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An Identification in Fish of the Genus *Puntius* Hamilton 1822 (Cypriniformes:Cyprinidae) of Some Wetlands in Northeast Thailand With the Use of Random Amplified Polymorphic DNA Technique

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Abstract: The experiment was carried out during the 2003 to 2006 at the Department of Fisheries, Khon Kaen University, Khon Kaen, Thailand in collaboration with the Department of Biosciences, the University of Hertfordshire, College Land, Hatfield, Herts, UK. Molecular RAPD technique was used for the determinations of DNA patterns of the fish genus *Puntius* Hamilton 1822. The fish samples of 1,500 individual fish were collected from fifteen wetlands in Northeast Thailand and they were used for DNA extraction. Before the experiment was carried out the fish samples were morphologically identified and it was found that the collected fish consisted of 9 species i.e., *Puntius altus*, *P. aurotaeniatus*, *P. binotatus*, *P. gonionotus*, (e) *P. leiacanthus*, *P. orphoides*, *P. partipentazona*, *P. schwanenfeldi* and *P. wetmorei*. Genomic DNAs were extracted from 5 mg of muscle tissues (skeleton muscles) with the use of PUREGENE™ DNA Isolation Kit for Laboratory Use, Gentra Systems, USA. Eighty decamer primers from four kits were subjected to a preliminary test. It was found that only 10 decamer primers were most suited for this PCR amplification. The results showed that genetic distant values being established among and between pairs of the fishes of the 9 fish species ranged from 0.191 to 0.456 for a pair between *Puntius gonionotus* and *Puntius altus* and a pair between *Puntius schwanenfeldi* and *Puntius leiacanthus*, respectively. Similarity coefficient values within the 9 fish species ranged from 0.109 to 0.231. The results on a Dendrogram of clusters showed that there were 5 minor groups of the 9 fish species but the 9 species could not be split or shifted into other genera of the fish due to small differences found within the values of similarity coefficients.

Key words: Fish species, genetic distances, *Puntius* Hamilton 1822, RAPD, similarity coefficients, wetlands

INTRODUCTION

With the work of Champasri *et al.* (2007) on fish of the genus *Puntius* Hamilton 1822 where the fish were morphologically identified and the results revealed that the fish of this genus could not be split or shifted into other genera as previously stated by Rainboth (1996a, b) where he did give out another three genera for the fish of the genus *Puntius* Hamilton 1822. In order to justify the results of the work being carried out by Rainboth (1996a, b) and Champasri *et al.* (2007), it may be of tangible value to identify the fish of this genus again with the use of a molecular technique (Random Amplified Polymorphic DNA Technique or RAPD) where DNAs of the fish are used in the laboratory in order to search for their identities in terms of similarities where the results could provide adequate information if the results found with the use of molecular technique could confirm the results being carried out by morphological technique as reported by Rainboth (1996a, b) and Champasri *et al.* (2007). Thus there is an urgent need to carry out more works on this particular respect. Long before RAPD technique was used, fish taxonomists have morphologically classified the various types of fish into their respective genera, families and species, i.e. they used, more or less, morphological characteristics of fishes as tools to identify fishes for their systematic identification, hence such information with respect to fish taxonomic identifications were accumulated.

For the past 30 years, it has been advocated that molecular technologies have contributed largely on a large number of DNA-based markers capable of revealing genetic variation in many species (Isabel *et al.*, 1999). It has been advocated that RAPD-PCR technique is a simple

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and straightforward method where the work based on amplification of discrete regions of genome by using arbitrary primers (Williams *et al*., 1990). Several authors have used RAPD markers to evaluate genetic variability and structure of a variety of species of Neotropical fish in the rivers of South America e.g., Almeida *et al.* (2001), Wasco and Galetti (2002), Leuzzi *et al.* (2004) and Matoso *et al.* (2004). Molecular markers could also be applied for use in identifying different types of populations of fish such as species, hybrid identification, phylogeny and many others (Almeida and Sodre, 2002). Other workers have also used Random Amplified Polymorphic DNA (RAPD) technique for their investigations such as Welsh and McClelland (1990), Williams *et al*. (1990), Hassanien *et al.* (2004) and Toth *et al.* (2005). Therefore, it may be of important value to investigate further if the results found with the use of RAPD technique could profoundly confirm the results being carried out with the use of morphological technique of Champasri *et al.* (2007) where they stated that the fish of the genus *Puntius* Hamilton 1822 could not be classified into other different genera as stated by Rainboth (1996a, b).

