

## Advances in molecular sexing of monomorphic birds in Indonesia: A scoping-review with potential application on the Bali starling (*Leucopsar rothschildi*) breeding

I Dewa Agung Panji Dwipayana<sup>1</sup>✉ , Ni Luh Putu Kayika Febryanti<sup>2</sup> ,  
I Ketut Ginantra<sup>1</sup> , Luh Putu Eswaryanti Kusuma Yuni<sup>1,4</sup> , and  
Ni Luh Watiniasih<sup>1,3</sup> 

<sup>1</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Udayana, Bali, Indonesia; <sup>2</sup>Department of Aquaculture, Faculty of Marine Science and Fisheries, Universitas Udayana, Bali, Indonesia; <sup>3</sup>Animal Taxonomy Laboratory, Universitas Udayana, Bali, Indonesia; <sup>4</sup>Ornithology Research Center, Universitas Udayana, Bali, Indonesia  
✉Corresponding author, E-mail: [panji.dwipayana@unud.ac.id](mailto:panji.dwipayana@unud.ac.id)

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**Abstract.** Accurate sex determination is essential for ecological research, captive breeding, and conservation of sexually monomorphic birds. In Indonesia, despite exceptional avian diversity and urgent conservation needs, the application of molecular sexing remains uneven across taxa. This scoping review synthesizes evidence from 35 studies published between 2015 and 2025, drawn from 176 records, that employed PCR amplification of chromo-helicase-DNA-binding (CHD) genes using various primer sets and sample types. Blood and feathers were the most commonly used materials (~80% of records), while swabs, eggshell membranes, and casque tissue were reported infrequently. Primer performance varied markedly among avian families and sample types. CHD1LF/CHD1LR and NP + P2 + MP consistently showed high amplification reliability, whereas P2/P8 and 1237L/1272H yielded inconsistent results. To enable objective comparison, we propose the Primer Performance Index (PPI), a composite metric integrating amplification success with

taxonomic breadth. PPI analysis demonstrated that primers with broad family coverage maintained high reliability after normalization, while taxa-restricted primers showed reduced performance. These findings support improved primer selection for conservation breeding, particularly for the critically endangered Bali Starling (*Leucopsar rothschildi*), and promote more standardized integration of molecular sexing into avian conservation practice in Indonesia.

**Keywords:** Bali Starling, conservation genetics, molecular sexing, Primer Performance Index.

## Introduction

Accurate sex determination in birds is a fundamental requirement for ecological research, captive breeding, and conservation management. In sexually monomorphic species, where males and females lack obvious external differences, traditional approaches such as behavioral observation or morphometrics are unreliable, particularly in juveniles (Petrou *et al.*, 2024). Gender misidentification can lead to skewed datasets in ecological studies and failed breeding programs (Hall *et al.*, 2025).

Molecular sexing, based on amplification of introns within the chromo-helicase-DNA-binding (CHD) genes on the Z and W chromosomes, offers a powerful solution. A range of primer sets has been developed to target these regions, including P2/P8, 2550F/2718R, CHD1F/CHD1R, CHD1LF/CHD1LR, and NP + P2 + MP. Reported success rates vary across taxa and sample types, suggesting that primer performance is context-dependent (Fitriana *et al.*, 2022; Ratri *et al.*, 2022; Fitriana *et al.*, 2023b; Fitriana *et al.*, 2023c). Blood is often considered the most reliable source of DNA, while feathers provide a non-invasive alternative but are prone to degradation. Other tissues such as swabs and eggshell membranes have been tested sporadically, but their limited representation raises questions about generalizability (Akrom *et al.*, 2020b; Yuda *et al.*, 2020; Vera *et al.*, 2021; Pamulang and Haryanto, 2021).

