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ORIGINAL PAPER

Diphyllobothrium nihonkaiense: wide egg size variation in 32 molecularly confirmed adult specimens from Korea

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Abstract The eggs of *Diphyllobothrium nihonkaiense* were reported to be smaller than those of the classical *Diphyllobothrium latum* in general. However, verification using a large number of adult tapeworms is required. We assessed the egg size variation in 32 adult specimens of *D. nihonkaiense* recovered from Korean patients in 1975–2014. The diagnosis of individual specimens was based on analysis of the mitochondrial cytochrome *c* oxidase 1 gene sequence. Uterine eggs (n=10) were obtained from each specimen, and their length and width were measured by micrometry. The results indicated that the egg size of *D. nihonkaiense* (total number of eggs measured, 320) was widely variable according to individual specimens, 54–76 μm long (mean 64) and 35–58 μm wide (mean 45), with a length-width ratio of 1.32–1.70 (mean 1.46). The worm showing the smallest egg size had a length range of 54–62 μm, whereas the one showing the largest egg size had a length range of 68–76 μm. The two ranges did not overlap, and a similar pattern was observed for the egg width. Mapping of each egg size (n=320) showed a wide variation in length and width. The widely variable egg size of *D. nihonkaiense* cannot be used for specific diagnosis of diphyllobothriid tapeworm infections in human patients.

Keywords *Diphyllobothrium nihonkaiense* · Diphyllobothriasis · Egg size variation · Korea

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Introduction

Diphyllobothriasis is an important fish-borne parasitic zoonoses caused by broad fish tapeworms such as *Diphyllobothrium latum* and *Diphyllobothrium nihonkaiense* with freshwater and anadromous fish as their second intermediate host, respectively (Chai et al. 2005). Infection with the latter species is reportedly on the rise in Asia, including Japan (Arizono et al. 2009a), China (Chen et al. 2014), and South Korea (= Korea) (Jeon et al. 2009; Park et al. 2013; Song et al. 2014; Kim et al. 2014; Shin et al. 2014). Sporadic infections with *D. nihonkaiense* were also detected in Europe (Yera et al. 2006; Wicht et al. 2007), America (Wicht et al. 2008), and New Zealand (Yamasaki and Kuramochi 2009), possibly due to an increased global consumption of pacific salmon, the major source of *D. nihonkaiense* infection (Arizono et al. 2009a). However, *D. nihonkaiense* is under-recognized because of its close morphologic similarities with the classical *D. latum*. Recent genetic studies in Asia (Korea, Japan, and China) suggested that all previous cases diagnosed as *D. latum* were in fact *D. nihonkaiense* (Jeon et al. 2009; Arizono et al. 2009a; Chen et al. 2014).

Several workers indicated that the eggs of *D. nihonkaiense* (53–65×35–45 μm) (Yamane et al. 1986; Ando et al. 2001; Yera et al. 2006; Wicht et al. 2007, 2008; Shimizu et al. 2008; Arizono et al. 2009b) were smaller, as compared to those of *D. latum* (58–76×41–57 μm) (von Bonsdorff 1977; Andersen and Halvorsen 1978; Beaver et al. 1984; Peduzzi and Boucher-Rodoni 2011; Lou et al. 2007; Wicht et al. 2007). However, diphyllobothriids show substantial variations in egg size, and a great size overlap exists between species, with resultant taxonomic limitation (Andersen and Halvorsen 1978). Despite the occurrence of only *D. nihonkaiense* in Korea (Jeon et al. 2009), some Korean diphyllobothriasis cases revealed eggs larger (Lee et al. 2007) than the reported values of *D. nihonkaiense* eggs, which highlights the need for precise differentiation between the two species and verification of their egg size range. Thus, in this study, we measured the length and width of eggs from 32 *D. nihonkaiense* (molecularly proven) adult specimens recovered from Korean patients to determine the egg size range of *D. nihonkaiense*.

Materials and methods

Specimens

In total, 32 *D. nihonkaiense* adult specimens referred to the Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine during 1975–2014 (Table 1) were included in the study. Most (28 of 32) specimens were collected between 1975 and 2002 (stored in formalin or ethanol) and primarily assigned as *D. latum* based on the morphological findings of the proglottids. Four specimens were collected between 2013 and 2014 and stored in ethanol. Molecular studies provided the diagnosis of *D. nihonkaiense* for all 32 specimens. Jeon et al. (2009) previously reported the molecular diagnosis of 28 specimens, and that of the remaining four specimens is reported in the current study. The study followed the ethical guidelines of Seoul National University College of Medicine, Seoul, Korea.

