Algae
Volume 21(4): 417-423, 2006

Morphology and Molecular Phylogeny of *Hypnea flexicaulis* (Gigartinales, Rhodophyta) from Korea

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Morphology and molecular phylogeny of a red algal species, *Hypnea flexicaulis* that is recently described from Japan, were investigated based on 23 collections from Korea (21), Taiwan (1), and the Philippines (1). *Hypnea flexicaulis* has percurrent axes with flexuous, antler-like branches which have wide branching angles, and abaxially curved ultimate branchlets. In order to study DNA divergence and phylogenetic relationships of the species, we determined plastid *rbc*L and mitochondrial *cox*1 sequences from the 23 collections. All 21 specimens from five different locations in Korea were almost identical to *H. flexicaulis* from Japan in *rbc*L sequences. Although there was a difference of three to five base pairs (bp) between samples from Korea and the Philippines or between the Philippines and Taiwan, Bayesian analyses of the *rbc*L data showed that all specimens from Korea, Japan, the Philippines, and Taiwan were strongly monophyletic. However, it is interesting that specimens from the Philippines differed by 31-34 base pairs in mitochondrial *cox*1 gene from those of materials from Korea and Taiwan, which differed by one to seven bp in *rbc*L between them. Although *H. boergesenii* is different from *H. flexicaulis* in having many antler-like branchlets, both appeared as sisters in all analyses of the *rbc*L data. This is the first report of *H. flexicaulis* from Korea based on morphology, *rbc*L, and *cox*1 gene sequences

Key Words: *cox*1, Hypnea, *H. flexicaulis*, morphology, phylogeny, *rbc*L, taxonomy

INTRODUCTION

Hypnea Lamouroux (1813) is an economically important red algal genus that produces carrageenan. The genus consists of about 53 species world-wide (Guiry *et al.* 2006) and abundant in tropical sea. In Korea, there are seven species of *Hypnea* as listed in Lee and Kang (2001) and Shin and Boo (1994): *H. boergesenii* Tanaka, *H. cervicornis* J. Agardh, *H. charoides* Lamouroux *H. japonica* Tanaka, *H. saidana* Holmes, *H. variabilis* Okamura, and *H. pannosa* J. Agardh. They are often abundant in the tidal zone of the southern coast of Korea during summer to autumn.

Recently, there has been an international effort to facilitate reliable and easier species identification which led to the use of DNA barcoding as a way to catalogue the diversity of organisms (Robba *et al.* 2006). A number of molecular markers have been used in red algal taxonomy (Yang and Boo 2004; Provan *et al.* 2004), but *rbc*L has been very commonly used in the taxonomy of red algae as proposed by Freshwater *et al.* (1994). Many mitochondrial genes have proven to be useful for the identification and phylogeny reconstruction of animals, but there are only a few reports thus far using protein-coding mitochondrial genes in red algae (Saunders 2005; Robba *et al.* 2006; Zuccarello *et al.* 1999).

There have been only two reports on the molecular taxonomy of *Hypnea*. Yamagishi & Masuda (2000) studied the genus from Japan and described *H. flexicaulis* Yamagishi *et* Masuda as a new species based on samples collected in Ikata, Japan. The species is distinguished by flexuous percurrent main axes and antler-like branches with wide branching angles showing abrupt abaxial bending. The same authors (Yamagishi *et al.* 2003) raised *H. cornuta* var. *stellulifera* J. Agardh to the rank of species as *H. stellulifera* (J. Agardh) Yamagishi et Masuda based on molecular evidence.

The goal of this paper was to describe the morphology of *Hypnea flexicaulis* based on our recent collections in Korea and to identify the species and to understand its phylogenetic position using *cox*1 and *rbc*L data. Specimens from Taiwan and the Philippines were also included in the present study. This is the first report of

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Table 1. Collection locations and date with voucher code, and GenBank accession numbers for taxa included in this study