MATERIALS AND METHODS

The experiment was carried out at the Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand in collaboration with the Department of Biosciences, University of Hertfordshire, UK during the 2003 to 2006. The fish samples were collected from fifteen different wetlands in Northeast Thailand. The fifteen locations include provinces of (1) Kalasin, (2) Yasothon, (3) Udon Thani, (4) Chaiya Phume, (5) Sisaket, (6), Mahasarakham, (7) Sakon Nakhon, (8) Roi-Et, (9) Nong Khai, (10) Nakhon Phanom, (11) Mukdaharn, (12) Nakhon Ratchasima, (13) Khon Kaen, (14) Nong Bua Lampoo and (15) Loei. The individual fish samples were collected with the use of trawl, gillnets, seine nets, hand-line and bamboo trap. The fish samples were morphologically identified and they consisted of nine species i.e., *P. altus*, *P. aurotaeniatus*, *P. binotatus*, *P. gonionotus*, *P. leiacanthus*, *P. orphoides*, *P. partipentazona*, *P. schwanenfeldi* and *P. wetmorei*. The collected fish samples were kept in liquid nitrogen containers with the use of the method of Chanpasri *et al.* (2007) and then the fish samples were used for DNA extraction. The laboratory works on DNA analysis were carried out at the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, 40002 Thailand. Genomic DNAs were extracted from 5 mg of muscle tissues (skeleton muscles) with the use of the method of PUREGENE™ DNA Isolation Kit for Laboratory Use, Gentra systems, USA (Gentra Systems Inc., 2000). DNAs were quantified using a spectrophotometer and then stored in a deep freezer at -20°C.

Polymerase Chain Reaction (PCR) amplification and gel electrophoresis: Initially, 80 decamer primers from four kits i.e., 20 decamer primers from each kit of A, C, E and Y of random sequences (Operon Technologies, Inc., Alameda, USA) were used. The work started from the screening of samples i.e., the searching for suitable primers where two randomly chosen fish samples from each population were subjected to PCR amplification. This was carried out in order to attain suitable polymorphic bands of DNAs for the nine fish species being used for this investigation (Bardakci and Skibinski, 1994; Barman *et al*., 2003; Liu and Cordes, 2004). It was found that only 10 decamer primers were suited most, thus the ten primers were used for this work. They include OPA-03, OPA-04, OPA-13, OPC-02, OPC-05, OPC-09, OPE-03, OPY-01, OPY-02 and OPY-11. RAPD amplification reactions were maintained followed the method of Williams *et al.* (1993). The RAPD profiles were generated from total genomic DNAs where PCR protocol for RAPD analysis was mixed with 1.0 unit of Ampli Taq DNA polymerase, 2.5 μL of 10x Taq buffer, 2.0 mM each of dNTPs (dATP, dCTP, dGTP and dGTP), 100 pmol (0.1 mM) of primer, 2.0 mM of $MgCl_2$ and 50 ng (1 μL) of genomic DNA to form a final volume of 25 μL, i.e., the samples were added with a high purification of distilled water (three times in distilling distilled water to attain the utmost water purification). PCR amplification was carried out in a Thermal Cycler machine (Corbett Research, Netherlands). Firstly preheated at 94°C for 2 min (step 1, denature) followed by 43 cycles of 1 min denaturation at 94°C and another 1 min at 36°C and then elongation or extension for 2 min at 72°C (step 2, annealing) and finally 7 min at 72°C was carried out to allow a complete extension of all amplified fragments (step 3, additional extension). For every PCR cycle, one PCR tube of negative control must not bear any DNA content (not being contaminated). This was carried out to avoid any possible contamination of other DNAs in each set of the PCR amplification.