Molecular genetic examinations

The procedures for molecular study of specimens were as follows: Briefly, genomic DNA was extracted using the Spin-Column Protocol of DNeasy® Blood & Tissue kit (QIAGEN, Hilden, Germany). Nested polymerase chain reaction (PCR) was then conducted using specific primers designed to amplify partial mitochondrial cytochrome *c* oxidase 1 (*cox1*) gene in diphyllobothriid species: Dl/nco1f1 (5′-TAGCTGCTGCTATACAATGTTGTTATT-3′) and Dl/nco1r1 (5′-ACGACGTGGTAAACCGCACACACCAAA-3′) for the first PCR of the outer region, subsequently followed by the second PCR of the inner region using Dl/nco1f2 (5′-GATCCTATATTATTTCAGCATATG-3′) and Dl/nco1r2 (5′-TAGAAACTATACAATGACATTGTA-3′). PCR products were sequenced using BigDye® Terminator v3.1 cycle sequencing kit by ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA).

The basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used in evaluation of genetic identity of the samples. Using the Geneious® version 6.1.6 (Biometers Ltd., Auckland, New Zealand), we aligned the obtained sequences with GenBank reference *cox1* sequences of diphyllobothriid species: AB573407 (Japan), AB375662 (Russia), AB684623 (China), AM412559 (Switzerland), AM778552 (Canada) for *D. nihonkaiense*, and DQ985706 (Russia), AB269325 (Japan), AB504899 (Chile), AM778554 (Italy), FM209180 (Switzerland) for *D. latum*. All 32 samples were more closely related to *D. nihonkaiense* (98.6–100 % identity) than to *D. latum* (92.8–92.9 % identity).

Morphometric examinations

The eggs of all 32 *D. nihonkaiense* specimens were measured to verify the claim that *D. nihonkaiense* has smaller eggs than *D. latum* (Yera et al. 2006; Wicht et al. 2007, 2008; Arizono et al. 2009b). We extracted the eggs from the uteri of at least two gravid proglottids of each tapeworm specimen (n=32) and measured the length and width of ten eggs per each specimen (total 320 eggs) by using an ocular micrometer under light microscopy (× 400 magnification).

Results

The eggs of *D. nihonkaiense* were morphologically not different from those of classical *D. latum*. They were ovoid to ellipsoidal, with relatively thick and refractile egg shell, and immature without a larval worm inside (Fig. 1). They were operculate with a wide operculum at the anterior end and equipped with a terminal knob at the posterior end.

The egg size of *D. nihonkaiense* varied remarkably according to individual specimens; however, within each specimen, the variation was generally not marked (Fig. 2). Some worms revealed smaller length and width (cases no. 7, 9, and 31), whereas others showed larger length and width (cases no. 1, 5, 29, and 32). Few specimens had a mixture of both smaller and larger eggs. Most other worms had medium length and width eggs (60–70 μm long and 40–50 μm wide). The worm showing the smallest egg size (by length) revealed a length range of 54–62 μm, whereas the one showing the largest egg size (by length) revealed a length range of 68–76 μm. These values were nonoverlapping (Fig. 2). A similar figure was seen for the egg width (Fig. 2).

When the size of 320 eggs from 32 specimens was plotted together, a wide variation in the egg size was apparent (Fig. 3). They were 54–76 μm (95 % confidence interval (CI), 56–73) in length and 35–58 μm (95 % CI, 37–52) in width, with the length-width ratio of 1.32–1.70 (95 % CI, 1.3–1.6). The mean values were 64 μm in length, 45 μm in width, and 1.46 in length-width ratio. The majority of eggs were within the size range of 60–70 μm in length and 40–50 μm in width (Fig. 3). In addition, the egg size range of *D. nihonkaiense* obtained in the study was broader than that reported previously for *D. nihonkaiense* or *D. latum* (Fig. 3).

