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Validation of a cheap and simple nondestructive method for obtaining AFLPs and DNA sequences (mitochondrial and nuclear) in amphibians

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Abstract

The use of nondestructive methods for obtaining DNA from amphibians (e.g. buccal swabs) allows genetic studies to be performed without affecting the survival of the studied individuals. In this study, we compared two methods of nondestructive DNA sampling, buccal swabs and interdigital membrane or toe-clipping, in several amphibian species of different size: *Rhinella spinulosa*, *R. atacamensis*, six species of the genus *Telmatobius* and *Pleurodema thaul*. We evaluated the integrity of the DNA extracted by sequencing fragments of mitochondrial and nuclear genes and by generating amplified fragment length polymorphisms markers (AFLPs). In all cases, we obtained an adequate amount of DNA (mean range 55–298 ng/μL). We obtained identical DNA sequences from buccal swab and interdigital membrane/toe-clip for all individuals. The differences in the coding of AFLP markers between the tissues were similar to those reported for replicas of the same type of sample in similar analyses in other species of amphibians. In conclusion, the use of buccal swabs is a trustworthy and inexpensive method to obtain DNA for mitochondrial and nuclear sequencing and AFLP analyses. Given the types of markers evaluated, buccal swabs may be used for phylogenetic, phylogeographic and population genetic studies, even in small amphibians (<33 mm).

Keywords: *12S*, *16S*, AFLPs, *rag1*, *rhodopsin*, amphibians, *control region*, noninvasive sampling

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Introduction

The use of noninvasive or nondestructive genetic sampling is a key factor in studies of molecular ecology and genetic conservation, when the purpose is to monitor wild populations without harming them (Taberlet *et al.* 1999; Beja-Pereira *et al.* 2009). However, in order to perform this kind of sampling, it is necessary to evaluate the reliability of the samples with pilot studies (Taberlet & Luikart 1999; Pidancier *et al.* 2003), which allow comparison with destructive sampling (Keyghobadi *et al.* 2009), identification of errors in genotyping (Taberlet & Luikart 1999; Pidancier *et al.* 2003; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009) and consideration of intraspecific variation, contrasting with known data from the geographic area which will be studied (Waits & Paetkau 2005).

To obtain genetic data, large quantities of fresh tissue are often required, which sometimes implies sacrifice of the individuals (Taberlet & Luikart 1999). Also, nonlethal alternatives for obtaining tissues are frequently used. The technique of toe-clipping is commonly used in amphibians (e.g. Funk *et al.* 2005; Spear *et al.* 2005), although the debate about whether this procedure affects the posterior survival of individuals is still open (McCarthy & Parris 2004; Phillott *et al.* 2007; Grafe *et al.* 2011; Perry *et al.* 2011). Alternatively, sampling a small piece of the interdigital membrane (e.g. Correa *et al.* in press) is a less invasive method than toe-clipping, because the latter destroys part of a bone. Recently, it has been proposed to use blood extraction via puncture of the abdominal medial vein to obtain DNA (Mendoza *et al.* 2012).

Another nondestructive method increasingly used to obtain DNA in amphibians is the buccal swab, which has several advantages: it does not affect individual survival (Poschadel & Möller 2004), it requires few field materials, and it is easy to apply and inexpensive compared to other described methods (Pidancier *et al.* 2003). Using buccal swabs, it has been feasible to amplify both mitochondrial (e.g. *cytochrome b*, Pidancier *et al.* 2003; *d-loop*, Poschadel & Möller 2004; Rovito 2010) and nuclear

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markers (e.g. microsatellites, Pidancier *et al.* 2003; Broquet *et al.* 2007; Spear & Storfer 2010). Buccal swabs have also been evaluated to determine the hybrids between species using restriction fragment length polymorphisms (RFLPs) (Patrelle *et al.* 2011).