Species	Voucher code	Location and date	*rbc*L	*cox*1
H. flexicaulis	PHBgam	Gampo, Gyeongju, Korea; 13.vii.2006	EF 136627	EF 136611
H. flexicaulis	PH68	Gijang, Busan, Korea; 17.x.2005	EF 136626	EF 136606
H. flexicaulis	PH48	Guryongpo, Pohang, Korea; 2.x.2005	EF 136624	EF 136596
H. flexicaulis	PH43	Wolpo, Pohang, Korea; 3.x.2005	EF 136623	EF 136610
H. flexicaulis	PH37	Wolpo, Pohang, Korea; 3.x.2005	EF 136630	EF 136609
H. flexicaulis	PH4	Gampo, Gyeongju, Korea; 2.x.2005	EF 136622	EF 136593
H. flexicaulis	PH6	Gampo, Gyeongju, Korea; 2.x.2005	EF 136625	EF 136607
H. flexicaulis	PH14	Gampo, Gyeongju, Korea; 2.x.2005	EF 136628	EF 136594
H. flexicaulis	PH17	Gampo, Gyeongju, Korea; 2.x.2005	EF 136629	EF 136595
H. flexicaulis	PH18	Gampo, Gyeongju, Korea; 2.x.2005	EF 136613	EF 136605
H. flexicaulis	PH20	Geomundo, Yeosu, Korea; 26.ix.2005	EF 136614	EF 136597
H. flexicaulis	PH21	Geomundo, Yeosu, Korea; 26.ix.2005	EF 136615	EF 136598
H. flexicaulis	PH23	Geomundo, Yeosu, Korea; 26.ix.2005	EF 136616	EF 136599
H. flexicaulis	PH25	Geomundo, Yeosu, Korea; 27.ix.2005	EF 136617	EF 136600
H. flexicaulis	PH30	Geomundo, Yeosu, Korea; 27.ix.2005	EF 136631	EF 136592
H. flexicaulis	PH31	Geomundo, Yeosu, Korea; 27.ix.2005	EF 136618	EF 136601
H. flexicaulis	PH32	Geomundo, Yeosu, Korea; 27.ix.2005	EF 136619	EF 136602
H. flexicaulis	PH33	Geomundo, Yeosu, Korea; 27.ix.2005	EF 136620	EF 136603
H. flexicaulis	PH34	Geomundo, Yeosu, Korea; 28.ix.2005	EF 136621	EF 136604
H. flexicaulis	PH0524	Daisanglan, Keelung, Taiwan; 31.iii.2006	EF 136612	EF 136608
H. flexicaulis	PH048	Dancalan, Bulusan, Philippines; 11.i.2006	EF 136632	EF 136591

H. flexicaulis outside Japan identified based on morphology and molecular markers. In the present study, the mitochondrial protein-coding *cox*1 gene proved to be useful for the identification of red algae.

MATERIALS AND METHODS

Collection and morphology

Specimens and their collection sites are listed in Table 1. Samples from the field were transported live back to the laboratory in sterilized seawater, cleaned, and sorted carefully under a dissecting microscope. Thalli were preserved in silica gel desiccant for DNA extraction. All voucher specimens were deposited in the herbarium of the Department of Biology (CNUK), Chungnam National University, Daejon, Korea.

Observations on ecological and growth habit were noted. Materials for morphological observations were preserved in 4% formaldehyde-seawater. Sections for microscopic observations were made by hand using a razor blade and stained with 1% aqueous aniline blue in a lactic acid/phenol/glycerol/water (1:1:1:1) solution and mounted in 50% glycerol/seawater on microscope slides. Photographs were made with a camera lucida attached to an Olympus microscope (VANOX AHBT3).

DNA extraction and sequencing

Genomic DNA was extracted from approximately 0.005 g of algal powder ground in liquid nitrogen using a DNeasy Plant Mini Kit (Qiagen) or Invisorb Spin Plant Mini Kit (Invitek), according to the manufacturer's protocols. PCR of the *rbc*L gene was done with specific primers such as F7, F645, R753, and R*rbc*S start (Freshwater and Rueness 1994; Lin *et al.* 2001; Gavio and Fredericq 2002). The PCR solution and reaction conditions followed those by Yang and Boo (2004, 2006). The mitochondrial *cox*1 sequences for *Chondrus crispus* Stackhouse (Z47547) by Leblanc *et al.* (1995) and Porphyra purpurea (Roth) C. Agardh (AF114794) by Burger *et al.* (1999) were aligned and used to devise specific primers to amplify this gene region for *Hypnea fexicaulis*: (upstream COXI43F - 5′ TCA ACA AAT CAT AAA GAT ATT GGW ACT 3′ and downstream COXI1549R - 5′ AGG CAT TTC TTC AAA NGT ATG ATA 3′). The PCR products of both genes were purified using a High Pure PCR Product Purification Kit (Roche) in accordance with the users guide. The sequences of the forward and reverse strands for *rbc*L and *cox*1 were determined using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). GenBank accession numbers are listed in Table 1.