Table 1 Cases analyzed in the study

Case no.	Collection date	Locality	Patient		Morphologic identification	Genetic identification	Fixatives
			Age	Sex			
1	Oct-1975	Seoul	10	M	*D. latum*	*D. nihonkaiense*	10 % formalin
2	Mar-1982	Seoul	48	M	*D. latum*	*D. nihonkaiense*	10 % formalin
3	Aug-1984	unknown	36	M	*D. latum*	*D. nihonkaiense*	10 % formalin
4	Oct-1986	Ulleungdo	20	M	*D. latum*	*D. nihonkaiense*	10 % formalin
5	Nov-1986	Ulleungdo	30	M	*D. latum*	*D. nihonkaiense*	10 % formalin
6	Apr-1987	Seoul	27	M	*D. latum*	*D. nihonkaiense*	10 % formalin
7	Jun-1987	Seoul	43	M	*D. latum*	*D. nihonkaiense*	10 % formalin
8	Jun-1987	Seoul	43	M	*D. latum*	*D. nihonkaiense*	10 % formalin
9	Dec-1991	Seoul	52	M	*D. latum*	*D. nihonkaiense*	10 % formalin
10	Feb-1994	Hoengseong	21	M	*D. latum*	*D. nihonkaiense*	10 % formalin
11	Jun-1995	Seoul	41	M	*D. latum*	*D. nihonkaiense*	10 % formalin
12	Jan-1996	Seoul	30	M	*D. latum*	*D. nihonkaiense*	10 % formalin
13	Jun-1996	Seoul	38	F	*D. latum*	*D. nihonkaiense*	10 % formalin
14	Oct-1997	Hwasun	27	M	*D. latum*	*D. nihonkaiense*	10 % formalin
15	Jan-1999	Hwasun	53	F	*D. latum*	*D. nihonkaiense*	10 % formalin
16	Jun-1999	Hwasun	33	M	*D. latum*	*D. nihonkaiense*	70 % ethanol
17	Aug-1999	Seoul	50	M	*D. latum*	*D. nihonkaiense*	70 % ethanol
18	Dec-1999	Seoul	37	F	*D. latum*	*D. nihonkaiense*	10 % formalin
19	Jul-2000	Seoul	45	M	*D. latum*	*D. nihonkaiense*	10 % formalin
20	Jul-2000	Seoul	7	F	*D. latum*	*D. nihonkaiense*	70 % ethanol
21	Feb-2001	Seoul	38	M	*D. latum*	*D. nihonkaiense*	10 % formalin
22	Sep-2001	Gunpo	43	F	*D. latum*	*D. nihonkaiense*	10 % formalin
23	Mar-2002	Seoul	59	M	*D. latum*	*D. nihonkaiense*	10 % formalin
24	Unknown	Seoul	31	M	*D. latum*	*D. nihonkaiense*	10 % formalin
25	Unknown	Unknown	40	F	*D. latum*	*D. nihonkaiense*	10 % formalin
26	Unknown	Unknown	26	M	*D. latum*	*D. nihonkaiense*	10 % formalin
27	Unknown	Unknown	–	–	*D. latum*	*D. nihonkaiense*	10 % formalin
28	Unknown	Unknown	–	–	*D. latum*	*D. nihonkaiense*	10 % formalin
29	Mar-2013	Seoul	56	M	*Diphyllobothrium* sp.	*D. nihonkaiense*	70 % ethanol
30	Mar-2014	Seoul	27	F	*Diphyllobothrium* sp.	*D. nihonkaiense*	70 % ethanol
31	Mar-2014	Seoul	25	M	*Diphyllobothrium* sp.	*D. nihonkaiense*	70 % ethanol
32	Mar-2014	Seoul	16	F	*Diphyllobothrium* sp.	*D. nihonkaiense*	70 % ethanol

Case nos. 1–28 were included among the cases reported by Jeon et al. [4]

