□ Parasitology □ Technical note

Duplex Real-time PCR Assay for the Detection of Two Intestinal Parasites, *Heterakis isolonche* and *Glaphyrostomum* sp., in Okinawa Rail (*Gallirallus okinawae*)

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ヤンバルクイナ (*Gallirallus okinawae*) の消化管内寄生虫2種 (*Heterakis isolonche* と *Glaphyrostomum* sp.) を検出する Duplex Real-time PCR Assay

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ABSTRACT. A duplex real-time polymerase chain reaction (PCR) assay was developed to decrease expended time for diagnosis as well as to improve on the sensitivity and specificity of current molecular diagnostic methods for the detection and differentiation of *Heterakis isolonche* and *Glaphyrostomum* sp. in Okinawa rail (*Gallirallus okinawae*). Novel PCR primers and probes were designed based on cytochrome c oxidase subunit I gene. Our results indicated that the assay could simultaneously detect both species without cross-amplification of Okinawa rail DNA. The assay detected DNA from a single parasite egg for both species and successfully amplified DNA extracted from feces. Thus, this new assay is an useful technique for monitoring the levels of parasite infection in Okinawa rail captive breeding program.

Key words : duplexreal-time PCR, intestinal parasites, Okinawa rail

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Okinawa rail (*Gallirallus okinawae*) was first described in 1981 and is an almost flightless avian species endemic to the northern part of Okinawa Island, where it is called "Yambaru area" [1]. The species is reported to be monogamous and territorial. Nests are constructed on ground during the breeding season in May and July and clutches of 2 - 4 eggs are incubated for 21 days [2, 3]. Wild populations of Okinawa rail are in decline due to habitat loss and predation by feral cats, dogs and also potentially Javan mongooses (*Herpestes javanicus*) [2]. Comparison of the distribution of this species between 1985 and 2004 revealed that the southern distribution border had moved 15 km northwards [3]. This indicates that there has been at least a 40% reduction in habitat since 1985. Furthermore, there was an estimated 1,500 to 2,100 birds in 1985, with a subsequent reduction in population size to 820 to 1,300 birds in 2006 [3]. Because of critical habitat loss and declining population size, Okinawa rail has been protected as a National Endangered Species since 1993. In 2006, this bird species was categorized as Critically endangered and represents one of the most endangered species in Japan.

Subsequently, the Ministry of Environment in Japan initiated a captive breeding project for the species in 2008 as a strategy to prevent its extinction. Infectious and parasitic disease monitoring is included as an important part of this captive breeding program to maintain the birds' health for successful reproduction. We previously developed a nested polymerase chain reaction (PCR) assay to detect *Heterakis isolonche* and

Glaphyrostomum sp., which are the main intestinal nematode and trematode, respectively, found in Okinawa rail [4, 5]. However, the nested PCR assay is not high enough sensitivity to detect single trematode egg, and thus it may give an inaccurate diagnosis result. Moreover, the nested PCR assay demanded twice amplifications: first by universal primers and second by species-specific primers, resulting in twice the time consumed for diagnosis. And after the second PCR, electrophoresis must be conducted to visualize PCR products after ethidium bromide staining. These procedures are time-consuming and laborious. Thus the need has arisen for developing a new diagnostic method to increase the sensitivity of identification and decrease expended time for diagnosis. Fluorescence-based real-time PCR has started to demonstrate its potential utility in the field of clinical parasitology [6-8]. In comparison to the PCR methods, main features of the new infectious disease diagnosis method are the shorter turn-around times and, very important, minimized risk of the confusion of PCR mixtures, since no post PCR amplification analysis. The goal of our present research was to improve on the previous diagnostic protocol by developing a duplex real-time PCR assay which can specifically and simultaneously detect and differentiate *H. isolonche* and *Glaphyrostomum* sp.

Primers and probes for the duplex real-time PCR assay were designed based on of the cytochrome c oxidase subunit I (*cox1*) gene sequence from *H. isolonche* (GenBank accession no. FJ009625, FJ009626, FJ009627) and *Glaphyrostomum* sp. (GenBank accession no. FJ713138). Sequences for the primers and probes are shown in Table 1.

