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Research Article

Molecular phylogeny of swallowtail butterflies (Lepidoptera: Papilionidae) based on mitochondrial cytochrome *c* oxidase I (*COI*) gene from Bangladesh

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Abstract

Swallowtail butterflies (Papilionidae) are among the best-known insects in the world. Mitochondrial cytochrome c oxidase I (*COI*, 642 bp) gene from nine species under four genera of swallowtail butterflies was sequenced and submitted to the GenBank. Basic local alignment search tool (BLAST) analysis showed that the *COI* gene sequences of these nine butterflies matched those of respective species from different geographical areas (99-96% similarity). The interspecific genetic divergence among swallowtail species was between 0.01–0.15%. Phylogenetic tree constructed using the maximum likelihood algorithm showed that the species of swallowtail butterflies were originated from a common ancestor where *Papilio polytes*, *Papilio nephelus*, *Papilio demoleus*, *Papilio memnon* and *Papilio polymnestor* belonged to a cluster. On the other hand, *Graphium agamemnon*, *Atrophaneura varuna* and *Pachliopta aristolochiae* belonged to another cluster. In the TCS haplotype network, considerable genetic divergence was observed among the nine species of swallowtail butterflies. Both phylogenetic and haplotype analyses showed that *Graphium doson* was genetically most divergent among the swallowtail butterflies studied in the present study. These results indicate that *Graphium* spp. might be the good model system to uncover many biological processes like color vision and image processing in organisms.

Keywords: Butterfly; DNA barcode; Evolutionary history; *Graphium* spp.; Species identification

1 | Introduction

Butterflies are diurnal insects and a crucial group of flower visitors in tropical ecosystems (Collins & Morris 1985; Bawa 1990). It is also considered as a good indicator of the health of any specified terrestrial ecosystem (Ghazoul 2002; Sharma & Sharma 2017) and therefore treated as an important group of organisms to study environmental and evolutionary processes (Regier et al. 2013). Among them, swallowtail butterflies belong to the Papilionidae family, which are large and colorful creature of Lepidopterans (Collins & Morris 1985). The butterflies of this family are the most well-known group of invertebrates that comprises over 570 species worldwide (Shen et al. 2015). While IUCN-Bangladesh assessed threat category of 305 species of butterflies, of which 25 species fall under the Papilionidae (IUCN Bangladesh 2015).

Swallowtail butterflies are recognized as model organisms in ecology, visual processing, evolutionary and behavioral biology (Collins & Morris 1985; Scriber et al. 1995; Zakharov et al. 2004; Arikawa 2017). Identification of butterflies, especially for swallowtails, the molecular methods is important since phenotypic variability caused by age, sexual dimorphism and

seasonal variations as well as convergent evolution cause more confusion to the species (Collins & Morris 1985; Kunte et al. 2011; Wilson et al. 2013). In this regard, DNA sequences of the mitochondrial cytochrome oxidase I (COI) gene can serve as a DNA barcode for identifying all kinds of animals (Hebert et al. 2003). On the other hand, comprehensive phylogenetic framework of butteries is lacking, which is crucial to understand evolutionary history and timing of ecological adaptation (Espeland et al. 2018). Therefore, the present work was conducted to generate COI gene sequences and analyse the phylogenetic relationship of swallowtail butterflies (Papilionidae) based on those sequences.

2 | Materials and methods

2.1 | Sample collection

Butterfly specimens were collected from various locations of Bangladesh (Table 1). The specimens were collected by an insect net in the field and preserved by dehydration in a small envelope. Butterflies were identified morphologically by following the keys described in Talbot (1939, 1947). Voucher specimens were prepared as per Brower (1996).