Discussion

The presence of diphyllobothriid tapeworm infections in Korea was briefly mentioned in several old reports based on recovery of eggs in human feces (Lee et al. 1983). However, the first report of an adult tapeworm from a human in 1971 was designated *D. latum* (Cho et al. 1971). Thereafter, approximately 110 cases of *Diphyllobothrium* sp. infection were documented, and the number of case reports has increased recently (Lee et al. 2007; Jeon et al. 2009; Park et al. 2013; Song et al. 2014; Kim et al. 2014; Shin et al. 2014). *D. nihonkaiense* was first characterized by molecular studies of the parasite gene in Korea (Jeon et al. 2009). Prior to this report, most of the diphyllobothriid tapeworms recovered from humans were simply assigned as *D. latum* based on morphology of adult tapeworms and eggs (Lee et al. 2007). Among other diphyllobothriid tapeworm infections, one case of *Diphyllobothrium yonagoense* infection (Lee et al. 1988) and two cases of *Diphyllobothrium parvum* infections (Lee et al. 1994) are documented. No imported cases of diphyllobothriid tapeworm infections have been reported in Korea. Thus, the present study strongly suggested that all previously reported cases of *D. latum* in Korea are actually *D. nihonkaiense* infection.

When *D. nihonkaiense* was first described as a new species in Japan (Yamane et al. 1986), the average egg size

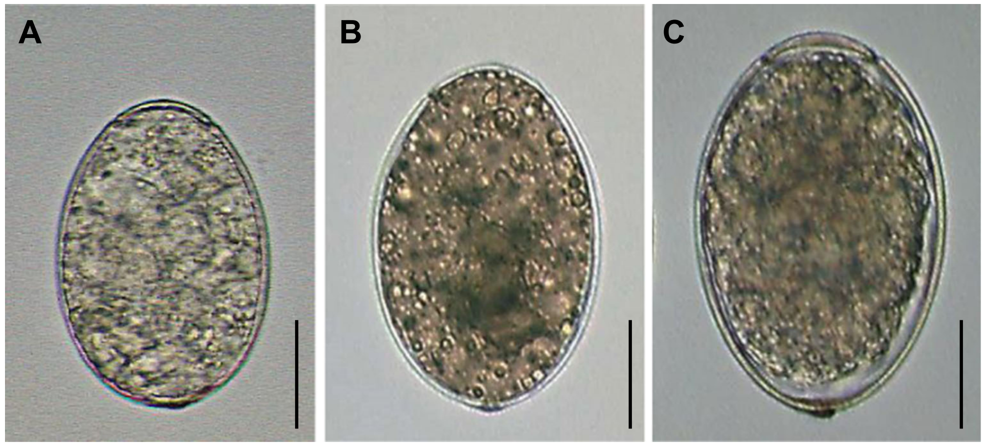

Fig. 1 **a–c** Eggs of *Diphyllobothrium nihonkaiense* showing variable sizes. They are ovoid to ellipsoidal, *brown*, and equipped with a wide operculum at the anterior end and a terminal knob at the posterior end. **a** An egg of small size from case no. 7 (56×39 μm). **b** An egg of medium size from case no. 4 (61×41 μm). **c** An egg of large size from case no. 5 (71×47 μm). *Scale bar*=20 μm

in the uterine loops of adult worms grown in experimental golden hamsters was 55.2×38.2 μm that was smaller than that of *D. latum* grown in the same experimental animals. However, the eggs from the strobilae of *D. nihonkaiense* and *D. latum* collected from human cases could not be differentiated from each other, and the sizes overlapped (Yamane et al. 1986). Later, the eggs (60×43 μm) of molecularly proven *D. nihonkaiense* worms (Ando et al. 2001) and those (60×41 μm) in the feces of a *D. nihonkaiense*-infected French woman were reported to be smaller than those of *D. latum* (75×55 μm) (Year et al. 2006). Subsequently, smaller egg size began to be used as a criterion to discriminate *D. nihonkaiense* from *D. latum* (Wicht et al. 2007, 2008). There was no significant difference between the length-width ratios of *D. nihonkaiense* and *D. latum* eggs (Table 2).

