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ABSTRACT

Over the last two decades, vocal and genetic data have been extensively used in avian studies addressing taxonomic and systematic issues. However, even when multiple lines of evidence lean toward lumping or splitting of species, some taxonomic committees refuse to acknowledge their validity until convincing genetic evidence is produced and integrated with other sources of data. As a case in point, the genus *Pseudocolaptes* (Furnariidae) consists of three mostly allopatric and overtly distinct neotropical taxa differing in plumage and vocal features: the Streaked Tuftedcheek (*P. boissonneautii*), the Buffy Tuftedcheek (*P. lawrencii*), and the contentious Pacific Tuftedcheek (*P. johnsoni*). In this study, we assemble already available mitochondrial and nuclear DNA sequences to assess their taxonomy and to provide appointed committees with specific proof to ascertain the number of *Pseudocolaptes* species. Phylogenetic inference and species delimitation analysis indicate three species equally divergent from each other. We hope that this work will contribute to resolve the long-lasting taxonomical confusion regarding this genus, and that proper future conservation plans will be adopted to preserve each species within the inhabited biodiversity hotspot.

Introduction

Referred to as a fundamental discipline of biology (Wilson et al. 2004), taxonomy relies on multiple lines of evidence spanning both phenotypic and genotypic information. Although initially based merely on morphological characters (Lis & Lis 2011, but see Kotov & Gololobova 2016), taxonomic studies started to incorporate also (if not entirely) genetic data since the 1990s (Vogler & Monaghan 2007). This additional independent source of evidence turned out to be extremely useful, in that the biological species concept, at the basis of traditional taxonomy, relies on the assumption of reproductive isolation, which nonetheless cannot be directly tested on allopatric populations (Sibley & Ahlquist 1990; Grant & Grant 1997). In some animal groups, bioacoustic data also represent an important diagnostic tool (Kvsn et al. 2020). Among passerine birds, vocalizations were first used on a broad scale to infer the taxonomy of suboscines such as Antbirds (Thamnophilidae) (Isler et al. 1998), Tapaculos (Rhinocryptidae) (Krabbe & Schulenberg 1997), and Tyrant Flycatchers (Tyrannidae) (Lanyon 1978), since in this group they were considerate innate and, hence, genetically based (Kroodsma 1984; Kroodsma & Konishi 1991).

Only gradual consensus grew that in oscine passerines vocal differences create reproductive barriers preventing gene flow and thus also bear taxonomic implications (Baptista 1996; Remsen 2005). Over the last two decades, the integration of vocal and genetic data has been used extensively in studies addressing the integrative taxonomy (e.g. Catchpole & Slater 2008; Weir & Wheatcroft 2010; Ng et al. 2018, 2020; Rheindt et al. 2020) and systematics (Alström 2001; Päckert et al. 2003; Farnsworth & Lovette 2008; Robin et al. 2017; Alström et al. 2018) of passerines (Rheindt et al. 2015; Sementili-Cardoso et al. 2018) and non-passerines (King 2002; Rheindt et al. 2011; Ng & Rheindt 2016; Ng et al. 2016). At present, vocal data are being used to tackle specific issues including taxonomic re-assessments (e.g. Rheindt et al. 2008), introgression (Cros & Rheindt 2017), and cryptic speciation (Rheindt et al. 2015; Gwee et al. 2019) as well as intraspecific variability (Boesman 2016; Forcina et al. 2019). In spite of the plethora of complementary tools in present-day taxonomy, the diversity of some avian groups remains largely unexplored, while in other cases the nonetheless multidisciplinary information available is incomplete or fragmentary. Even if several lines of evidence lean toward the distinctiveness of

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⊕ Supplemental data for this article can be accessed here.

one or more species, some taxonomy committees refuse to acknowledge their validity until ad hoc studies assembling different sources of supporting information and/or producing pertinent genetic evidence are carried out (e.g. Saitoh et al. 2015; Ng et al. 2017; Rheindt et al. 2017; Lim et al. 2019; Tyler et al. 2020).