Complementary chromatograms were aligned and reconciled using Sequence Navigator v. 1.0.1 (Applied

Table 2. List of *Hypnea* used in *rbc*L analyses from GenBank with corresponding accession numbers

Species or variety	Collection site (reference)	GenBank accession Number
Hypnea charoides Lamouroux	Nabeta, Shimoda, Shizuoka Prefecture, Japan (Yamagishi and Masuda 2000)	AB033159
H. chordacea Kützing	Shirahama, Shimoda, Shizuoka Prefecture, Japan (Yamagishi and Masuda 2000)	AB033160
H. cornuta (Kützing) J. Agardh	Teguma, Nagasaki, Nagasaki Prefecture, Japan (Yamagishi and Masuda 2000)	AB033161
H. cornuta (Kützing) J. Agardh	Sukuji, Ishigaki Island, Okinawa Prefecture, Japan (Yamigishi *et al.* 2003)	AB095911
H. cornuta (Kützing) J. Agardh	Taranto, Italy (Yamigishi *et al.* 2003)	AB095912
H. stellulifera J. Agardh	Pulau Besar, Melaka, Malaysia (Yamigishi *et al.* 2003)	AB095913
H. stellulifera J. Agardh	Pulau Sipadan, Sabah, Malaysia (Yamigishi *et al.* 2003)	AB095914-5
H. flagelliformis J. Agardh	Fukaura, Aomori Prefecture, Japan (Yamagishi and Masuda 2000)	AB033162
H. flexicaulis Yamagishi *et* Masuda	Shirahama, Shimoda, Shizuoka Prefecture, Japan (Yamagishi and Masuda 2000)	AB033163
H. japonica Tanaka	Banshobana, Ei, Kagoshima Prefecture, Japan (Yamagishi and Masuda 2000)	AB033164
H. musciformis (Wulfen) Lamouroux	New Hanover Co., North Carolina, USA (Hommersand & Fredericq 2001)	U04179
H. pannosa J. Agardh	Hedo-misaki, Okinawa Prefecture, Japan (Yamagishi and Masuda 2000)	AB033165
H. yamadae Tanaka	Nomozaki, Nagasaki Prefecture, Japan (Yamigishi *et al.* 2003)	AB095916
Hypnea sp.	Izumozaki, Kushimoto, Wakayama Prefecture, Japan (Yamagishi and Masuda 2000)	AB033167
H. spinella (C. Agardh) Kützing	Sesoko Island, Okinawa Prefecture, Japan (Yamagishi and Masuda 2000)	AB033166
H. spinella (C. Agardh) Kützing	Florida, United States of America (Hommersand & Fredericq 2001)	AF385635
H. volubilis Searles	Los Angeles, United States of America (Hommersand & Fredericq 2001)	AF385636
H. boergesenii T. Tanaka	Taiwan (Hommersand & Fredericq 2001)	AF385634

Table 3. Pairwise divergence in *rbc*L sequences between specimens* of *Hypnea flexicaulis* and *H. boergesenii* used in this study. Each number indicates absolute distances (below diagonal) and uncorrected *p*-distances (above diagonal)

PH048	—	0.00201	0.00268	0.00335	0.00268	0.00216	0.00875
PH30	3	—	0.00067	0.00134	0.00067	0.00070	0.00732
PH14	4	1	—	0.00201	0.00134	0.00143	0.00804
PH0524	5	2	3	—	0.00201	0.00214	0.00585
PH43	4	1	2	3	—	0.00144	0.00803
AB033163	3	1	2	3	2	—	0.00808
AF385634	12	10	11	8	11	11	—

*Specimens PH4, PH6, PH20, PH21, PH23, PH25, PH31, PH32, PH33 and PH34 are identical to PH30; specimens PH17, PH48, PH18, PH68, PH37 and PHBgam are identical to PH14.