2.2 | DNA extraction, amplification and sequencing

Genomic DNA was extracted from these nine swallowtail butterflies from the legs of adult using the Wizard Genomic@ DNA Purification Kit (Promega, Madison,WI, USA). PCR amplification of 642 bp long segment of the mitochondrial cytochrome c oxidase I (COI) gene region was performed using the primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Hebert et al. 2003). PCR was performed in 20 μL of Q2 Green PCR MasterMix (Promega, Madison, WI, USA) in a thermal cycler (Vetteii, USA). The cycle conditions were as follows: initial denaturation (94°C for 5 min), 35 cycles of denaturation (94°C for 1 min), primer annealing (49°C for 1 min), and primer extension (72°C for 1 min), and a final extension (72°C for 4 min). The success of amplification was evaluated by 1% agarose gel electrophoresis under ultraviolet light (Bio Analyzer). The amplification product was sequenced using an ABI 3500 sequencer.

2.3 | Phylogenetic analysis

All The sequences of these nine butterfly species were edited using Chromas version 2.6.2. The assembled sequences were aligned using the ClustalW multiple alignment function in BioEdit version 7.0 (Hall 1999).

Nucleotide compositions were calculated and summarized, and pairwise distances were estimated using the Kimura 2 Parameter (K2P) model with the MEGA10 program (Kimura 1980; Kumar et al. 2018). Phylogenetic trees were constructed in MEGA10 using the K2P model and 1000 bootstrap replications with the maximum likelihood methods (Kumar et al. 2018). *Orthetrum sabina* from Bangladesh was used in phylogenetic analyses as an outgroup (Table 1).

2.4 | Haplotype network

Haplotype network was constructed using the program POPART 1.7 (Leigh & Bryant 2015) based on TCS network (Clement et al. 2000). The haplotype network identifies the mutational relationship among haplotypes**.**

Table 1. List of taxa, GenBank accession numbers and the GPS coordinates of Papilionidae species used in this study

Species Name	GPS Coordinates	Date of collection	Voucher No.	GenBank Accession No.
Papilio polytes	23.8725 N 90.2675 E	06-07-2017	BBV 0237	MG892099
Papilio demoleus	23.8758 N 90.2683 E	18-07-2017	BBV 0238	MF775396
Papilio memnon	24.3278 N 91.7836 E	12-05-2018	BBV 0236	MK014745
Papilio polymnestor	23.8758 N 90.2683 E	02-11-2017	BBV 0241	MK014746
Papilio nephelus	24.3278 N 91.7836 E	30-04-2017	BBV 0239	MH019969
Graphium agamemnon	23.87442 N 90.26814 E	24-08-2017	BBV 0230	MH019968
Graphium doson	23.87442 N 90.26814 E	18-07-2017	BBV 0228	MK014744
Atrophaneura varuna	24.32647 N 91.78356 E	16-03-2018	BBV 0226	MH807562
Pachliopta aristolochiae	23.8758 N 90.2683 E	26-02-2018	BBV 0235	MH807561
*Orthetrum sabina**	23.872742 N 90.267578 E	14-06-2017	DRBV 029	MF784360

* *Orthetrum sabina* is a dragonfly and used as out-group in the phylogenetic analysis

3 | Results

Nine swallowtail butterflies' mitochondrial cytochrome c oxidase I (COI) gene sequences were generated and they were subsequently submitted to NCBI's GenBank. The accession numbers are listed in Table 1. BLAST results showed that the COI gene sequences of nine species matched (99% to 96%) with those species in the NCBI's GenBank submitted from different geographical areas.