As a case in point, the genus *Pseudocolaptes* (Furnariidae) consists of three overtly distinct groups which differ in plumage and vocal features: the Streaked Tuftedcheek (*P. boissonneautii*), occurring in the Andes from Venezuela to Bolivia; the Pacific Tuftedcheek (*P. johnsoni*), patchily distributed along the western slopes of Colombian and Ecuadorian Andes; and the Buffy Tuftedcheek (*P. lawrencii*), narrowly distributed in the mountains of Costa Rica and Panama (del Hoyo & Collar 2016) (Figure 1). Their taxonomic history is, however, particularly complex (Ridgely & Tudor 1994), with *P. johnsoni* long confused with *P. boissonneautii*, thought to be merely the immature plumage of the latter or lumped with *P. lawrencii* (Figure 2). A more careful examination of morphological features first (Robbins & Ridgely 1990) and vocalizations later spurred the IOC World Bird list and HBW (The Handbook of the Birds of the World) with BLI (BirdLife International) to treat *P. lawrencii* and *P. johnsoni* as two distinct species as currently acknowledged by the IUCN (International Union for Conservation of Nature). Specifically, the morphological differences pointing to the validity of *P. johnsoni* were summarized in del Hoyo and Collar (2016) as follows: 'Until recently, normally considered conspecific with *P. lawrencii*, but differs in its stronger rufous (less buff-tinged) mantle and back; rich rufous vs grey-streaked cream-buff underparts; rufous-tan vs grey-
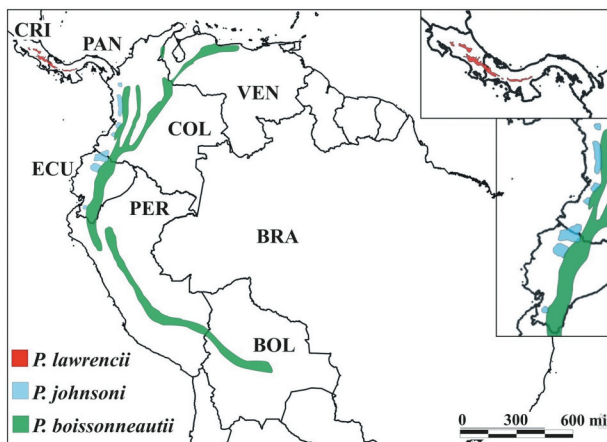

Figure 2. Pictures of the *Pseudocolaptes* species in first basic plumage: a. *P. boissonneautii* (Jardín, Colombia. Photo credit: Juan José Arango); b. *P. johnsoni* (Tangaras Reserve, El Carmen de Atrato, Choco, Colombia. Photo credit: Tom Friedel); c. *P. lawrencii* (Panama. Photo credit: Miguel Rios).

Figure 1. Distribution map of *Pseudocolaptes*. Country codes: CRI: Costa Rica; PAN: Panama; VEN: Venezuela; COL: Colombia; ECU: Ecuador; PER: Peru; BOL: Bolivia; BRA: Brazil. The magnified insets on the right refer to the distribution of *P. lawrencii* (above) and *P. johnsoni* (below).

black outer vanes of primaries; wing-coverts dark grey-brown with vague rufous edges vs blackish with strong rufous tips; shorter tail (sample size too small, but

evidence indicative)' (Supplementary Figure 1). The vocal differences, already treated by Spencer (2011) and Boesman (2016), were summarized in del Hoyo and Collar (2016) as follows: 'divergent song, being a high-pitched rattled series of notes, slowing into stuttering and ending (always) with a characteristic high-pitched down-slurred note, vs several well-spaced staccato notes followed by a trill, which usually ascends in pitch and then descends while slowing in pace.' Moreover, the playback response was evaluated (even though in just one direction) by both Spencer (2011) and, more systematically, Freeman and Montgomery (2017), who found no response of *P. lawrencii* to playbacks of *P. johnsoni*. As far as genetic data are concerned, a first study based on mitochondrial and nuclear DNA markers found *P. boissonneautii* to be sister to the Rusty-winged Barbtail (*Premnornis guttuligera*) (Pérez-Emán et al. 2010). Subsequently, Derryberry et al. (2011) realized the first comprehensive study on the adaptive radiation of Neotropical ovenbirds and woodcreepers, using an expanded panel of DNA loci and a robust dataset of almost 300 species including *P. lawrencii* and *P. boissonneautii*. They proved the monophyly of the genus *Pseudocolaptes* and confirmed its sister relationship to *Premnornis guttuligera*. Nonetheless, a genetic investigation comparing representatives of the three morphological groups is still missing, since Derryberry and coauthors did produce genetic data from an individual of the contentious *P. johnsoni* but did not use such information in their phylogeny nor for any comparative analyses. In spite of the overt differences in phenotypes, the SACC (South American Classification Committee) along with the NACC (North American Classification and Nomenclature Committee) of the AOS (American Ornithological Society) and, as a consequence, the eBird and Clements checklists still treat *P. johnsoni* and *P. lawrencii* as conspecifics. In its most recent assessment, SACC motivated its decision on the basis of the fragmentarity of phenotypic information and the lack of a comprehensive genetic study pointing to the validity of *P. johnsoni* and, hence, the occurrence of three vs two *Pseudocolaptes* species (Remsen 2003). A later proposal to SACC still lacking genetic information (Freeman 2018) remains undecided to date.