Indonesia has extraordinary avian diversity and pressing conservation challenges. It provides a critical context for evaluating molecular sexing. Despite the country's high bird richness and the urgent need for effective breeding management in threatened species, molecular sexing has been applied unevenly, with fragmented coverage across families and limited integration into conservation practice (Purwaningrum *et al.*, 2019, Ratri *et al.*, 2022). The critically endangered Bali Starling (*Leucopsar rothschildi*) exemplifies this gap (IUCN, 2021). Although extensive rehabilitation and release efforts have been undertaken (Sutomo *et al.*, 2023), molecular sexing has rarely been incorporated into breeding programs.

This review seeks to synthesize current evidence on molecular sexing of monomorphic birds in Indonesia, with particular emphasis on blood and feather samples as the most extensively tested and practical materials. It aims to identify patterns in how different sample types and primer sets influence amplification success across avian families. A new framework named the Primer Performance Index (PPI), is introduced to normalize success rates and provide a more balanced comparison of primer reliability. The resulting insights are then applied to explore opportunities for strengthening the breeding management of the critically endangered Bali starling (*Leucopsar rothschildi*).

## **Materials and methods**

### ***Methods***

This scoping review followed the methodological and reporting standards outlined in the PRISMA-ScR (Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews) framework as established (Tricco *et al.*, 2018). The entire document, including the flow diagram presented in Figure 1, was structured to comply with all 22 items specified in the PRISMA-ScR checklist.

### ***Protocol and Registration***

All methodological procedures and decisions are comprehensively detailed within the body of this manuscript.

### ***Inclusion Criteria***

This scoping review applied a set of predefined inclusion and exclusion criteria to identify relevant studies on molecular sexing of monomorphic birds in Indonesia, with particular emphasis on the Bali starling (*Leucopsar rothschildi*). Studies were first de-duplicated using Zotero, then included if they met all of the following conditions: (1) they were accessible primary research articles, published in peer-reviewed journals or institutional repositories; (2) they focused on the sexing of bird species found in Indonesia, including but not limited to the Bali starling; (3) they involved monomorphic birds, which are species lacking external sexual dimorphism; (4) they were written in English or Bahasa Indonesia and published between January 2015 and October 2025; and (5) they reported at least one technical detail such as sample type, gene target, primer identity, PCR conditions, or sexing accuracy.

### ***Exclusion Criteria***

Studies were excluded if they focused on sexually dimorphic birds or non-avian taxa, relied solely on morphological, behavioral, or hormonal sexing methods without molecular data, or lacked sufficient methodological detail. Review articles, opinion pieces, conference abstracts, and theses without full-text access or complete workflows were also excluded. Additionally, studies conducted entirely outside Indonesia with no relevance to Indonesian bird species or populations were not considered.

### ***Information Sources***

A comprehensive literature search was carried out in November 2025 across four electronic databases: Google Scholar, PubMed, the Directory of Open Access Journals (DOAJ), and GARUDA, an Indonesian journal indexing platform. To supplement the database search, reference lists from key publications were manually examined to identify additional supportive studies.