To our knowledge, there are no reports to date that have evaluated the use of buccal swabs to obtain DNA fragments with the amplified fragment length polymorphism (AFLP) technique. This technique can be used with the DNA of any species and is ideal when there is no previous information on nucleotide sequences (Vos *et al.* 1995), such as for nonmodel species. It is based on the detection of genomic restriction fragments by amplification using the polymerase chain reaction (PCR). However, a restriction and challenge to its application is that a large quantity of highly pure and nondegraded DNA is necessary to generate reliable and comparable profiles, in contrast to mitochondrial DNA (mtDNA) or microsatellites. The technique requires a digestion step with restriction enzymes that cut DNA in specific places and a ligation step with ligation enzymes that add adapters to the blunt ends of the generated fragments, and PCR amplification (Vos *et al.* 1995; Meudt & Clarke 2007). In addition, the nuclear DNA has two copies per cell, unlike mtDNA that has hundreds or thousands of copies (Birky *et al.* 1989).

The aim of this study was to validate a cheap and simple nondestructive method for obtaining DNA for mitochondrial and nuclear sequencing from buccal swabs of amphibians of different sizes. Accordingly, we used *Rhinella atacamensis*, *R. spinulosa*, *Pleurodema thaul* and individuals of six species of the genus *Telmatobius.* We first evaluated the quantity of DNA obtained from buccal mucosa and from a piece of the digital membrane/toe-clip from the same individuals. We then evaluated the integrity of the DNA by amplifying a nuclear (*rag1* or *rhodopsin*) and a mitochondrial (*control region*, *16S* or a fragment including *12S-tRNAval-16S*) gene fragment and generated AFLP markers from both types of tissue. Finally, we compared the genetic data with available information for these species.

Materials and methods

Species studied

We used adult specimens of nine species which together cover a wide range of sizes. We used *Rhinella spinulosa* (snout-vent length (SVL) range = 85–100 mm), *R. atacamensis* (SVL = 65–78 mm), individuals of six species of the genus *Telmatobius* (SVL = 40–67 mm) and *Pleurodema thaul* (SVL = 26–33 mm). The capture sites and the sample size per taxon are given in Fig. 1 and Table 1, respectively. All individuals were

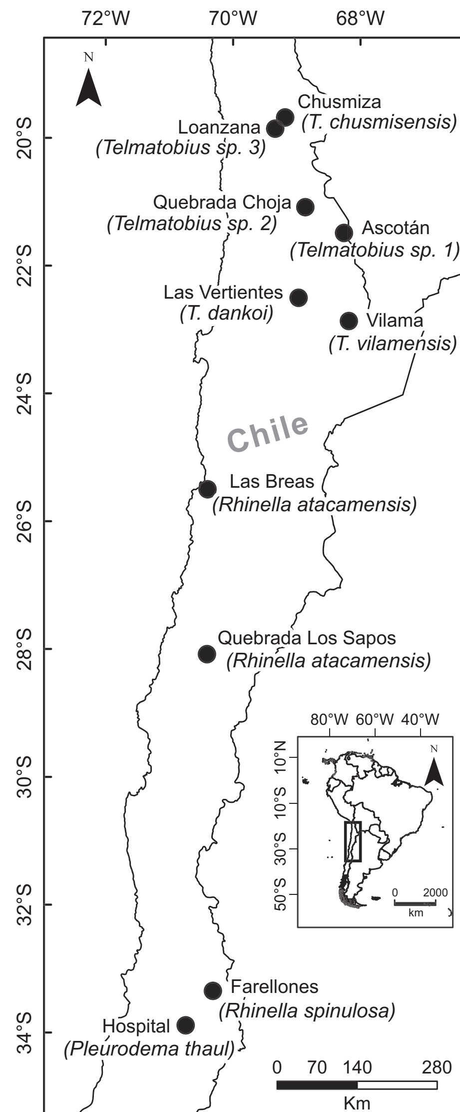

Fig. 1 Geographic location of the sampling localities.

released after taking the samples in the same locations they were collected.