In this study, we assemble available genetic data as a completion of multiple lines of evidence to provide taxonomic committees with all the tools needed for making a sound decision about the number and identity of *Pseudocolaptes* species, thus eliminating the confusion arising from conflicting taxonomic treatments and assisting future conservation actions.

Materials and methods

We used the *Pseudocolaptes* sequences from Derryberry et al. (2011) and downloaded the concatenated alignment for the entire Furnariidae family and four genera of its sister clade, Philydorini, as outgroup. Despite having sequenced representatives of both *P. lawrencii* and *P. johnsoni*, the same authors only included the former in their final analyses. We compared these sequences to others available in GenBank (Supplementary information Table 1). The alignment consisted of NADH dehydrogenase subunit 3 (ND3; 351 bp), cytochrome oxidase subunit 2 (CO2; 683 bp), NADH dehydrogenase subunit 2 (ND2; 1,041 bp), recombination activating gene 1 (RAG-1; 2,872 bp), recombination activating gene 2 (RAG-2; 1,152 bp), and the β-fibrinogen intron 7 (Bf7; ∼852 bp). Sequences were concatenated and aligned in Seaview v4.2.11 (Gouy et al. 2010). The complete DNA matrix included 41 taxa. Analyses were performed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods. We implemented the most appropriate substitution model for each gene fragment as determined by the Bayesian Information Criterion in PartitionFinder v2 (Lanfear et al. 2017) to choose the optimal partitioning strategy for both phylogenetic analyses (Supplementary information Table 2). MrBayes v3.2 (Ronquist & Huelsenbeck 2003) was used to construct a concatenated Bayesian Inference tree under the best-fitting substitution model for each gene partition. We used default priors and Markov chain settings, and searches were performed with random starting trees. Each run consisted of four chains of 30,000,000 generations, sampled every 3,000 generations. ML searches were conducted in RAxML v7.0.4 (Silvestro & Michalak 2012) using partition data sets under default settings and support was assessed by using 1,000 bootstrapped replicates (GTRGAMMAI). Trees were visualized and edited in FigTree v1.4 (Rambaut 2010). All phylogenetic analyses were performed in the CIPRES platform (Miller et al. 2010). We ran the Poisson Tree Processes (PTP) method as implemented in the bPTP web server for the ML and BI trees (Zhang et al. 2013) (Figure 3).