Approximately 10 μL of amplification product plus 3 μL of loading dye were resolved in 2.0% agarose gel and then the sample was assayed by electrophoresis in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA) with pH value of 8.0 at 100 volt for 40 min. Finally, the gels were stained with ehtidium bromide (0.5 μg mL^{-1}) for 10 min and then viewed under UV light gel-document (SYNGENE, The United Kingdom) where the GeneSnap software (installed within the UV light gel-document) was used to identify photographs of gels and then the software gave out scores of DNA bands. DNA bands were recorded (the score of 1 was used for the presence of

DNA band and the score of 0 was used for the absence) and then transformed the reading scores of DNA bands from GeneSnap to XLS file and finally the file was subjected to NTSYS-pc 2.10 programme (Rohlf, 2000) for further calculations to attain similarity index values and genetic distances. At the final stage of calculations, both similarity index values and genetic distances were calculated further through a SAS Computer Programme (Littell *et al.*, 2002) for multivariate analysis of variance (MANOVA) where the attained statistical values were used for discriminatory effectiveness of the analysis.

RESULTS

The nine collected species of fish of the genus *Puntius* Hamilton 1822: The collected species of fish from 15 different sites (provinces) in Northeast Thailand consisted of 9 nominal species. The 9 nominal species were identified into their respective species along with names of the authors of the published works and their respective numbers of the harvested locations (Fig. 1-9), i.e., 1 = *Puntius altus*, 2 = *P. aurotaeniatus*, 3 = *P. binotatus*, 4 = *P. gonionotus*, 5 = *P. leiacanthus*,