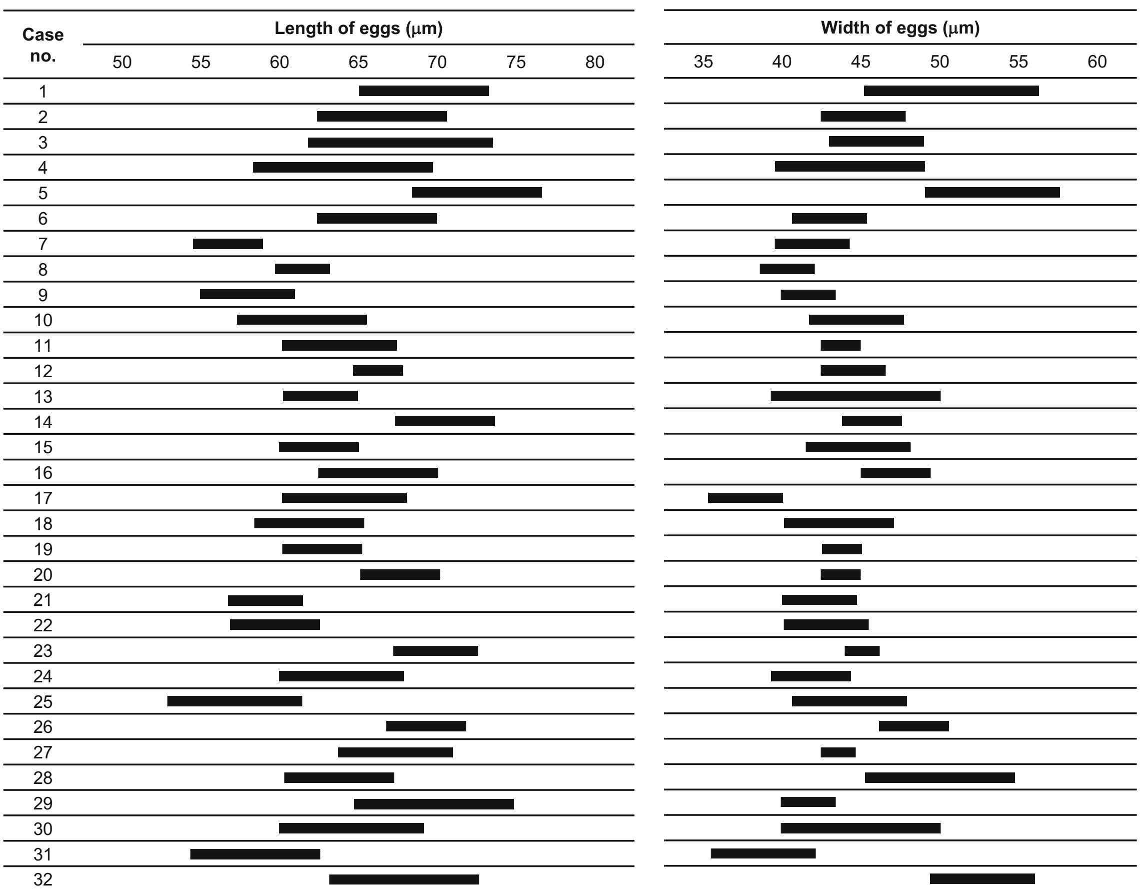

Fig. 2 Egg size range (length and width) of molecularly confirmed *D. nihonkaiense* adult tapeworms (n=32) recovered from Korean patients. Uterine eggs were taken from at least two gravid proglottids of each specimen, and 10 eggs were measured for each specimen. The egg size appeared to vary remarkably by individual specimens, from those having small-sized eggs to those having medium- or large-sized eggs