Sampling method

To obtain samples of buccal mucosa, we followed the method of Poschadel & Möller (2004), rubbing the swab gently several times over the interior wall of the buccal cavity. We used commercial cotton swabs, which were first wrapped individually in Kraft paper and sterilized in an autoclave. We then cut the tip of the swab and placed it in buffer solution (Tris–HCl 100 mM pH 7.5, EDTA 100 mM, NaCl 100 mM and SDS 0.5% w/v) to assure the preservation of the DNA (Pidancier *et al.* 2003). In all sampling procedures, we used latex gloves in order to control for contamination. All swabs were

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Table 1 Species studied, sample size (*n*) and quantification of the amount of DNA obtained from the samples of buccal swabs and interdigital membranes (or toe-clipped in the case of *Pleurodema thaul*)

Species	*n*	Type of sample	Mean amount of DNA [ng/μL]	Range of DNA [ng/μL]
Rhinella spinulosa	13	Swab	169.29	114.88–220.38
		Membrane	215.50	34.40–472.79
Rhinella atacamensis	5	Swab	95.90	42.49–144.78
		Membrane	55.07	42.23–92.37
Pleurodema thaul	18	Swab	99.31	64.48–167.02
		Toe-clipped	259.43	79.21–408.2
Telmatobius	10	Swab	296.51	136.22–512.84
		Membrane	148.61	66.25–262.62

stored at 4 °C until extraction. We used a smaller cotton swab for individuals of *Pleurodema thaul* due to their small size. As a control, we used a section of tissue of the interdigital membrane (approximately 2 mm^2) of the same individuals in all species, except for *P. thaul*, where we used a toe-clip because it was not possible to obtain tissue from the interdigital membrane.

DNA extraction and quantification

The DNA of all samples was recovered with a salt extraction method modified from Jowett (1986). DNA was quantified by spectrophotometry (OD260; NanoDrop® ND-1000 Spectrophotometer; Thermo Scientific, Wilmington, DE, USA), and the quality was visualized in a 1.5% agarose gel stained with GelRed™ nucleic acid gel stain (Biotium, Hayward, CA, USA).

Nucleotide sequences

We amplified fragments of a nuclear and a mitochondrial gene for each species. The primers and protocols used to amplify these genes are described in Tables 2 and 3, respectively. Amplification products were sequenced in both directions in an ABI13730XL automatic sequencer (Applied Biosystems, Carlsbad, CA, USA) at Macrogen Inc. sequencing service (Seoul, Korea). The resulting sequences were edited and aligned in BioEdit v.7.0.9.0 software (Hall 1999), compared with each other and with other sequences obtained previously from other individuals of the same species.

AFLP markers

To obtain AFLP markers, we used a modified protocol of Vos *et al.* (1995), which was adapted to use selective fluorescent primers. The reaction mixtures for all species in the steps of digestion, ligation, preselective and selective PCR are detailed in Table S2 (Supporting information). The digestion of DNA was performed at 37 °C for 3 h, after which the enzymes were inactivated at 70 °C for 15 min. Ligation of the adaptors was performed at 16 °C for 10 h. The digested and ligated DNA product was diluted 10 times to perform the preselective PCR. The product of the preselective PCR was diluted 25 times before use in selective PCR. The thermal profile for the preselective PCR was as follows: 94 °C for 2 min, followed by 28 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 2 min, with a final extension of 72 °C for 5 min. The thermal profile for the selective PCR was as follows: 94 °C for 2 min, followed by 12 cycles of 94 °C for 45 s, 65–57 °C for 45 s (decreasing by 0.7 °C in each cycle) and 72 °C for 2 min, followed by 28 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 2 min, with a final extension of 72 °C for 5 min. To choose the primers to be used in selective PCR, previously we performed a screening with 16 combinations of the four primers EcoRI-ACX and the four primers MseI-CAX, where X represents A, C, G or T. Primer EcoRI-ACX has a fluorescent marker in its 5′ extreme (fluorophores 6FAM, VIC, NED or PET; Applied Biosystems). The selective amplification products were separated by capillary electrophoresis in an ABI13730XL automatic analyzer (Applied Biosystems) with G5 filter at Macrogen Inc. sequencing service. The AFLP electropherograms were visualized and processed with the GeneMarker v.1.85 program (SoftGenetics, LLC, State College, PA, USA). We recognized as bands only those signals with intensity above 200 relative fluorescence units (rfu), as long as this value was greater than the background noise. The analysis was restricted to bands of between 55 and 450 bp, depending upon the primer combination (Table S3, Supporting information). We selected only the combinations that showed the strongest signals. According to this criterion, we used four primer combinations for *Rhinella spinulosa*, eight for *R. atacamensis*, five for *Pleurodema thaul* and three for species of genus *Telmatobius* (Table S3, Supporting information). We performed a semi-automatic scoring, generating a binary matrix of presence (1) and absence (0) of bands for each of the defined loci according to the defined