Biosystems). The alignment of each gene sequence was based on the alignment of the inferred amino acid sequence and was refined by careful visual inspection. There were no gaps in the alignments of *rbc*L and *cox*1. The final alignment is available from the corresponding author upon request.

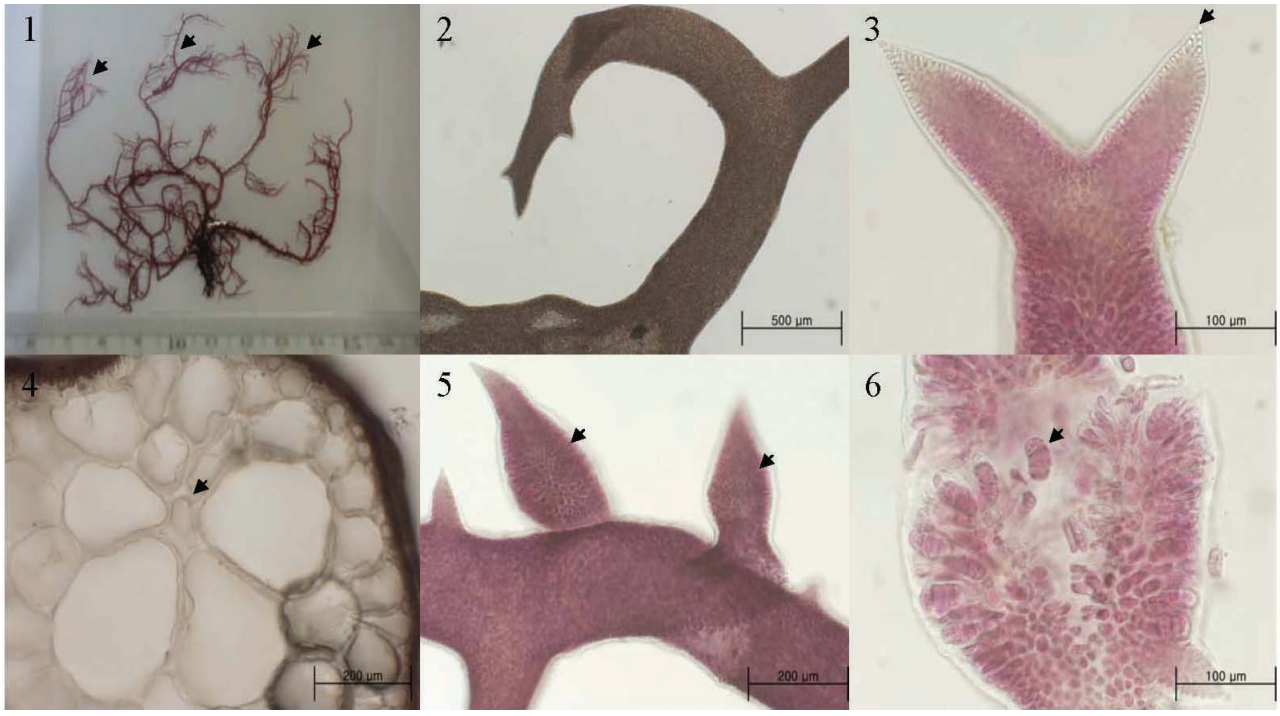
Phylogenetic analyses

Bayesian analyses, as a proxy for maximum likelihood topology, with MrBayes v 3.0b4 (Huelsenbeck and Ronquist 2003) were conducted to determine the nucleotide substitution model that best fits each individual data. GTR+Γ+I was the best model for *rbc*L data set. Four independent Markov Chain Monte Carlo were run twice simultaneously for 2,000,000 generations and trees were sampled every 100th generation. 2,000 generations

Table 4. Pairwise divergence in *cox*1 sequences between specimens* of *Hypnea flexicaulis* used in this study. Each number indicates absolute distances (below diagonal) and uncorrected *p*-distances (above diagonal)

PH048	—	0.02348	0.02391	0.02180	0.02447
PH30	33	—	0.00427	0.00355	0.00507
PH14	34	6	—	0.00352	0.00072
PH0524	31	5	5	—	0.00432
PH43	34	7	1	6	—

*Specimens PH4, PH6, PH20, PH21, PH23, PH25, PH31, PH32, PH33 and PH34 are identical to PH30; specimens PH17, PH48, PH18, PH68, PH37and PHBgam are identical to PH14.