We used the BEAST v2.6.3 (Bouckaert et al. 2019) suite together with the bModelTest package (Bouckaert & Drummond 2017) to simultaneously estimate the phylogeny, substitution model, and divergence times in BEAUti, setting clock and substitution models independently for each partition and linking the tree model. We used a relaxed lognormal distributed clock model, choosing an uninformative uniform prior for
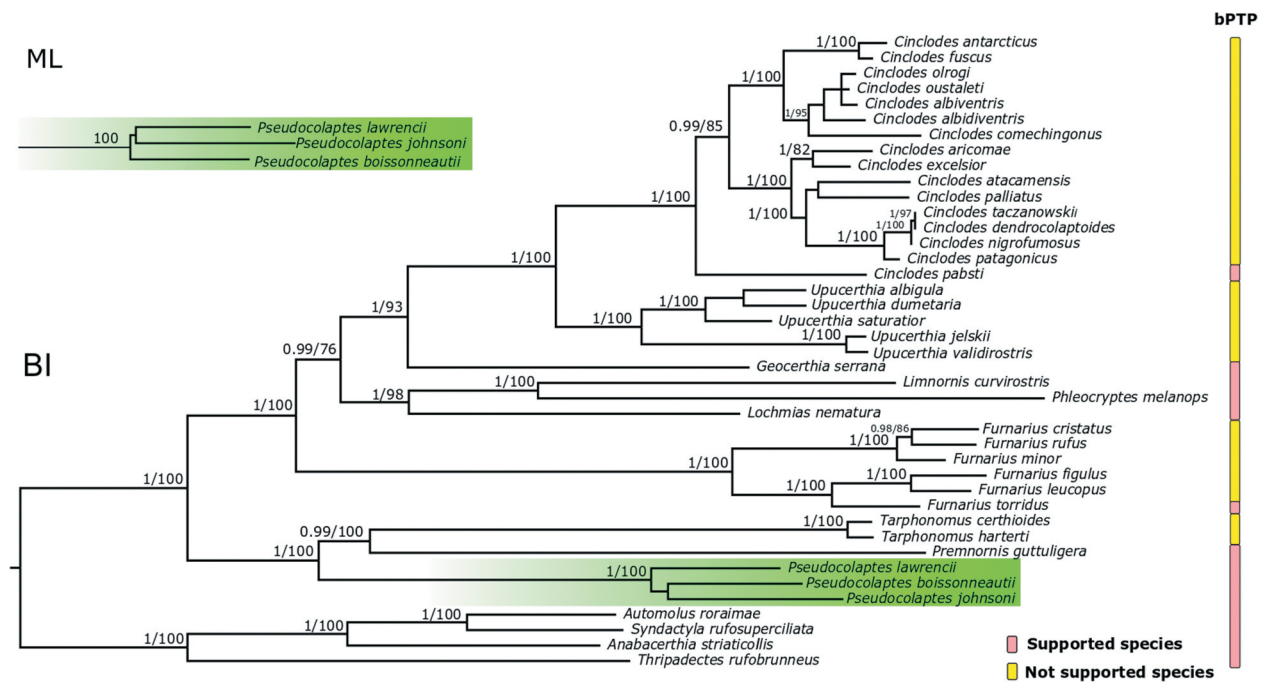
Figure 3. Bayesian tree inferred in MrBayes based on the data set of Derryberry et al. (2011). Values by nodes at the left and right side of the dash (/) represent Bayesian posterior probabilities (>90%) and Maximum Likelihood (ML) bootstrap values (>75%), respectively. The upper left inset evidence the different topology obtained for *Pseudocolaptes* species (highlighted in green) in the ML tree.

the mean rate parameter. Mitochondrial loci were calibrated based on Lerner et al. (2011) estimates (COII: 0.019, ND2: 0.029, ND3: 0.024 and 10% standard deviation) (Figure 4). All other parameters were set to default values. For each analyses, two BEAST Markov Chain Monte Carlo (MCMC) chains were run independently with 50 million generations each, sampling every 5,000 states. Convergence and parameter mixing were verified with Tracer v1.7 (Rambaut et al. 2018), ensuring consistency across runs and that most parameters had sufficient effective sample sizes (>200). Trees and logfiles of both runs were then combined using LogCombiner v2.5.0, and TreeAnnotator v2.5.0 was used to summarize estimates into a maximum-clade-credibility (MCC) tree. Trees were visualized and edited in FigTree v.1.4 (Rambaut 2010).

Results

We recovered the same overall tree topology for the Furnariidae as Derryberry et al. (2011), with most nodes highly supported in both phylogenetic analyses. However, the phylogenetic relationships internal to *Pseudocolaptes* did change between the ML and BI analyses, with *P. lawrencii* + *P. johnsoni* being sister taxa in the ML and *P. boissonneautii* + *P. johnsoni* in the BI analyses, but low node support in both cases

(Figure 3). The PTP species delimitation analysis identified three putative species within *Pseudocolaptes* (0.84 and 0.9 support for *P. lawrencii* along with *P. johnsoni* and *P. boissonneautii*, respectively, in the ML tree; 0.91 and 0.85 support for *P. lawrencii* and *P. johnsoni* along with *P. boissonneautii*, respectively, in the BI tree).

