusum*, which was not completely consistent with the morphological data analysis described below.

Morphology and size of *T. castaneum* and *T. confusum* are most similar in the *Tribolium* flour beetles (Bousquet, 1990). The destructive flour beetle *T. destructor* is similar in appearance to the red flour beetle *T. castaneum* and the confused flour beetle *T. confusum*, but *T. destructor* is much darker than both them (Walter, 1990). In contrast, the flour beetle *T. freemani* is significantly different from *T. castaneum*, *T. confusum* and *T. destructor* by its large size: it is almost three times as massive as them (Brownlee and Sokoloff, 1988; Wade *et al.*, 1994).

CONCLUSIONS

T. castaneum and *T. confusum* are genetically distinct from each other even though their morphology and size are very similar, and the COI and Cytb regions are very useful for quantifying the genetic differentiation and phylogenetic relationships between species, especially morphologically similar species. In addition, the molecular phylogeny is not completely consistent with the phylogenetic relationships inferred from morphological data. In order to reveal the "true" phylogeny of this important flour beetle-genus, we strongly hope that this study will serve as a stimulus for the *Tribolium*-oriented community to initiate a more thorough phylogeny investigation by integrating molecular, morphological and reproductive isolation data.

ACKNOWLEDGMENTS

We thank Yong-Hong Pang for assistance in this work and Dr. Thomas W. Phillips (Kansas State University) for helpful comments on the manuscript. This work was supported by the National Natural Science Foundation of China (Grant No. 31172159), the Scientific Research Foundation for the Returned Overseas Chinese Scholars of State Education Ministry and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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(Received 30 December 2014, revised 22 May 2015)