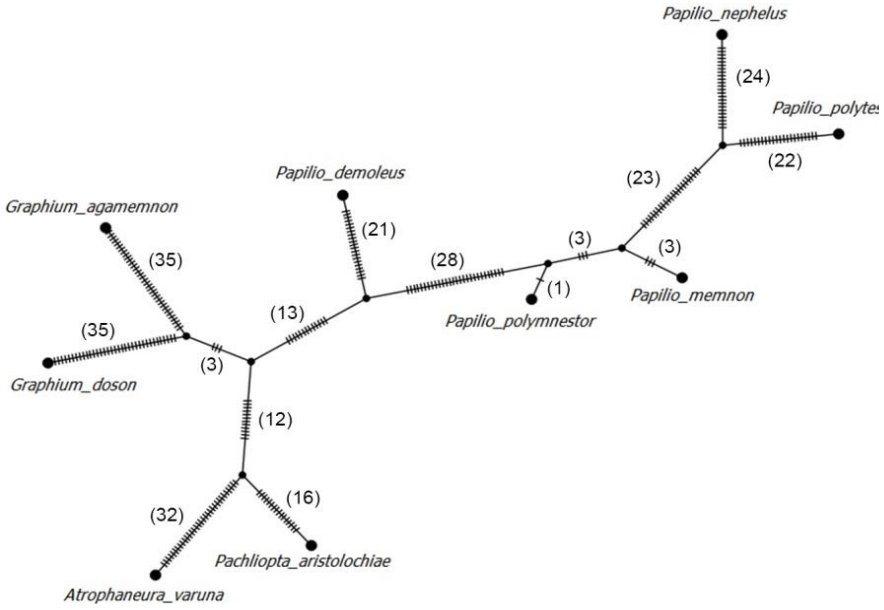

Figure 2. TCS haplotype network of Swallowtail butterflies based on mitochondrial *COI* gene (642 bp). Large circles represent the haplotype and small circles represent the immediate common ancestors. Mutational steps are presented by the hatch marks and numbers.

3.1 | Genetic distance analysis

Nucleotide divergence values of the nine species based on the Kimura-2 parameter model ranged from 0.01 to 0.15 (Table 2). The shortest distance of 0.01 was obtained between *P.polymnestor* and *P.memnon*, indicating that these two species are the closest in their genetic makeup. The highest distance of 0.15 was obtained between *A.varuna* and *P.memnon*, *A.varuna* and *P.polymnestor* as well as *A.varuna* and *P. nephelus*, respectively.

3.2 | Phylogenetic analysis

The maximum likelihood phylogenetic tree of the nine species showed monophyletic entity and shared a common ancestor (Fig. 1). Phylogenetic tree revealed two main clades, A and B. Clade A consisted of six species of one genus that includes *Papilio polytes, Papilio nephelus*, *Papilio demoleus, Papilio memnon* and *Papilio polymnestor,* while Clade B consisted of three species of three genera that includes *Graphium agamemnon, Atrophaneura varuna* and *Pachliopta aristolochiae* (Fig. 1). On the other hand, *Graphium doson* situated distantly compared to these two clades.

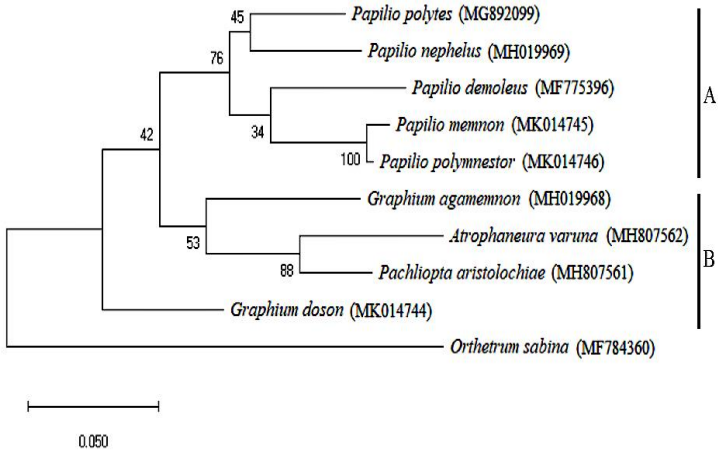

Figure 1. Maximum likelihood tree of the swallowtail butterflies based on partial sequences (642 bp) of mitochondrial COI gene. Bootstrap values are shown at the branching points. *O. sabina* was used as the out-group.

3.3 | Haplotype network

The TCS haplotype network of mitochondrial *COI* gene of nine butterflies showed mutational relationship among them (Fig. 2). *P. polytes* and *P. nephelus* separated from its immediate common ancestor by 22 and 24 mutational steps, respectively. These two species were separated again from *P. memnon* by 23 mutational steps. On the other hand, *P. polymnestor*, *P. demoleus, P. aristolochiae* and *A. varuna* were diverged from its immediate common ancestors by 1, 21, 16 and 32 mutation steps respectively. *G. agamemnon* and *G. doson* showed highest number of mutated sites among other swallowtail species and genetically distant from its common ancestors by 35 mutational numbers.