Runs showed high effective sample size (ESS) convergence (>2,000), indicating adequate sampling of the posterior distribution. The *Pseudocolaptes* clade dated to 1.8 Mya (1.5–2.1, 95% HPD) and the internal node remained unresolved to infer accurate divergence estimates; however, its dating is highly similar to that of the *Pseudocolaptes* clade (1.7 Mya), suggesting high genetic divergence between all three taxa (Figure 4).

Discussion

Taxonomic information is of key importance for a number of applied disciplines in biology. Monitoring ecosystems and the models predicting future impacts on biodiversity rely, for instance, on the completeness and correctness of taxonomic knowledge (Coleman 2015). This is particularly true for areas hosting exceptionally high proportions of endemic species yet experiencing fast-increasing rates of habitat loss and fragmentation. These high priority areas for conservation biologists, often referred to as biodiversity hotspots (Myers et al.
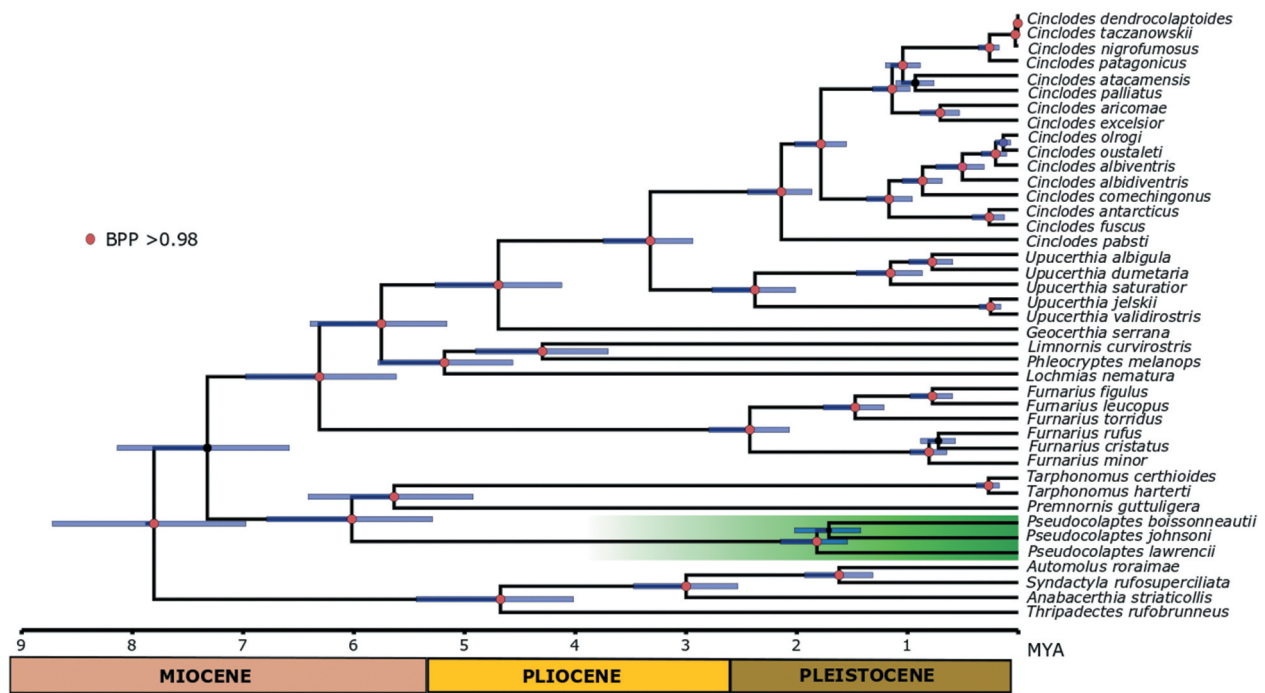
Figure 4. Bayesian time tree as inferred in BEAST based on part of the alignment from Derryberry et al. (2011) under Lerner et al. (2011) estimate rates for the thee mitochondrial loci. Red nodes denote posterior probabilities higher than 0.98. Blue bars on nodes are 95% highest posterior density ranges (HPD). The *Pseudocolaptes* clade is highlighted in green.