Amplification for the duplex real-time PCR assay involved an initial denaturation step at 95℃ for 30 sec and 40 cycles of denaturation at 95℃ for 5 sec, annealing at 55℃ for 20 sec and extension at 72℃ for 15 sec. Assays were conducted using a Smart Cycler® System (Takara Bio Inc., Shiga Prefecture, Japan) with threshold value set at 30. PCR reaction mixtures were prepared using CycleavePCR ™ Core Kit CY501 (Takara Bio Inc.) in a final volume of 25 μl, containing 1 μl of template deoxyribonucleic acid (DNA), 2.5 μl of 10 × Cycleave PCR Buffer, 5 μl of 25 mM Mg^{2+}solution, 3 μl of 2.5 mM each dNTP mix, 0.5 μl of 200U/μl TliRNase H II, 0.25 μl of 5U/μl *TaKaRa Ex*Taq ™ HS, 0.5 μl of each primer specific to *H. isolonche* (20 μM), 0.25 μl of each primer specific to *Glaphyrostomum* sp. (20 μM) and 1 μl of each probe (5 μM) and up to 25 μl of dH_2O.

Genomic DNA of *H. isolonche* (n=21), *Glaphyrostomum* sp. (n=11) and Okinawa rail (blood: n=2, intestine: n=2) were extracted using a QIAamp® DNA Mini Kit (Qiagen, Tokyo, Japan)following the manufacturer's protocol. All DNA isolates were stored at −30℃ until used for PCR amplification. In addition, DNA samples were prepared from two fecal samples containing both *H. isolonche* and *Glaphyrostomum* sp. eggs, as confirmed by microscopic observation. A QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan) was used for fecal DNA extraction. Extracted DNA was used for specificity testing of the primers and probes (Table 2).

Eggs of *H. isolonche* and *Glaphyrostomum* sp. were collected from mature adult parasites using a glass micropipette. DNA from 50 eggs of *H. isolonche* and 100 eggs of *Glaphyrostomum* sp. was extracted using a QIAamp DNA Stool Mini Kit following the manufacturer's protocol. Then 7.8 ng/μl of DNA extraction in 50 eggs of *H. isolonche* and 8.3 ng/μl in 100 eggs of *Glaphyrostomum* sp. were obtained. Subsequently, a 50 times dilution of *H. isolonche* extraction and 100 times dilution of *Glaphyrostomum* sp. extraction were performed to create aliquots containing DNA amounts equivalent to 1 egg/μl of *H. isolonche* and *Glaphyrostomum* sp. These extracted DNA samples were used for sensitivity testing of the PCR assay.

Specificity testing demonstrated an appropriate fluorescence signal without the interference of any non-specific signals was only observed when relevant DNA template, primers and probe were added to the real-time PCR reaction mixtures (PCR mixture 1-6, Table 2). Then a duplex real-time PCR assay (PCR mixture 7-9, Table 2) was performed and also observed an appropriate fluorescence signal without the interference of any non-specific signals (Fig.1). We further applied the duplex real-time PCR assay to other genomic DNA samples from *H. isolonche* (n=20), *Glaphyrostomum* sp. (n=10) and fecal DNA containing eggs from both species. Corresponding fluorescence signals were observedfor each of these samples using the duplex real-time PCR assay. In addition, DNA isolates from two blood samplesand two intestine samples of Okinawa rail were also tested with the duplex real-time PCR assay

Table 1 Primers and probes designed for the duplex real-time PCRassay

	Heterakis isolonche	Glaphyrostomum sp.
Primer	5' -CTYTRGGWATRGTWTATGCTATTTTAA-3'	5' -AAGCTTATGGCTGTTGTTACCTTCA-3'
	5' -CAAWAMAGGTTGAAAAACYATYTTAG-3'	5' -CGAAAGTCCAGCCAAATGTAATG-3'
Probe	5' (Eclipse)-GTAGTTTGGGCT (ROX)-3'	5' (Eclipse)-AAAGTTCACCCC (FAM)-3'

Table 2 Preparation of specificity testing for primers and probes.
("○" means mixed materials in PCR mixture, "+"means positive result, "−"means negative result)