4 | Discussion

DNA sequences of the mitochondrial cytochrome oxidase I (*COI*) gene can serve as

Table 2. Interspecific genetic distance among Papilionidae species based on Kimura 2 Parameter on *COI* gene fragment (642 bp)

Species Name	1	2	3	4	5	6	7	8
Papilio polytes								
Papilio demoleus	0.10							
Papilio memnon	0.08	0.09						
Papilio polymnestor	0.08	0.08	0.01					
Papilio nephelus	0.07	0.09	0.09	0.08				
Graphium agamemnon	0.13	0.12	0.14	0.14	0.13			
Graphium doson	0.12	0.12	0.14	0.13	0.12	0.12		
Atrophaneura varuna	0.13	0.14	0.15	0.15	0.15	0.13	0.13	
Pachliopta aristolochiae	0.12	0.10	0.12	0.12	0.12	0.11	0.11	0.08

a DNA barcode for identifying all kinds of animals, especially cryptic species in tropical regions (Hebert et al. 2003, 2004a b; Ward et al. 2005; Hajibabaei et al. 2006). In the present study, *COI* gene sequences of nine species of swallowtail butterflies were generated and submitted to NCBI's GenBank. BLAST results showed that the *COI* gene sequences of nine species matched with the *COI* sequences of respective species, which already deposited in NCBI GenBank databases. These sequences will help to identify such butterflies effectively in future studies including monitoring of iodiversity, elucidation of cryptic species and new species descriptions.

Besides taxon identification by means of *COI* gene sequences, systematic and phylogenetic analysis using the gene sequences were carried out by several workers in different groups of insects including swallowtail butterflies (Simonsen et al. 2011; Rukhsana et al. 2014; Ruihua et al. 2018). In the present study, phylogenetic relationship of the swallowtail butterflies determined by the ML method using partial sequences of the *COI* gene, which is generally supported the results obtained by traditional classification methods. Here, phylogenetic analysis showed that there are two clades where *G. doson* situated distantly among all swallowtail butterflies. In many behavioral patterns including foraging and color discrimination, *Graphium* spp. showed distinguished responses than any other butterflies including *Papilio* (Chen et al. 2016). Moreover, in the haplotype analysis highest mutational steps (35 steps) were observed both for *G. agamemnon* and *G. doson* respectively (Fig. 2). These results indicate that genetic makeup of these butterflies is unique than any other studied swallowtail species. In addition, these results are also supported by phylogenetic tree analysis in the present study as well. These genetic variations of *Graphium* spp. are caused by the mutation which is fundamental to evolutionary process. However, animal with a higher rate of mutation is beneficial for faster adaptation to its environment (Sprouffske et al. 2018). As mentioned previously that the behavioral and physiological responses of *Graphium* spp. is notable in nature, as they are very much fast flier and have remarkable color vision capacity than any other butterflies including *Papilio* (Chen et al. 2016). In color vision, *Papilio xuthus* has six classes of photoreceptors in its compound eye whereas *Graphium sarpedon* remarkably has 15 classes of photoreceptors (Arikawa 2017; Chen et al. 2018). Taken together, it is evident that genetic makeup of *Graphium* spp. is different and unique among all butterflies, and hence might be the promising model organism to solve many biological processes in future.

5 | Conclusions

The present study reveals DNA barcoding in the identification of nine swallowtail butterflies and develops a comprehensive DNA barcode database for these butterfly species from Bangladesh. The present study also elucidates the molecular phylogenetic relationship among swallowtail butterflies. Among the studied species, *Graphium doson* is unique in its genetic makeup and this could be a good model system to uncover many biological processes in future.

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Authors' contributions

Ananna Ghosh and Muhammad Sohel Abedin collected and identified the specimens; Ananna Ghosh, Fahmina Sarkar Borsha and Md Khayrul Hasan were performed laboratory work; Md Monwar Hossain supervised the research work. Md Monwar Hossain and Abdul Jabber Howlader wrote the paper. All authors approved the final manuscript for publication.