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Table 2 Primers used to amplify nuclear and mitochondrial gene fragments in the different amphibian species studied

Species	*Rhinella spinulosa* and *R. atacamensis*	*Pleurodema thaul*	*Telmatobius* sp.
Nuclear			
Gene	*rag1*	*rhodopsin*	*rag1*
Primer (5′–3′)	Rag1Cm (GGAGAYGTAAGT GAGAAACATGG)	Rhod1Af (ACCATGAACGGAACA GAAGGYCC)	Rag1F (AGCTGCAGYCARTACC AYAARATGTA)
	Rag1Em (TCKGCAGCATTYCCA ATGTCACAG)	Rhod1Dr (GTAGCGAAGAARC CTTCAAMGTA)	Rag1Em (TCKGCAGCATTYCCA ATGTCACAG)
Reference	Modified from Biju & Bossuyt (2003)	Bossuyt & Milinkovitch (2000)	San Mauro *et al.* (2004)/Biju & Bossuyt (2003)
Mitochondrial			
Gene	*Control region*	*12S-tRNAval-16S*	*16S*
Primer (5′–3′)	CytbA-L(GAATYGGRGGWCAACC AGTAGAAGACCC)	1216LN(CCAAYACGTCAGGTCA AGGTG)	16Sar-L (CCGGTCTGAACTCAGA TCACGT)
	ControlP-H (GTCCATAGATTCAST TCCGTCAG)	1216H (TGATTACGCTACCT TYGCACGGT)	16Sbr-H (CCTGTTTATCAAAAACAT)
Reference	Goebel *et al.* (1999)	Modified from Goebel *et al.* (1999)	Palumbi *et al.* (1991)

Table 3 Reaction mixtures and cycling profiles for PCRs of nuclear and mitochondrial markers in each species. Reaction mixtures are given as the final concentration or amount

		Rhinella spinulosa		*Rhinella atacamensis*		*Pleurodema thaul*		*Telmatobius* sp.	
		D-loop	*rag1*	*D-loop*	*rag1*	*12S-tRNAval-16S*	*rhodopsin*	*16S*	*rag1*
Reaction mixture	mgCl$_2$ (mM)	3	3	3	3	3	3	2.5	3
	dNTPs (mM)	0.167	0.1	0.167	0.4	0.6	0.4	0.1	0.1
	Primer forward (μM)	0.2	0.12	0.2	0.12	0.12	0.13	0.67	0.12
	Primer reverse (μM)	0.2	0.12	0.2	0.12	0.12	0.13	0.67	0.12
	Taq polymerase (U)	1.25	1	1.25	1.5	1.25	1	1	1
Cycling reaction	Number of cycles	42	40	42	40	37	37	35	45
	Denaturation (min/°C)	0:30/94	1:00/94	0:30/94	0:30/94	0:35/94	0:45/94	0:30/94	0:40/94
	Annealing (min/°C)	0:45/56	0:45/55	0:45/56	0:45/55	0:45/56	0:55/52	0:45/58	0:50/55
	Extension (min/°C)	1:30/72	1:00/72	1:30/72	1:00/72	1:30/72	0:45/72	0:45/72	1:00/72

parameters; then, this matrix was revised and edited by reviewing each AFLP profile by eye. We calculated the error rate between replicates (swab and membrane/toe-clipped samples) using the mean Jaccard distance (Holland *et al.* 2008). The same person performed all matrix analyses.