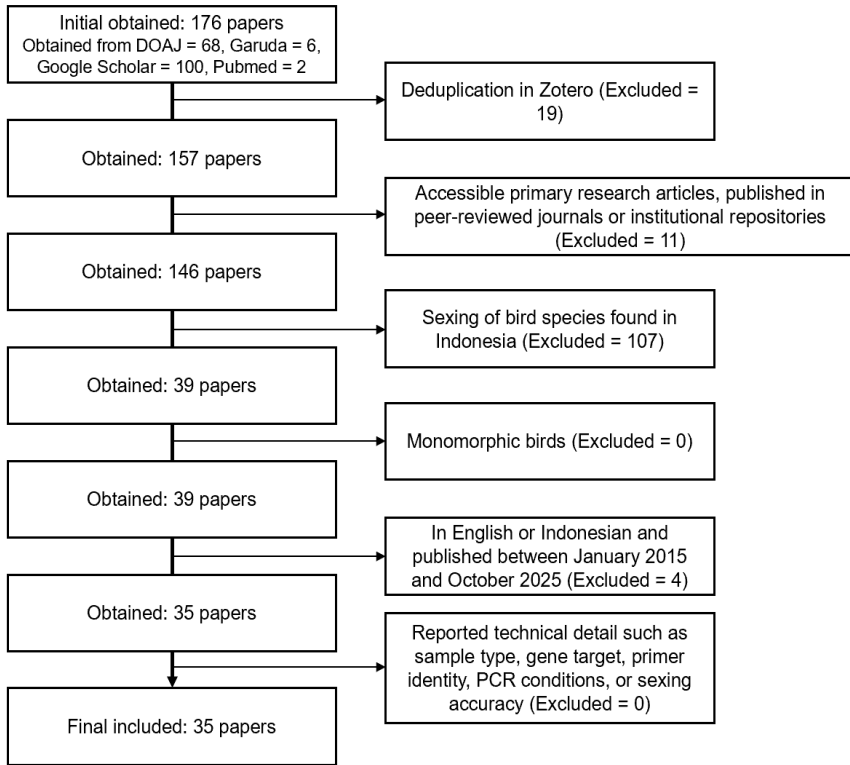
### ***Search Strategy***

To maintain relevance and reduce retrieval of low-quality or random results, the search was restricted to the first ten pages of each keyword query across all databases. The complete search strings are documented in Supplementary Material Table S1 in (<https://osf.io/hwbru/files/osfstorage/69b206346d96b8672c0514e9>).

Example for Google Scholar: "Bird" AND ("Indonesia" OR "Bali") AND ("molecular sexing" OR "PCR sexing") AND ("Primer").

### ***Selection of Sources of Evidence***

The titles and abstracts of all 176 records were independently screened by the first and second authors in accordance with the predefined eligibility criteria. Discrepancies identified during either the initial screening or the full-text assessment were addressed through discussion and, when required, resolved collectively by all authors. The third author subsequently verified and finalized the extracted metadata against the original publications to ensure accuracy and consistency. The complete study selection process is illustrated in the PRISMA-ScR flow diagram shown in Figure 1.



**Figure 1.** PRISMA-ScR flow diagram. The initial papers obtained were 176, the final included papers were 35.

### ***Data Charting Process***

A standardized data-charting template was created in Excel and initially piloted on five journals to ensure consistency. Following this calibration, data extraction was carried out independently by the first and second authors, with any discrepancies resolved through consensus discussions involving the third author.

### ***Data Items***

For each included study, we extracted bibliographic details (authors, year, journal name), species, family, and geographic origin. Molecular sexing details included sample type (blood, feather, swabs), DNA extraction method/kit, primer name, primer sequence, gene target name, PCR conditions (e.g. annealing temperature, cycle number), and gel electrophoresis resolution. We recorded sexing success rate, false positives/negatives, and comparison method (e.g., against behavioral method).

### ***Synthesis of Result***

All primers from the collected studies were consolidated and plotted against the families in which they were applied. When the same primer appeared in multiple studies targeting the same family, its success rates were averaged to produce a single representative value for that primer-family combination. For each primer within each sample type category (blood, feathers, oral swabs, anal swabs, casque tissue), two values were calculated: the average success rate across individuals, and the number of families represented. These values were used to generate a standardized measure of primer efficiency through the Primer Performance Index (PPI), which integrates raw success with taxonomic breadth. By applying this procedure separately for each sample type, the synthesis produced a structured dataset in which primer performance could be compared across tissues and families on a normalized basis.

In this index, primer success rate is weighted by the proportion of avian families in which the primer was tested. This adjustment is necessary because a primer that shows 100% success in only one family should not be interpreted as equally reliable as a primer that maintains high success across many families. Therefore, PPI gives higher comparative value to primer sets that combine high amplification success with broader taxonomic coverage.

Primer Performance Index (PPI) is calculated by:

$$PPI = (SR \times 0.5) + \left(\frac{NF}{TF} \times 0.5\right)$$

where:

- SR = Primer success rate for a certain sample across families
- NF = Number of families tested by this primer
- TF = Total number of families included in this study

### **Results**

A complete extracted data is available in the Supplementary Material Table S2 (<https://osf.io/hwbbru/files/osfstorage/69b206346d96b8672c0514e9>).