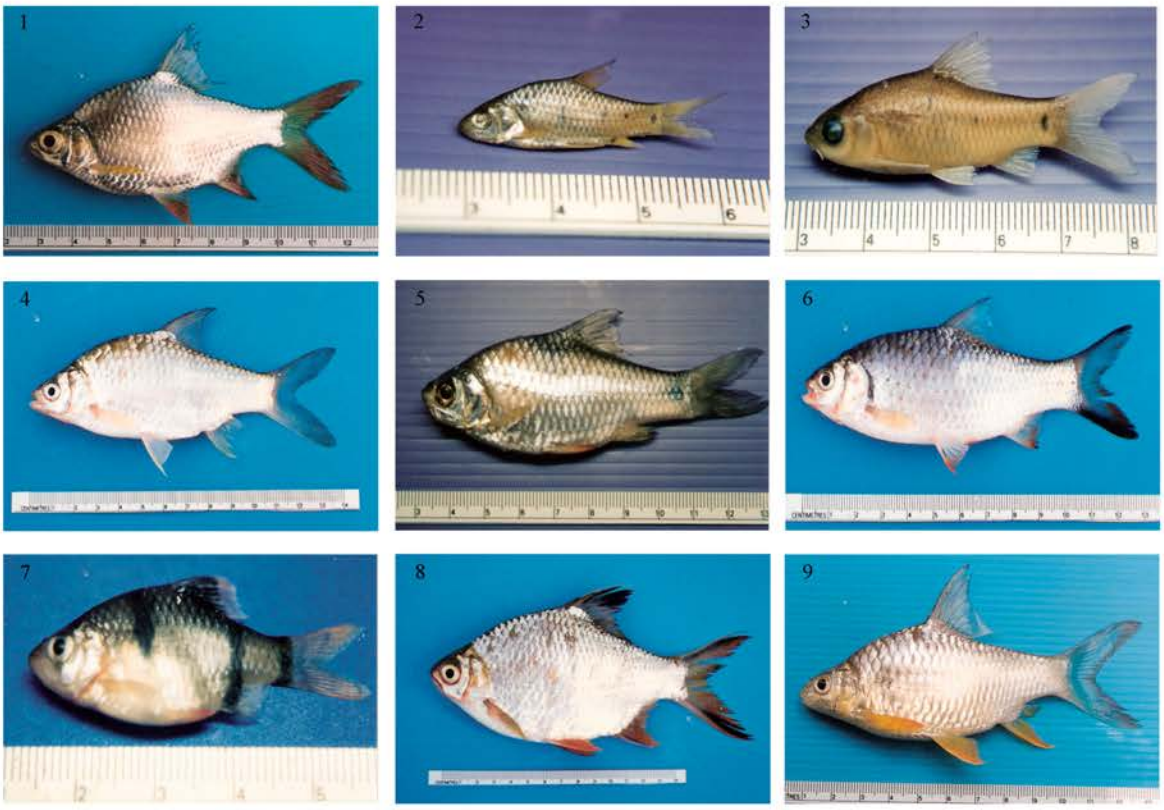

Fig. 1-9: Photographs of the nine species of fish, genus *Puntius* Hamilton 1822 with their respective numbers of the locations of the 15 wetlands (provinces) in Northeast Thailand: 1 = *Puntius altus* (Gunther, 1868), harvested from locations 3, 9, 10, 11 and 13; 2 = *Puntius aurotaeniatus* (Tirant, 1885) harvested from locations 1, 3, 6, 11 and 15; 3 = *Puntius binotatus* (Valenciennes, 1842) harvested from locations 3, 7, 9, 11 and 14; 4 = *Puntius gonionotus* (Bleeker, 1850) harvested from locations 1, 3, 6, 9 and 13; 5 = *Puntius leiacanthus* (Bleeker, 1860) harvested from locations 2, 4, 5, 7 and 9; 6 = *Puntius orphoides* (Valenciennes, 1842) harvested from locations 6, 8, 12, 14 and 15; 7 = *Puntius partipentazona*. (Fowler, 1934) harvested from locations 1, 3, 4, 8 and 13; 8 = *Puntius schwanenfeldi* (Bleeker, 1853) harvested from locations 3, 9, 10, 11 and 13; 9 = *Puntius wetmorei* (Smith, 1931) harvested from locations 1, 9, 10, 11 and 13. Numbers of locations: 1 = Kalasin; 2 = Yasothorn; 3 = Udonthani, 4 = Chaiyaphum, 5 = Srisaket, 6 = Mahasarakham, 7 = Sakonnakhon, 8 = Roi-Et, 9 = Nong Khai, 10 = Nakhonphanom, 11 = Mukdahan, 12 = Nakhonratchasima, 13 = Khon Kaen, 14 = Nongbualumphu and 15 = Loei

Table 1: Genetic distances among the nine fish species of the genus *Puntius* Hamilton 1822 being harvested from fifteen wetlands in Northeast Thailand during the years 2003 to 2006

Fish species	1	2	3	4	5	6	7	8	9
1	0.0000								
2	0.1989	0.0000							
3	0.2896	0.1809	0.0000						
4	0.3397	0.2895	0.3117	0.0000					
5	0.2461	0.1911	0.2971	0.3979	0.0000				
6	0.3145	0.2640	0.3016	0.4316	0.3383	0.0000			
7	0.3295	0.2430	0.2758	0.3605	0.3630	0.3064	0.0000		
8	0.3468	0.2834	0.3659	0.3579	0.3560	0.3614	0.3137	0.0000	
9	0.3793	0.2421	0.3376	0.4076	0.3311	0.4562	0.3670	0.3616	0.0000

Where 1 = *Puntius wetmorei*, 2 = *P. altus*, 3 = *P. aurotaeniatus*, 4 = *P. binotatus*, 5 = *P. gonionotus*, 6 = *P. leiacanthus*, 7 = *P. orphoides*, 8 = *P. partipentazona* and 9 = *P. schwanenfeldi*

6 = *P. orphoides*, 7 = *P. partipentazona*, 8 = *P. schwanenfeldi* and 9 = *P. wetmorei*. These nine nominal fish species possess a similar body structure with respect to their morphological identifications where the identification followed the method of Smith (1945), Rainboth (1996a, b) and Champasri *et al.* (2007) although the sizes of the fish were not of the same sizes.

Genetic distances of the nine fish species: With the results on genetic distances (Nei and Li, 1979), the nine fish species possessed genetic distant values ranged from 0.191 to 0.456 for two pairs of fishes i.e., between *Puntius gonionotus* and *Puntius altus* and between *Puntius schwanenfeldi* and *Puntius leiacanthus*, respectively (Table 1). The differences among the nine species were relatively small and not statistically significant.