	Genomic DNA		Primer set	
	Heterakis isolonche	*Glaphyrostomum* sp.	*H. isoloncheе*	*Glaphyrostomum* sp.
PCR mixture1	○		○	
PCR mixture2	○			○
PCR mixture3		○		○
PCR mixture4		○	○	
PCR mixture5	○	○	○	
PCR mixture6	○	○		○
PCR mixture7	○		○	○
PCR mixture8		○	○	○
PCR mixture9[1)]	○	○	○	○

	Probe		Result	
	H. isoloncheе (ROX)	*Glaphyrostomum* sp. (FAM)	*H. isoloncheе* (ROX)	*Glaphyrostomum* sp. (FAM)
PCR mixture1	○		+	−
PCR mixture2		○	−	−
PCR mixture3		○	−	+
PCR mixture4	○		−	−
PCR mixture5	○		+	−
PCR mixture6		○	−	+
PCR mixture7	○	○	+	−
PCR mixture8	○	○	−	+
PCR mixture9[1)]	○	○	+	+

[1)] The result was shown in Fig.1.

and no cross-amplification of this DNA was observed. These results indicated that the novel duplex real-time PCR assay was specific and did not amplify Okinawa rail DNA.

Assessment of the sensitivity of the duplex real-time PCR assay using DNA dilutions containing equivalent to each single egg of *H. isolonche* and *Glaphyrostomum* sp. indicated that this assay was highly sensitive and could simultaneously detect a single egg of both parasites (Fig. 2).

The goal of our research was to improve on the current parasite diagnostic protocolused for the Okinawa rail. We previously developed a nested PCR assay to detect *Heterakis isolonche* and *Glaphyrostomum* sp. [5]. The nested PCR assay was required twice of PCR and electrophoresis in each species. And at least five *Glaphyrostomum* sp. eggs were required for positive result. On the other hand, our present results suggest that the newly developed duplex real-time PCR assay can specifically and simultaneously detect and differentiate *H. isolonche* and *Glaphyrostomum* sp. in Okinawa rail. This assay can successfully detect DNA from a single egg and can also be applied to feces. Our novel assay can therefore be used for differentiating parasite species based on even one parasite egg.

H. isolonche infection results in the development of nodular granulomas and nodular typhlitis in pheasants and turkeys [9-12], while the pathogenicity of *Glaphyrostomum* sp. is currently unclear. There are currently no reports on the pathogenicity, including morbidity and mortality, of *H. isolonche* and *Glaphyrostomum* sp. infection in wild Okinawa rails. However, it might be possible that these parasite species produce clinical conditionsin captive Okinawa rail as observed for other captive birds due to captive environment stress, especially when wild individuals are introduced into a captive breeding population [13, 14]. Implementation of a quarantine procedure and regular parasite checks using fecal samples are therefore important to control parasite load in captive populations. Our newly developed duplex real-time PCR assay can be applied to fecal samples and thus, offers an effective