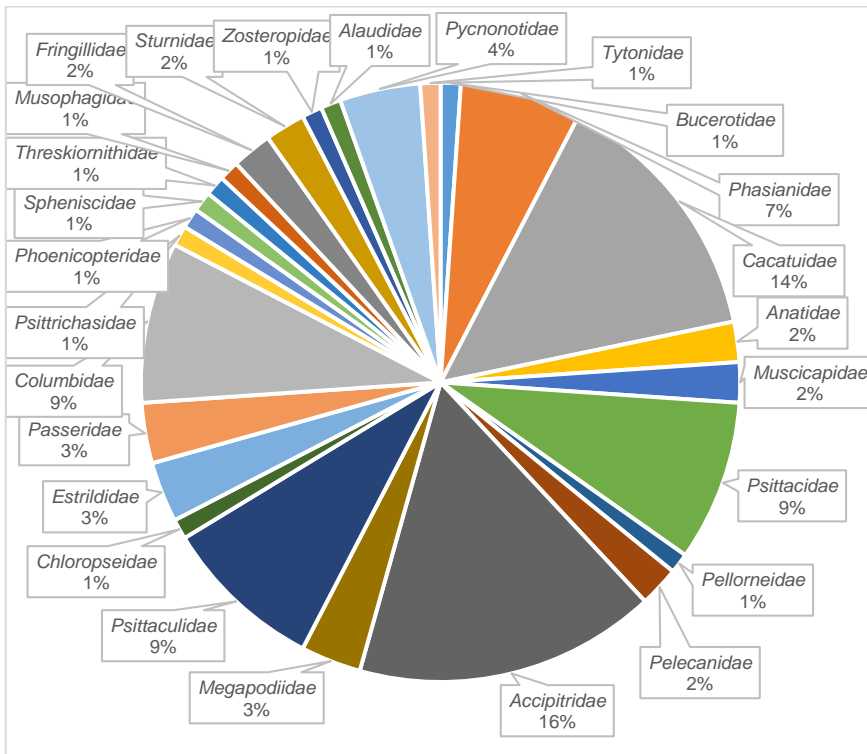
#### ***Selection of Sources of Evidence***

All titles and abstracts were independently assessed by two reviewers in accordance with the predefined eligibility criteria outlined in the Method section.

**Characteristics of Sources of Evidence**

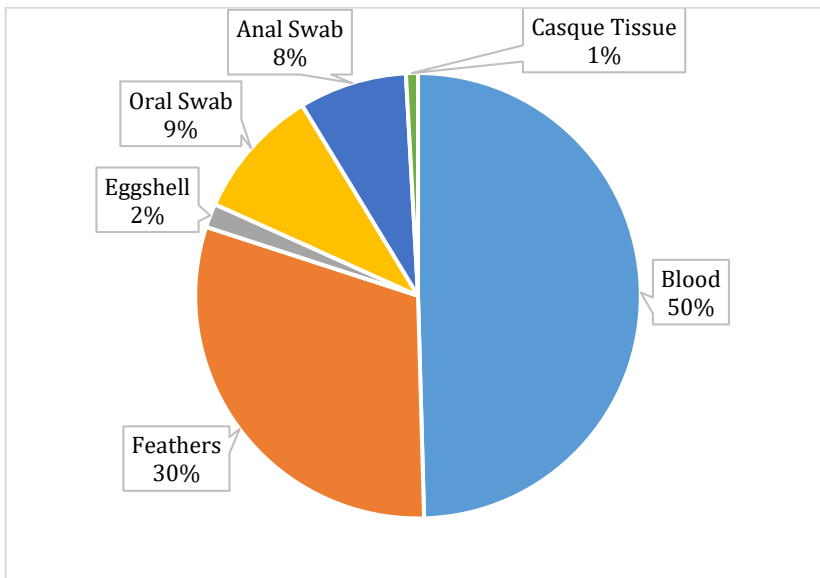
Family distribution from the obtained studies were calculated for each species included. Sample type distribution is calculated by multiplying each species with number of sample types taken from them. Thus, the number will be higher than the total species included because one individual could have multiple sample types (e.g. blood, feather) taken from them.