2000), usually host high cryptic diversity (e.g. Angulo & Icochea 2010). A rigorous taxonomic assessment of local species is a fundamental prerequisite for the implementation of successful conservation policies in biodiversity hotspots. Even though vocalizations gain increasing attention as a tool to untangle cryptic avian diversity (also in a conservation perspective: Lewis et al. 2020), many taxonomy authorities require a combination of morphological and genetic evidence for assessing the validity of cryptic species. Molecular data have indeed revealed an astonishing yet neglected diversity in avian radiations of tropical biodiversity hotspots (e.g. Lohman et al. 2010).

In this study, we focused on the paradigmatic example of *Pseudocolaptes*, a genus displaying morphological and vocal diversity suggesting the occurrence of three species but with genetic data produced (and used in a phylogeny: Derryberry et al. 2011) only for two of them. Our goal was to present ultimate evidence to assess the validity of the contentious species-level taxon *P. johnsoni* and put an end to the discrepancies in taxonomic treatments by different authorities. We focused our attention on this genus as an exemplary case of cryptic species complex from well-known biodiversity hotspots, namely Mesoamerica (*P. lawrencii*), the Choco Darien Western Ecuador (*P. johnsoni*) and Tropical Andes (*P. boissonneautii*). These areas,

mapped by Myers et al. (2000), have been confirmed as hosting an astonishingly high vertebrate diversity in a more recent and exhaustive assessment focusing on mammals, amphibians and birds (Jenkins et al. 2013). The Andes in particular are renowned for being home to several cryptic avian species (Graves 1987; Mendoza et al. 2016; Krabbe et al. 2020).

The genus *Pseucodolaptes* as a whole is strongly supported, and the unclear phylogenetic relationship between *P. lawrencii* and *P. johnsoni* points toward the occurrence of three equally divergent taxa within this taxon (Figure 3). Under the scenario that *P. lawrencii* and *P. johnsoni* were the same species, we would have expected to recover strongly supported monophyly for these two taxa, but this is not the case. Moreover, the values of the PTP species delimitation analysis (0.84 to 0.91) are remarkably higher than those generally observed among Furnariidae (which are lower than 0.6, not shown). In other words, denying the species status for *P. lawrencii* and *P. johnsoni* would imply rejecting that of several other taxa within this avian family, provided that either equal or very similar evolutionary rates occur. Genetic estimates suggest divergences dating to around the Late Pleistocene (Figure 4), indicating likely changing climatic conditions in the region that fragmented forests during dry periods and resulted in vegetation re-expansions during humid phases, which promoted

cladogenetic events. Our time estimates remain shorter than those of Lerner et al. (2011) likely due to differences in calibration estimates (fossil vs substitution rates) and different data sets (number of taxa). However, as in that study, it is noteworthy that the genetic divergence between all three *Pseudocolaptes* taxa remains much deeper than the majority of divergence times within the genera *Cinclodes*, *Upucerthia*, *Furnarius* and *Tarphonomus*, again suggesting a specific distinction. Taken together with morphological and vocal differences (Spencer 2011; Boesman 2016; del Hoyo & Collar 2016; Supplementary Figure S1), these genetic data provide convincing evidence for the validity of *P. johnsoni* as a species. Importantly, it is worth mentioning that the three groups display a mostly allopatric distribution (Figure 1), with isolation on both sides of the Darien gap (well known as the biogeographical barrier: Bagley & Johnson 2014; Mendoza et al. 2019; Pérez-Escobar et al. 2019) representing the most likely speciation driver. Moreover, *P. boissonneautii* and *P. johnsoni* are locally parapatric (the latter occurring at lower elevations than the former), but nowhere sympatric (Ridgely & Tudor 1994).

We conclude that this study represents a concrete contribution to the resolution of the taxonomic discrepancy inherent to the cryptic diversity in biodiversity hotspots. By using genetic evidence already produced but never used in combination with other analyses, we have shed definitive light on the validity of the contentious species. Our results are expected to get to a consensus among the taxonomic treatment of this species complex, which will ultimately aid conservation and management actions in high biodiversity priority areas. We call for similar investigations addressing other analogous case studies in order to eliminate taxonomic uncertainty and warrant the smooth implementation of concerted policies for the preservation of wildlife in an epoch of increasing global change yet also of limited resources for biodiversity research.

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Disclosure statement

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