Results and discussion

The amounts of DNA obtained from buccal swab, interdigital membrane and toe-clipped samples (Table 1, Table S1, Supporting information) were within the ranges recommended for AFLP finger printing method (Vos *et al.* 1995; Marnik *et al.* 2007). The mitochondrial and nuclear gene fragments were amplified in all samples of *Rhinella spinulosa*, *R. atacamensis*, *Pleurodema thaul* and species of *Telmatobius*; there were no differences between sequences obtained with buccal swab and membrane/toe-clip tissues. Additionally, the sequences corresponded to known data for the species studied [i.e. the same haplotypes and nuclear genotypes previously observed by our group (Correa *et al.* 2010, in press)].

Buccal swabs are susceptible to contamination, and using nonspecific methods such as AFLPs, it would not be possible to distinguish this contamination from real variation (Dyer & Leonard 2000). However, the AFLP genotypes obtained from buccal swabs and membranes were very similar (Fig. 2a,b). The coding error among genotypes for the same individual is given in Table 4; briefly, they were 1.6% for *R. spinulosa*, 0.85% for *R. atacamensis*, 1.74% for *P. thaul* and 1.4% for *Telmatobius*. These values are similar to coding errors reported in other studies of AFLPs in amphibians, 2% in *Rana temporaria* (Bonin *et al.* 2004) and 0.8% in *Calotriton asper* (Milá *et al.* 2010), and in other taxa using nonlethal sampling, for example the lepidopterans *Satyrium behrii* (1.7%) and *Apodemia mormo* (1.8%) (Keyghobadi *et al.* 2009).

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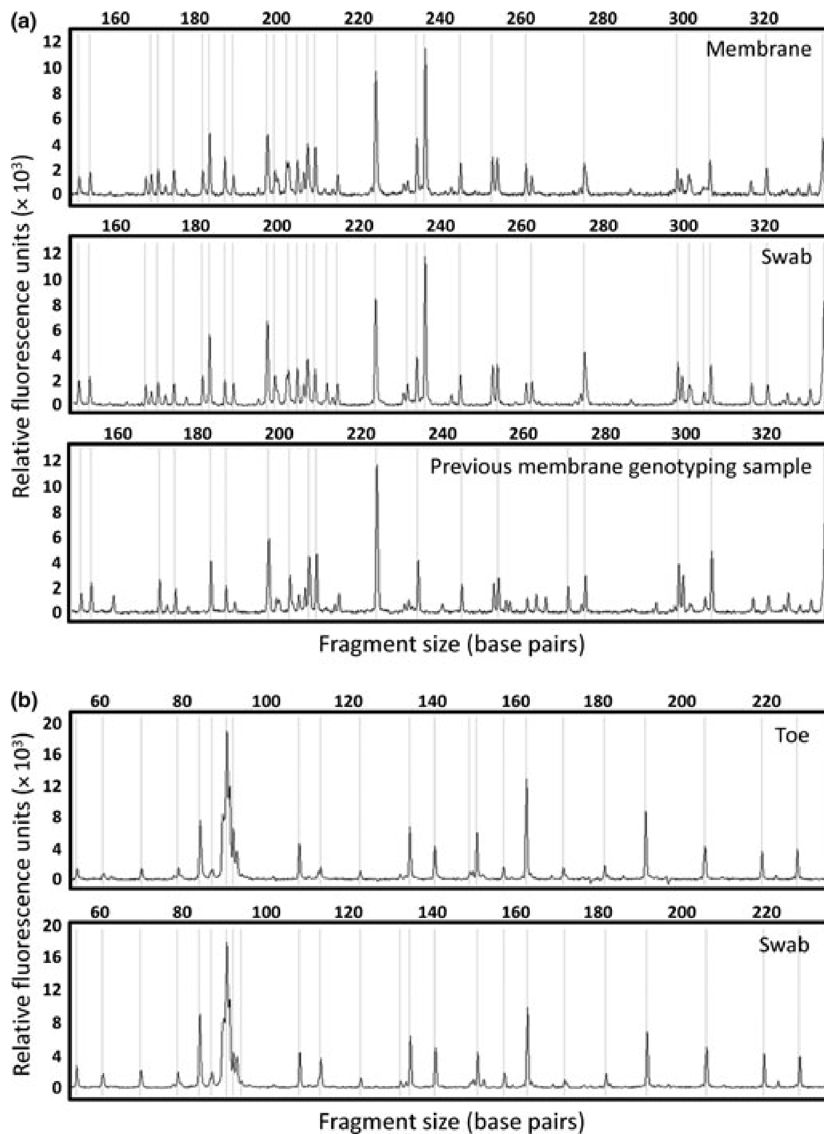