Distribution of similarity coefficients of the nine fish species: Amongst the nine fish species of the genus *Puntius* Hamilton 1822, when displayed separately into species, it was found that the highest value of similarity coefficient was found with *Puntius orphoides* followed by *Puntius aurotaeniatus*, *Puntius wetmorei*, *Puntius gonionotus*, *Puntius schwanenfeldi*, *Puntius binotatus*, *Puntius leiacanthus*, *Puntius altus* and *Puntius partipentazona* with values of 0.231, 0.214, 0.188, 0.175, 0.170, 0.152, 0.143, 0.132 and 0.109, respectively (Fig. 10). Similarity coefficients of individual fish species were relatively small and not statistically significant.

Dendrogram structure of clusters of the nine fish species: The results of UPGMA (un-weighted pair-group method) revealed that there were five different groups of clusters of the nine fish species i.e., group 1 consisted of *Puntius gonionotus*, group 2 include *Puntius leiacanthus* and *Puntius orphoides*, group 3 include *Puntius partipentazona* and *Puntius schwanenfeldi*, group 4 consisted of *Puntius altus* and *Puntius binotatus* and finally group 5 include *Puntius wetmorei* and *Puntius aurotaeniatus*. Nonetheless, similarity coefficient values

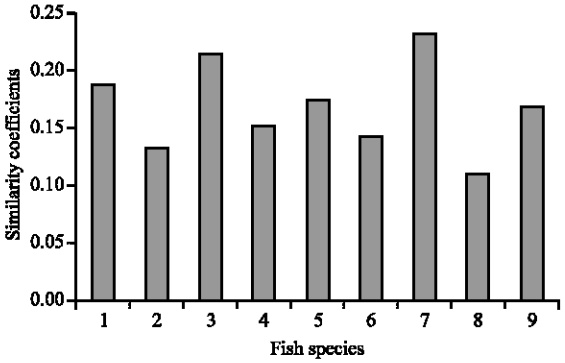

Fig. 10: The distribution of similarity coefficients of the nine fish species of the genus *Puntius* Hamilton 1822 being harvested from fifteen wetlands (provinces) in Northeast Thailand. Where 1 = *Puntius wetmorei*, 2 = *P. altus*, 3 = *P. aurotaeniatus*, 4 = *P. binotatus*, 5 = *P. gonionotus*, 6 = *P. leiacanthus*, 7 = *P. orphoides*, 8 = *P. partipentazona* and 9 = *P. schwanenfeldi*

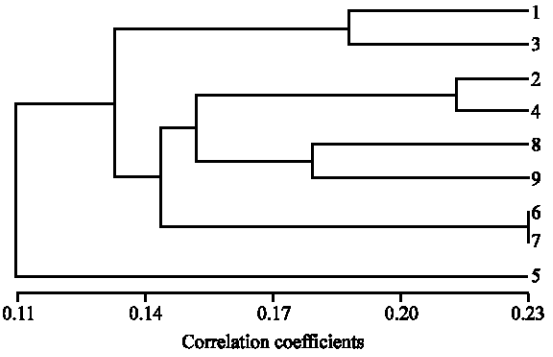

Fig. 11: A dendrogram structure showing five fish clusters and correlation coefficients of the nine fish species being harvested from fifteen wetlands (provinces) in Northeast Thailand. Where 1 = *Puntius wetmorei*, 2 = *P. altus*, 3 = *P. aurotaeniatus*, 4 = *P. binotatus*, 5 = *P. gonionotus*, 6 = *P. leiacanthus*, 7 = *P. orphoides*, 8 = *P. partipentazona* and 9 = *P. schwanenfeldi*

attained for all of the nine fish species were relatively small and not statistically significant where the values ranged from 0.109 to 0.231 for *Puntius partipentazona* and *Puntius orphoides*, respectively (Fig. 11).