The dataset in Figure 2 includes molecular sexing records from 26 avian families and 67 species. *Accipitridae* accounted for the largest proportion of samples (16%), followed by *Cacatuidae* (14%). *Columbidae*, *Psittaculidae*, and *Psittacidae* represented 9% each, while *Phasianidae* contributed 7%. The remaining families, each only accounted for < 5% of the dataset. This distribution is not perfectly equal, but it is sufficiently balanced to allow robust amplification trends to be identified in well-sampled groups, while still capturing comparative insights across many others.



**Figure 2.** Family-level representation of birds examined in molecular sexing studies included in this review. Percentages indicate the proportion of extracted family records across the included studies. Studies involving more than one family were counted separately for each family

A total of six biological sample types were used for molecular sexing, with blood samples comprising the majority (50%) of the dataset (Figure 3). Feathers accounted for 30%, reflecting their practical use in non-invasive sampling. Swab-based methods contributed a combined 17%, split between oral swabs (9%) and anal swabs (8%), while eggshell membranes (2%) and casque tissue (1%) were used in specialized cases. This distribution highlights a strong reliance on blood and feather samples for routine sexing, while also demonstrating the feasibility of alternative sources in forensic or conservation contexts.



**Figure 3.** Distribution of biological sample types used for molecular sexing in the studies included in this scoping review. Percentages indicate the proportion of extracted sample-type records across the included studies. When a study used more than one sample type, each sample type was counted separately

### ***Results of Individual Sources of Evidence***

Among the evaluated primer sets, the CHD1F/CHD1LR primer pair achieved 100% PCR-based molecular sexing success rates across seven families (*Phasianidae*, *Anatidae*, *Muscicapidae*, *Chloropseidae*, *Psittacidae*, *Estrildidae*, and *Passeridae*) and maintaining values above 90% in blood samples overall (Table 1). CHD1F/CHD1R primer set also performed strongly, with complete success in *Sturnidae*, *Phasianidae*, *Muscicapidae*, and *Passeridae*, though values

dropped to 40% in *Columbidae*. The widely used 2550F/2718R primer set achieved 100% success in *Accipitridae*, *Megapodiidae*, and *Psittaculidae*, but showed reduced amplification in *Columbidae* (40%) and mixed outcomes in *Muscicapidae* (50%). NP + P2 + MP primer set was particularly effective in captive birds such as parrots, lovebirds, and cockatoos, reaching 100% in multiple *Psittacidae*, *Psittaculidae*, and *Cacatuidae* species, though feather success rates varied from 20% to 100%. InSex-F/InSex-R primer set achieved 100% in *Accipitridae* and *Phasianidae*, but dropped to 84.6% in degraded eggshell samples. In contrast, 1237L/1272H and CHDZ/CHDW primer sets frequently failed, with success rates mostly ranging from 0-50% depending on family, and P2/P8 primer set produced inconsistent values, from 0% in several *Psittacidae* species to 100% in, for example, *Zosteropidae*, *Alaudidae*, *Psittaculidae*, *Cacatuidae*, and *Pellorneidae*.

A complete list of extracted data is available in Supplementary Material Table S2, but a snip is presented below.

**Table 1.** Molecular sexing success rate according to species, family, sample type, and primer set in the included studies.