Conflicts of interest

Authors declare no conflict of interest.

References

Arikawa, K. 2017. The eyes and vision of butterflies. Symposium Review, The Journal of Physiology **595**(16):5457–5464. https://doi.org/10.1113/JP273917

Bawa, K. S. 1990. Plant-pollinator interactions in tropical rain forests. Annual Review of Ecology and Systematics **21**:399–422. https://doi.org/10.1146/annurev.es.21.110190.002151

Brower, A. V. Z. 1996. A new mimetic species of *Heliconius* (Lepidoptera: Nymphalidae), from southeastern Colombia, as revealed by cladistic analysis of mitochondrial DNA sequences. Zoological Journal of the Linnean Society **116:**317–332. https://doi.org/10.1111/j.1096-3642.1996.tb00126.x

Chen, Pei-Ju., Awata, H., Matsushita, A., Yang, E. C. and Arikawa, K. 2016. Extreme Spectral Richness in the Eye of the Common Bluebottle Butterfly, *Graphium sarpedon*. Frontiers in Ecology and Evolution **4** (18): 1–12. https://doi.org/10.3389/fevo.2016.00018

Clement, M., Posada, D. and Crandall, K. A. 2000. TCS: A computer program to estimate gene genealogies. Molecular Ecology **9**: 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Collins, N. M. and Morris, M. G. 1985. Threatened Swallowtail Butterflies of the World: The IUCN Red Data Book. Gland, Switzerland. pp 1-401. https://portals.iucn.org/library/sites/library/files/documents/RD-1985-002.pdf

Espeland, M., Breinholt, J., Willmott, K. R., Warren, A. D., Vila, R., Toussaint, E. F. A. et al. 2018. A Comprehensive and Dated Phylogenomic Analysis of Butterflies. Current Biology **28** (5): 770–778. https://doi.org/10.1016/j.cub.2018.01.061

Ghazoul, J. 2002. Impact of logging on the richness and diversity of forest butterflies in a tropical dry forest in Thailand. Biodiversity and Conservation **11**: 521–541. http://dx.doi.org/10.1023/A:1014812701423

Hall, T. A. 1999. Bioedit, a user-friendly biological sequences alignment editor and analysis program for windows95/98/NT. Nucleic Acids Symposium Series **41:** 95–98. https://www.scinapse.io/papers/1483247593

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W. and Hebert, P. D. 2006. DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences USA **103**: 968–971. https://doi.org/10.1073/pnas.0510466103

Hebert, P. D., Cywinska, A., Ball, S. L. and deWaard, J. R. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences **270**: 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P. D., Stoeckle, M. Y., Zemlak, T. S. and Francis, C. M. 2004a. Identification of birds through DNA barcodes. PLoS Biology **2**: e312. https://doi.org/10.1371/journal.pbio.0020312

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H. and Hallwachs, W. 2004b. Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences USA **101**: 14812–14817. https://doi.org/10.1073/pnas.0406166101

IUCN Bangladesh. 2015. Red List of Bangladesh Volume 7: Butterflies. IUCN, International Union for Conservation of Nature, Bangladesh Country Office, Dhaka Bangladesh, pp 1-400. https://www.iucn.org/resources/research-publication/red-list-bangladesh-volume-7-butterflies

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution **16**:111–120. https://doi.org/10.1007/BF01731581

Kumar, S., Stecher, G., Li M., Knyaz, C. and Tamura, K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution **35**:1547–1549. https://doi.org/10.1093/molbev/msy096

Kunte, K., Shea, C., Aardema, M. L., Scriber, J. M., Juenger, T. E., Gilbert, L. E. and Kronforst, M.R. 2011. Sex Chromosome Mosaicism and Hybrid Speciation among Tiger Swallowtail Butterflies. PLoS Genetics **7** (9): e1002274. https://doi.org/10.1371/journal.pgen.1002274