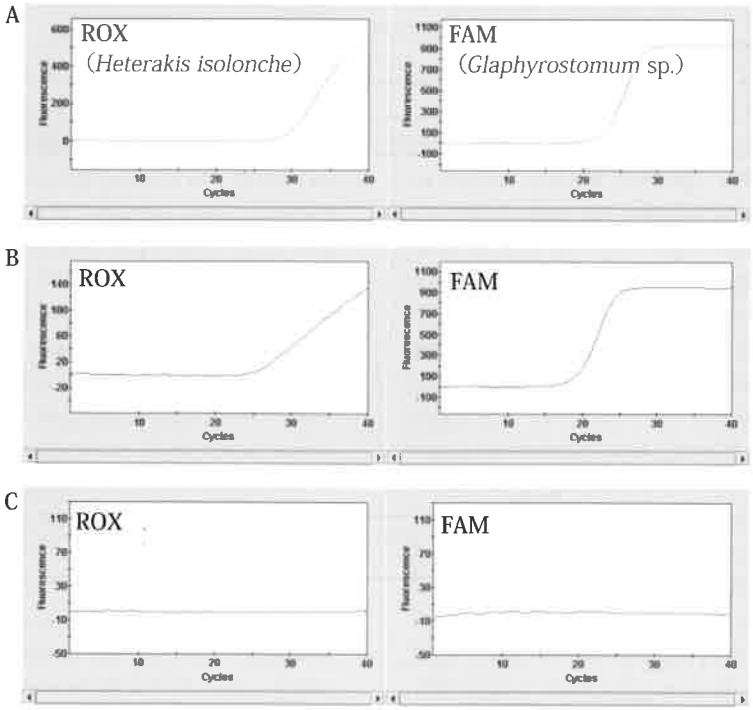

Fig. 1 A result for the newly developed duplex real-time PCR assay

A : PCR mixture containing genomic DNA of *Heterakis isolonche* and *Glaphyrostomum* sp, primer sets for both parasites and probes for both parasites

B : Positive control

ROX : PCR mixture containing genomic DNA of *H. isolonche* only, primer sets for both parasites and probes for both parasites

FAM : PCR mixture containing genomic DNA of *Glaphyrostomum* sp. only, primer sets for both parasites and probes for both parasites

C : Negative control

strategy for monitoring of parasite load in captive Okinawa rail. It is possible that other parasite species could be found in Okinawa rails when further researches are conducted. Thus false positive could occure due to the genome from the other parasite. However other real time PCR base species identification technique, called high-resolution DNA analysis (HRM) [15], could be applied with the same primers which were used in the present study. Thus further researches on parasite species identification using HRM in Okinawa rails must be conducted.

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要　　約

ヤンバルクイナ (*Gallirallus okinawae*) の消化管内寄生虫２種について Duplex real-time PCR assay を開発した。本法はヤンバルクイナ由来の DNA の影響を受けずに２種を同時に検出できることを確認した。また，寄生虫卵１個分の DNA 量も検出でき，糞便にも応用可能であった。本法はヤンバルクイナ飼育個体群を対象として寄生虫検査を糞便サンプルで実施する場合に特に有用な手法である。

キーワード：ヤンバルクイナ，消化管内寄生虫，Duplex real-time PCR assay

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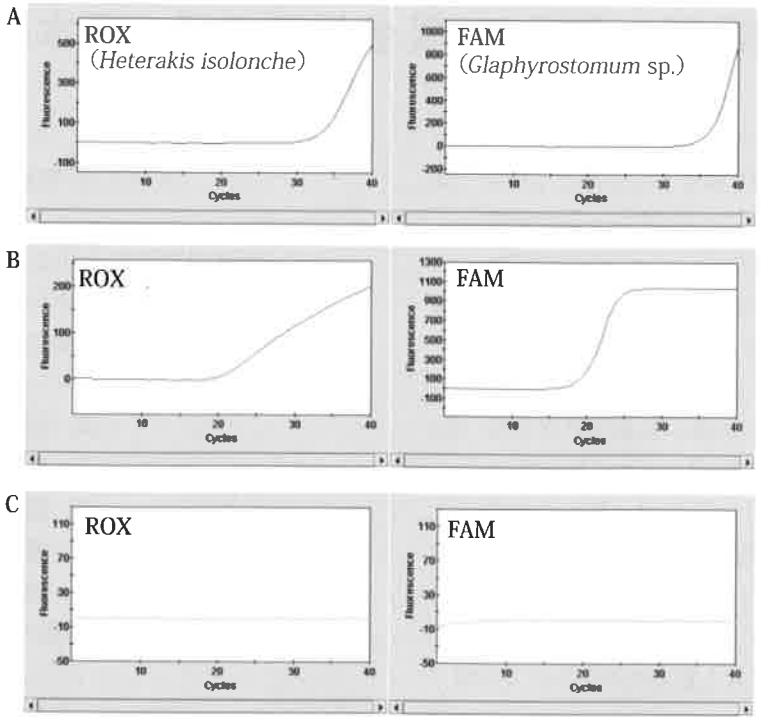

Fig. 2 Sensitivity testing of the duplex real-time PCR assay using parasite eggs.
A : DNA mixture of *Heterakis isolonche* and *Glaphyrostomum* sp. single-egg
B : Positive control
C : Negative control

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