Fig. 2 Electropherograms showing examples of comparison of AFLP markers from membrane/toe-clip and buccal swabs. (a) *Rhinella atacamensis* with the combination EcoRI-ACT/MseI-CAC (fluorophore 6-FAM). Genotypes previously observed from membrane samples are also shown (Correa *et al.* in press). (b) *Pleurodema thaul* with combination EcoRI-ACT/MseI-CAG (fluorophore 6-FAM).

Table 4 Sample size, number of AFLP markers obtained and differences in the coding (presence/absence) of AFLP markers for swab and membrane/toe-clip samples for each species

Species	*n*	Number of AFLP markers	Error rate (%)
Rhinella spinulosa	13	174	1.60
Rhinella atacamensis	5	536	0.85
Pleurodema thaul	18	372	1.74
Telmatobius	10	121	1.40

Although our estimates of coding error are comparable, caution should be taken in the case of species where the sampling size was small, because coding error values could be biased. However, in the case of *R. atacamensis*, one of species with the small sample size (n = 5), our data were similar to the patterns obtained previously by Correa *et al.* (in press). In that study, they used DNA obtained from muscle, interdigital membrane, toes and larvae, and the sample size was larger (n = 16) for the same locality (Quebrada Los Sapos, see Fig. 1, see also Fig. 2a for a comparison of the interdigital membrane results between that study and our data).

According to our results, extracting DNA from buccal swabs allowed us to obtain high-quality DNA, mitochondrial and nuclear DNA sequences and reliable AFLP genotyping. In the case of AFLPs, this is an auspicious result, considering that this technique provides a sweep of the whole genome allowing it (i) to be applied in genetic studies both at the interspecific and at intraspecific level and (ii) to be used especially for amphibians with conservation problems, including species of small size.

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C.C., P.M., L.P., C.G., and P.S. collected the samples, C.C., P.M., L.P., and P.S. performed PCRs, C.G. and L.P. edited nuclear and mitochondrial sequences and C.G. analyzed AFLPs. C.G., M.A.M., and C.C. wrote the manuscript.

Data Accessibility

GenBank (http://www.ncbi.nlm.nih.gov/genbank/) accession numbers for nucleotide sequences obtained

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using swabs: JX442284-JX442301 (12S-tRNAval-16S, *Pleurodema thaul*), JX442302-JX442319 (rhodopsin, *Pleurodema thaul*), JX442320-JX442324 (control region, *Rhinella atacamensis*), JX442325-JX442329 (rag1, *Rhinella atacamensis*), JX442330-JX442342 (control region, *Rhinella spinulosa*), JX442343-JX442355 (rag1, *Rhinella spinulosa*), JX442356-JX442365 (16S, *Telmatobius*), JX442366-JX442375 (rag 1, *Telmatobius*). The DNA concentrations obtained for each extraction are given in Table S1 (Supporting information).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Quantification of the amount of DNA obtained for each individual from the samples of buccal swabs and interdigital membranes (or toe-clipped in the case of *Pleurodema thaul*). Code sample of Herpetological Collection of the Departamento de Biología Celular y Genética de la Universidad de Chile (DBGUCH).

Table S2 AFLP protocols for *Rhinella spinulosa*, *R. atacamensis*, *Pleurodema thaul* and *Telmatobius*. One-Phor-All buffer plus (10X) = 100 mM Tris–acetate, pH 7.5, 100 mM Mg-acetate, 500 mM K-acetate. NEB = New England Biolabs. C_f = final concentration.

Table S3 Primer combinations used in selective PCRs in the AFLP protocol for *Rhinella spinulosa, R. atacamensis, Pleurodema thaul* and *Telmatobius.* The range of fragment sizes assessed in the profiles obtained with each combination is indicated.

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