DISCUSSION

It has been advocated that in Thailand the fish of the genus *Puntius* Hamilton 1822 comprises of 36 nominal species (Smith, 1945). However, with the results of Champasri *et al.* (2007) they did carry out research on this genus of fish of fifteen wetlands in Northeast Thailand and reported that only nine fish species were available and caught by fishing tools of different kinds. It could have been possible that some other fish species, apart from the nine species, may be found in other natural inhabitation such as rivers, swamps and other sources of reservoirs, thus only nine species were collected. Some wetlands in Northeast Thailand have its natural connection with some rivers e.g., Ubon Ratana Dam at Khon Kaen Province has its initial connection with Chi River then Mun River and Khong River eventually (Suksri, 1999) where some other fish species of the same genus may be found in different years and perhaps they may be found in other omitted locations since there are approximately 549 locations of its kind in Northeast Thailand such as Kang Lawa at Manjakiri District, Khon Kaen; Bueng Khong Long at Roi-Et province and Bueng Tam at Mukdaharn province and many others (Anonymous, 2005). The published results of Champasri *et al.* (2007) on morphological identification revealed that the nine fish species being harvested from fifteen wetlands in Northeast Thailand did not provide any information where the fish species could be classified into other different genera as stated by Rainboth (1996a, b). Thus there is an urgent need to investigate the nine fish species again with the use of molecular technique hence this work was carried out. The actual investigation being carried out in laboratory with the use of molecular identification where DNA bands of the fish samples were used in the various processes where the results reflected, more or less, high accuracy and the technique has been recognized and accepted by a number of authors such as Williams *et al.* (1993), Isabel *et al.* (1999), Almeida *et al.* (2001), Almeida and Sodre (2002), Wasco and Galetti (2002), Leuzzi *et al.* (2004), Matoso *et al.* (2004) and Toth *et al.* (2005).

With the present work, the results on the determinations of genetic distances revealed that genetic values being established among the nine fish species and between pair of the fishes ranged from 0.191 to 0.456 for a pair between *Puntius gonionotus* and *Puntius altus* and a pair between *Puntius schwanenfeldi* and *Puntius leiacanthus*, respectively. The differences found were relatively small and not statistically significant. The results indicated that the nine fish species did not differ largely among themselves hence the fish could not be categorized into other genera as reported by Rainboth (1996a, b). Similarly, the results derived from similarity coefficient values of the fish where the work was calculated through NTSYS-pc 2.10 Computer Programme (Rohlf, 2000), it was found that the attained values ranged from 0.109 to 0.231 for the nine fish species. This narrow range of similarity coefficient values indicated that the nine tested fish species possess a similar DNA band pattern in spite of some minor differences on physical appearances of the fish. With the results found with a Dendrogram structure of clusters, although the nine fish species gave small differences in values of similarity coefficients, the nine fish species could still be identified into five minor groups i.e., group 1 consists of *Puntius gonionotus* alone, group 2 includes *Puntius leiacanthus* and *Puntius orphoides*, group 3 includes *Puntius partipentazona* and *Puntius schwanenfeldi*, group 4 consists of *Puntius altus* and *Puntius binotatus* and finally group 5 includes *Puntius wetmorei* and *Puntius aurotaeniatus*. The results indicated that even the Dendrogram structure of clusters were produced and able to separate the fish into five different groups but the fish of the nine species could not be split or shifted into other genera due to its small values of similarity coefficients (0.111-0.231). Salini *et al.* (2004) stated that correlation coefficient values being calculated for the search in differentiating fish not exceeded a value of 0.78 could be considered to be insignificant.

CONCLUSIONS

The Random Amplified Polymorphic DNA (RAPD) Fingerprints Technique was used in identifying the fish of genus *Puntius* Hamilton 1822 being collected from different wetlands in Northeast Thailand. The results revealed that 1,500 individual fish samples were harvested and collected from the fifteen wetlands (provinces), i.e., (1) Kalasin, (2) Yasothon, (3) Udon Thani, (4) Chaiya Phume, (5) Sisaket, (6), Mahasarakham, (7) Sakon Nakhon, (8) Roi-Et, (9) Nong Kai, (10) Nakhon Phanom, (11) Mukdaharn, (12) Nakhon Ratchasima, (13) Khon Kaen, (14) Nong Bua Lampoo and (15) Loei. All of the individual fish samples were identified into its respective species and it was found, after the use of the various complicated calculations on the results derived from morphological identifications, the collected fish samples were categorized into nine fish species only. The results with

respect to RAPD analysis showed that the nine fish species should only belong to the same genus of *Puntius* Hamilton 1822. Thus the use of the RAPD molecular technique was able to produce the ultimate results with high effectiveness in identifying the fish species, particularly the fish of the genus *Puntius* Hamilton 1822. Therefore, the nine fish species could not be split or shifted into other genera as reported by Rainboth (1996a, b).

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