Figs 1-6. *Hypnea flexicaulis* from Gampo, Pohang, Korea (13.vii.2006). **1.** Antler-like upper branches (arrowhead). **2.** Curved branchlet. **3.** Apex of a branch showing a distinct apical cell (arrowhead). **4.** Cross-section of a lower axis showing the central axis (arrowhead). **5.** Tetrasporangial branchlet (arrowhead). **6.** Zonately divided tetrasporangia (arrowhead).

burn-in after the likelihood values reached stationary at approximately 200,000 generations. Consensus tree searches from two sets with 50% majority rule of the 18,000 trees were performed in constructing trees and resulted in the Bayesian posterior probabilities (BPP) for all data set. To test the interspecific variation of both *rbc*L and *cox*1, absolute and uncorrected-*p* pairwise distance matrix was constructed (Tables 3 and 4).

RESULTS

Morphology

Hypnea flexicaulis grows gregariously in the lower intertidal to upper subtidal zones on somewhat sheltered coasts in southern Korea. Thalli are fleshy or subcartilaginous, greenish-yellowish or brownish-red, with an entangled base of creeping branches. Axes and first-order branches are flexuous, and upper branches are shaped like antlers (Fig. 1). Main axis is terete throughout, obscurely percurrent, branching in an alternate-spiral manner at an angle. However, axes grow in the opposite direction to the branches, turning away from them so that the branching angles become wider. Upper ordinary branchlets and adventitious branchlets are sometimes curved or hooked to abaxial or irregular directions (Fig. 2). Axes and branches of lower orders produce short, simple or long, divided adventitious branchlets, usually at right-angles. All branches have apical cells (Fig. 3). Periaxial cells are elliptical or broadly obovate in cross-section (Fig. 4). Axial cells are elongated and slender in longitudinal section. Each periaxial cell and cortical cell has secondary pit-connections, without lenticular thickenings on its walls. Tetrasporangia formed in the proximal, middle, or sometimes distal swollen sori of ultimate branchlets (Fig. 5). Mature tetrasporangia have zonately arranged spores (Fig. 6). Cystocarps and spermatangia were not found.

Molecular phylogeny

The *rbc*L sequences analyzed from 23 specimens of *Hypnea flexicaulis* were 1491 nucleotides (nt) long. All 21 specimens from five different locations in Korea were very similar to *H. flexicaulis* from Shimoda, Japan in terms of their *rbc*L sequences (Table 3) and with a difference of 1-2 base pairs (bp). There was a difference of three bp between materials from Japan and the Philippines or between materials from Japan and Taiwan; 3-5 bp between materials from Korea and the Philippines or materials from the Philippines and Taiwan.

Fig. 7. Bayesian *rbc*L tree for *Hypnea flexicaulis* using *Grateloupia* species as outgroup. Numbers above the branches indicate Bayesian posterior probabilities. Jp = Japan, Kr = Korea, Ph = Philippines, and Tw = Taiwan.

The phylogenetic tree obtained from Bayesian analysis of the *rbc*L data is shown in Fig. 7. The 44 aligned *rbc*L sequences had 1092 constant characters, 95 parsimony-uninformative characters, and 304 parsimony-informative characters. All specimens from Korea, Japan, the Philippines, and Taiwan were strongly monophyletic and well supported (100% BP). *H. boergesenii* appeared as a sister taxon to *H. flexicaulis* in all analyses of *rbc*L data (96% BP).

The *cox*1 sequences from 21 specimens of *H. flexicaulis* were 1422 nucleotides (nt) long. In pairwise distance matrix for the *cox*1 (Table 4), there was a difference of 1-7 bp between samples from Korea and Taiwan. Specimens from the Philippines differed by 31-34 bp from those of

Korea and Taiwan.