Title	Species	Family	Sample	Sexing Success Rate
(Fitriana <i>et al.</i> , 2020)	<i>Rhinoplax vigil</i>	<i>Bucerotidae</i>	Casque tissue	- CHD1F/CHD1R: 80.88% - 2550F/2718R: 51.47% - P2/P8: 20.58%
	<i>Leucopsar rothschildi</i>	<i>Sturnidae</i>	- Blood - Feathers	CHD1F/CHD1R: - Blood: 100% - Feather: 100% 2550F/2718R: - Blood: 100% - Feathers: 100% P2/P8: - Blood: 40.9% - Feathers: 0%
(Pambuko <i>et al.</i> , 2023)	<i>Gallus gallus domesticus</i>	<i>Phasianidae</i>	Blood	- CHD1F/CHD1R: 100% - AV2550F/AV2718R: 100% - AVP2/AVP8: 60% - AV1237L/AV1272H: 60% - CHDZ/CHDW: 0%
(Zein <i>et al.</i> , 2017)	<i>Cacatua galerita</i>	<i>Cacatuidae</i>	Blood	- 2550F/2718R: 100%
	<i>Cacatua sulphurea</i>	<i>Cacatuidae</i>		
	<i>Cacatua alba</i>	<i>Cacatuidae</i>		
	<i>Cacatua moluccensis</i>	<i>Cacatuidae</i>		

Title	Species	Family	Sample	Sexing Success Rate
	<i>Cacatua goffiniana</i>	<i>Cacatuidae</i>		
	<i>Probosciger aterrimus</i>	<i>Cacatuidae</i>		
(Fitriana <i>et al.</i> , 2023a)	<i>Gallus gallus domesticus</i>	<i>Phasianidae</i>	Blood	- CHD1LF/CHD1LR: 100% - CHD1F/CHD1R: 100% - 2550F/2718R: 100% - 1237L/1272H: 0% - P2/P8: 0%
	<i>Anas platyrhynchos domesticus</i>	<i>Anatidae</i>		-CHD1LF/CHD1LR: 100% - CHD1F/CHD1R: 100% - 2550F/2718R: 50% - 1237L/1272H: 0% - P2/P8: 0%
	<i>Brachypteryx leucophrys</i>	<i>Muscicapidae</i>		- CHD1LF/CHD1LR: 100% - CHD1F/CHD1R: 100% - 2550F/2718R: 50% - 1237L/1272H: 0% - P2/P8: 0%
	<i>Agapornis fischeri</i>	<i>Psittacidae</i>		- CHD1LF/CHD1LR: 100% - CHD1F/CHD1R: 0% - 2550F/2718R: 100% - 1237L/1272H: 0% - P2/P8: 0%
(Ó Marcaigh <i>et al.</i> , 2021)	<i>Pellorneum celebense</i>	<i>Pellorneidae</i>	Feathers	- P2/P8: 100% - CHD1F/CHD1R: 0%
(Dharmayanthi <i>et al.</i> , 2021)	<i>Pelecanus conspicillatus</i>	<i>Pelecanidae</i>	Blood	- 2550F/2718R: 100%
(Annisa <i>et al.</i> , 2021)	<i>Nisaetus cirrhatus</i>	<i>Accipitridae</i>	Blood	- 2550F/2718R: 100%
(Yuda and Saputra, 2021)	<i>Macrocephalon maleo</i>	<i>Megapodiidae</i>	Eggshell membrane	- 2550F/2718R: 100%
(Nugroho and Zein, 2015)	<i>Lorius lory</i>	<i>Psittaculidae</i>	Feathers	- 2550F/2718R: 100%
(Fuadah <i>et al.</i> , 2025)	<i>Copsychus malabaricus</i>	<i>Muscicapidae</i>	Blood	- CHD1LF/CHD1LR: 100% - CHD1F/CHD1R: 100% - P2/P8: 100% - 2550F/2718R: 50% - 1237L/1272H: 50%