Leigh, J,W. and Bryant, D. 2015. POPART: Full-feature software for haplotype network construction. Methods in Ecology and Evolution **6**(9):1110–1116. https://doi.org/10.1111/2041-210X.12410

Regier, J. C., Mitter, C., Zwick, A., Bazinet, A. L., Cummings, M. P., Kawahara, A. Y., et al. 2013. A Large-Scale, Higher-Level, Molecular Phylogenetic Study of the Insect Order Lepidoptera (Moths and Butterflies). PLoS ONE **8**(3): e58568. https://doi.org/10.1371/journal.pone.0058568

Ruihua, Z., Ping, J., Chuanbo, S., Deyong, S., Feng, Z. and Chaochao, H. 2018. The analysis of genetic variation in the mitochondrial genome and its application for the identification of *Papilio* species. 2018. Mitochondrial DNA Part B: Resources **3** (2): 687–690. https://doi.org/10.1080/23802359.2018.1481776

Rukhsana, K., Akhilesh, V. P. and Sebastian, C. D. 2014. Deciphering the molecular phylogenetics of the Asian honey bee, *Apis cerana* (Hymenoptera:Apidae) and inferring the phylogeographical relationships using DNA barcoding, Journal of Entomology and Zoology Studies **2** (4): 218–220.

Scriber, J. M., Lederhouse, R. C. and Dowell, R. V. 1995. Hybridization studies with North American swallowtails. In ''Swallowtail Butterflies: Their Ecology and Evolutionary Biology (J. M. Scriber, Y. Tsubaki, and R. C. Lederhouse, Eds.), Scientific Publication, Gainesville, FL. pp 269–281.

Sharma, M. and Sharma, N. 2017. Suitability of Butterflies as Indicators of Ecosystem Condition: A Comparison of Butterfly Diversity across four habitats in Gir Wildlife Sanctuary. International Journal of Advanced Research in Biological Sciences **4** (3): 43–53.

Shen, J., Cong, Q. and Grishin, N. V. 2015. The complete mitochondrial genome of *Papilio glaucus* and its phylogenetic implications. Meta Gene **5**: 68–83. https://doi.org/10.1016/j.mgene.2015.05.002

Simonsen, T. J., Zakharov E. V., Djernaes, M., Cotton, A. M., Vane-Wright, R. I. and Sperling, F.A.H. 2011. Phylogenetics and divergence times of Papilioninae (Lepidoptera) with special reference to the enigmatic genera *Teinopalpus* and *Meandrusa*. Cladistics **27**:113–137. https://doi.org/10.1111/j.1096-0031.2010.00326.x

Sprouffske, K., Aguilar-Rodríguez, J., Sniegowski, P. and Wagner, A. 2018. High mutation rates limit evolutionary adaptation in *Escherichia coli*. PLoS Genetics **14(**4): e1007324. https://doi.org/10.1371/journal.pgen.1007324

Talbot, G. 1939. The Fauna of British India, including Ceylon and Burma. Butterflies. I. (Papilionidae & Pieridae). (ed. 2), Taylor and Francis Ltd., London. xxxix + 600. https://doi.org/10.1080/00222939009460807

Talbot, G. 1947. The Fauna of British India including Ceylon and Burma - Butterflies vol. II.Taylor and Francis Ltd. London, pp1-506. https://doi.org/10.1080/00222930709487349

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R. and Hebert, P. D. 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society London B **360**: 1847–1857. https://doi.org/10.1098/rstb.2005.1716

Wilson, J. J., Sing, K. W. and Sofian-Azirun, M., 2013. Building a DNA barcode reference library for the true butterflies (Lepidoptera) of Peninsula Malaysia: what about the subspecies? PLoS ONE **8** (11): e79969. https://doi.org/10.1371/journal.pone.0079969

Zakharov, E. V., Caterino, M. S. and Sperling, F. A. 2004. Molecular phylogeny, historical biogeography, and divergence time estimates for swallowtail butterflies of the genus *Papilio* (Lepidoptera: Papilionidae). Systematic Biology **53**:193–215. https://doi.org/10.1080/10635150490423403