DISCUSSION

Our recent collections of *Hypnea flexicaulis* from various sites along the southern coasts of Korea corresponds in their habit, branching, and tetrasporangia to the description of Yamagishi and Masuda (2000). According to Yamagishi and Masuda (2000), *H. flexicaulis* has been erroneously referred to as *H. cervicornis* J. Agardh in Japan (Tanaka 1941), but the latter species was reduced by Haroun and Prud'homme van Reine (1993) to the synonymy of *H. spinella* (C. Agardh) Kützing. It was shown that there is a distinct transition of typical *H. spinella*, showing a compact form in habitats with rough wave action, into typical *H. cervicornis*, a more elongate growth form found in habitats with less wave action. However, *H. flexicaulis* is entirely different from *H. spinella* and that no species with comparable morphology similar to it has been found among some 50 hitherto described species of *Hypnea* (Yamagishi and Masuda 2000).

Another query is on the relationship between *H. charoides* from Korea (Shin and Boo 1994) and *H. flexicaulis*. According to Womersley (1994) and Yamagishi and Masuda (2000), *H. charoides* is characterized by slender spinous branchlets that are mostly directed at right-angles from the parent branch and becoming abruptly curved towards the axis with age and then growing straight like the ordinary branches and adventitious branchlets. That is not the case in reference to the illustrations of *H. charoides* from Korea as described by Shin and Boo (1994), but the illustrations rather resemble the descriptive morphology of *H. flexicaulis* from Japan.

The present phylogenetic analyses of the *rbc*L data further support the occurrence of *H. flexicaulis* outside of Japan. Bayesian analyses of all 23 specimens from Korea, Taiwan, and the Philippines, including *H. flexicaulis* from Japan, formed a well-supported monophyletic clade (100% BP). There was no substantial base pair (bp) difference detected among samples, with pairwise distance of only one to five bp differences between samples. The neighbor-joining tree generated by Yamagishi and Masuda (2000) shows *H. flexicaulis* was positioned as a sister to the clade including *H. cornuta*, *H. musciformis*, *H. chordacea* and *H. flagelliformis*. Although the topology is similar to our phylogenetic tree, however, *H. boergesenii*, which was not included in their analysis, appeared as a sister taxon to *H. flexicaulis* in all analyses of the *rbc*L data. *H. boergesenii* (AF385634) from Taiwan studied by Hommersand and Fredricq (2001) is molecularly closely related to *H. flexicaulis* from Taiwan as revealed in the present study. Further study on the taxonomic relationship between *H. boergesenii* and *H. flexicaulis* should be carried out.

The *cox*1 analysis of 23 specimens of *H. flexicaulis* was conducted to serve as additional and independent data to be used in conjunction with morphology and *rbc*L data analyses. Although no phylogenetic tree using *cox*1 sequences was generated because of the paucity of the analyzed data, most of the samples from Korea and Taiwan differed just by 1-7 bp. However, it is interesting that the specimen from the Philippines differed by 31-34 bp from those of Korea and Taiwan, whereas the divergence of *rbc*L sequences between samples from the Philippines and Korea/Taiwan is in a range of that seen between samples from Korea and Taiwan. The results hypothesize that, based on a higher interspecific divergence of the *cox*1 gene in red algae (Robba *et al.* 2006; Saunders 2005), the Philippines specimen may be biologically different from *H. flexicaulis* from Korea and Taiwan. Despite the close proximity between the Philippines and Taiwan, the Philippines is more tropical than Taiwan. The Philippine sample was collected from southeastern Luzon Island which is characterized by a very rich marine red algal flora (Kraft *et al.* 1999). The eastern coast of the northern half of the Philippines is affected by the Kuroshio Current which flows north towards the eastern coast of Taiwan all the way up to the eastern coast of Honshu, Japan, bringing with it seaweed propagules, among other organisms. During the cooler months of the year, many subtropical species found in Taiwan may be encountered in northeastern Philippines as well. Likewise, some distinctively tropical species of algae can also be found in some areas of northern Taiwan (Yang *et al.* 1994), where *H. flexicaulis* used in the present study was collected. A marine phytogeographic study of the seaweeds along the coastal areas affected by the Kuroshio Current would be an interesting subject for future studies.

ACKNOWLEDGEMENTS

We thank Dr. Myung Sook Kim and Il Ki Hwang for sharing their collections from Gijang and Geomundo, Korea and Dr. Lawrence M. Liao for critical reading of the manuscript. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion

Fund, KRF-2006-211-C0051) and by a grant from Korean Science and Engineering Foundation (R0120060001020702006) to S.M. Boo.

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Received 9 September 2006
Accepted 10 November 2006