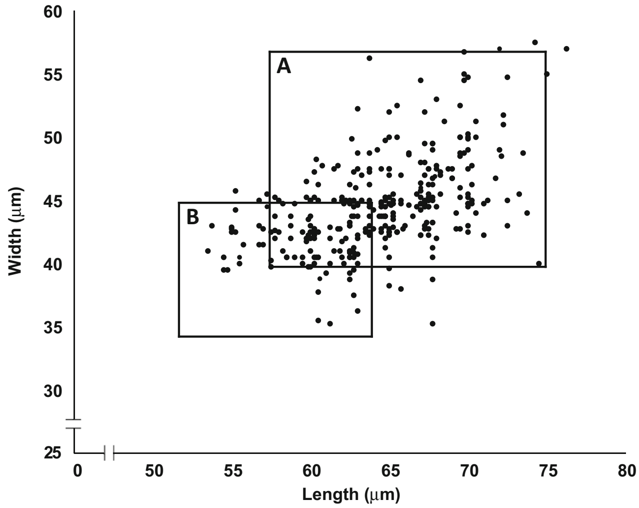

Fig. 3 Distribution of the egg size of molecularly confirmed *D. nihonkaiense* adult tapeworms (10 eggs each were measured from 32 specimens, totaling 320 eggs). The mean egg size was 64×45 µm, and the range was 54–76×35–58 µm (95 % CI, 56–73×37–52 µm). *Box* "*A*" indicates the range of egg sizes of previously reported *D. latum* (von Bonsdorff 1977; Andersen and Halvorsen 1978; Beaver et al. 1984; Peduzzi and Boucher-Rodoni 2011; Lou et al. 2007; Wicht et al. 2007), whereas *box* "*B*" represents the range of previously reported *D. nihonkaiense* egg size (Yamane et al. 1986; Ando et al. 2001; Yera et al. 2006; Wicht et al. 2007, 2008; Shimizu et al. 2008; Arizono et al. 2009b)

We demonstrated that the egg size of 32 molecularly confirmed *D. nihonkaiense* specimens from Korea varied more greatly than that previously reported for *D. nihonkaiense* (Ando et al. 2001; Yera et al. 2006; Wicht et al. 2007, 2008; Shimizu et al. 2008; Arizono et al. 2009b) (Table 2, Fig. 3). Moreover, the egg size range of *D. nihonkaiense* was wide enough to overlap the range of the previously reported *D. latum* eggs (von Bonsdorff 1977; Andersen and Halvorsen 1978; Beaver et al. 1984; Peduzzi and Boucher-Rodoni 2011; Lou et al. 2007; Wicht et al. 2007). Thus, egg size appears to be an inadequate distinguishing feature between the two diphyllobothriid species recovered from human infections, in agreement with Yamane et al. (1986). In addition, our findings supported the necessity of molecular analysis for specific diagnoses of *D. nihonkaiense* and *D. latum* infection in humans.

Wild pacific salmon was suggested as the most important source of infection with *D. nihonkaiense* in Japan and other countries (Arizono et al. 2009a). However, several Korean cases had consumed fish other than the salmon, which included the trout, perch, and mullet (Lee et al. 2007). Therefore, further study on the source of human infection with *D. nihonkaiense* in Korea is required. The annual clinical incidence of *D. nihonkaiense* infection in two Japanese institutions in Tokyo and Kyoto showed an apparent surge in the recent years (Arizono et al. 2009a). Similarly, in Korea, *D. nihonkaiense* cases have also been on the rise since 2013 (Park et al. 2013; Song et al. 2014; Kim et al. 2014; Shin et al. 2014). Case occurrence, as well as the source of diphyllobothriasis infection in Korea, requires careful monitoring.

Table 2 Comparative egg sizes of *Diphyllobothrium nihonkaiense* and *Diphyllobothrium latum* from humans among the literature

Reporter (year)	*D. nihonkaiense*			*D. latum*		
	Length (µm)	Width (µm)	L/W ratio	Length (µm)	Width (µm)	L/W ratio
von Bonsdorff (1977)				65	45	
Andersen and Halvorsen (1978)				64 (±1.7)	46 (±1.6)	1.37 (±0.03) (hamster)
Andersen and Halvorsen (1978)				66 (±1.0)	49 (±0.6)	1.35 (±0.03) (arctic fox)
Beaver et al. (1984)				66 (58–76)	44 (40–51)	
Peduzzi and Boucher-Rodoni (2011)				70	50	
Yera et al. (2006)				75	55	
Lou et al. (2007)				68 (64–74)	47 (45–49)	1.4
Wicht et al. (2007)				72 (±1.2)	57 (±2.3)	
Yamane et al. (1986)	55 (±1.3)	38 (±1.5)	1.45 (±0.06)			
Ando et al. (2001)	60	43				
Yera et al. (2006)	60	41				
Wicht et al. (2007)	57 (±0.6)	45 (±0.9)				
Wicht et al. (2008)	53–59	35–40				
Shimizu et al. (2008)	58–65	40–43				
Arizono et al. (2009b)	61 (55–65)	39 (33–44)				
Jeon et al. (2009)	56 (±1.0)	41 (±1.5)	1.37±0.06			
Present study	64 (54–76)	45 (35–58)	1.46 (1.3–1.7)			

Conflict of interest The authors declare that they have no conflict of interest related to this study.

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