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Title	Species	Family	Sample	Sexing Success Rate
	<i>Chloropsis sonnerati</i>	<i>Chloropseidae</i>		- CHD1LF/CHD1LR: 100% - CHD1F/CHD1R: 50% - P2/P8: 0% - 2550F/2718R: 50% - 1237L/1272H: 0%
(Fitriana <i>et al.</i> , 2023b)	<i>Coturnix coturnix</i>	<i>Phasianidae</i>	Blood	CHD1LF/CHD1LR: 100%
	<i>Melopsittacus undulatus</i>	<i>Psittacidae</i>		
	<i>Estrildid finches</i>	<i>Estrildidae</i>		
	<i>Passer montanus</i>	<i>Passeridae</i>		
(Fitriana <i>et al.</i> , 2023c)	<i>Coturnix coturnix</i>	<i>Phasianidae</i>	Blood	- CHD1F/CHD1R: 100% - 2550F/2718R: 100% - 1237L/1272H: 50% - P2/P8: 50%
	<i>Estrildid finches</i>	<i>Estrildidae</i>		- CHD1F/CHD1R: 100% - 2550F/2718R: 50% - 1237L/1272H: 0% - P2/P8: 0%
	<i>Passer montanus</i>	<i>Passeridae</i>		- CHD1F/CHD1R: 100% - 2550F/2718R: 50% - 1237L/1272H: 0% - P2/P8: 0%
	<i>Melopsittacus undulatus</i>	<i>Psittacidae</i>		- CHD1F/CHD1R: 100% - 2550F/2718R: 100% - 1237L/1272H: 0% - P2/P8: 0%
(Fitriana, <i>et al.</i> , 2022)	<i>Columba livia</i>	<i>Columbidae</i>	Blood	- CHD1LF/CHD1LR: 90% - P2/P8: 20% - 2550F/2718R: 40% - CHD1F/CHD1R: 40% - 1237L/1272H: 50%
	<i>Streptopelia chinensis</i>	<i>Columbidae</i>		
	<i>Treron griseicauda</i>	<i>Columbidae</i>		
	<i>Spilopelia chinensis</i>	<i>Columbidae</i>		
	<i>Geopelia striata</i>	<i>Columbidae</i>		
(Ratri <i>et al.</i> , 2022)	<i>Cacatua sulphurea</i>	<i>Cacatuidae</i>	- Blood - Feathers	NP + P2 + MP (combined): - Blood: 100% - Feathers: 25%
(Savitri <i>et al.</i> , 2021)	<i>Cacatua galerita</i>	<i>Cacatuidae</i>	- Blood - Feathers	NP + P2 + MP (combined): - Blood: 100% - Feathers: 100%
(Hidayat <i>et al.</i> , 2021)	<i>Cacatua goffiniana</i>	<i>Cacatuidae</i>	- Blood - Feathers	NP + P2 + MP (combined): - Blood: 100% - Feathers: 20%
(Argarini <i>et al.</i> , 2020)	<i>Agapornis fischeri</i>	<i>Psittaculidae</i>	Feathers	NP + P2 + MP (combined): 100%

Title	Species	Family	Sample	Sexing Success Rate
(El Islami <i>et al.</i> , 2021)	<i>Agapornis personata</i>	<i>Psittaculidae</i>	Feathers	NP + P2 + MP (combined): 100%
(Sitohang, 2017)	<i>Nisaetus bartelsi</i>	<i>Accipitridae</i>	Blood	- 2550F/2718R: 100%
	<i>Nisaetus cirrhatus</i>	<i>Accipitridae</i>		- 2561/2728: 100% - P2/P8: 0% - 1237L/1272H: 0%
(Purwaningrum <i>et al.</i> , 2019)	<i>Electus roratus</i>	<i>Psittaculidae</i>	Feathers	NP + P2 + MP (combined): 100%
	<i>Cacatua sanguinea</i>	<i>Cacatuidae</i>		
	<i>Probosciger aterrimus</i>	<i>Cacatuidae</i>		
	<i>Lorius lory</i>	<i>Psittaculidae</i>		
	<i>Psittrichas fulgidus</i>	<i>Psittrichasidae</i>		
	<i>Pelecanus onocrotalus</i>	<i>Pelecanidae</i>		
	<i>Cygnus atratus</i>	<i>Anatidae</i>		
	<i>Phoenicoparrus minor</i>	<i>Phoenicopteridae</i>		
	<i>Ara ararauna</i>	<i>Psittacidae</i>		
	<i>Eolophus roseicapilla</i>	<i>Cacatuidae</i>		
<i>Spheniscus demersus</i>	<i>Spheniscidae</i>			
<i>Psittacus erithacus</i>	<i>Psittacidae</i>			
<i>Amazona amazonica</i>	<i>Psittacidae</i>			
<i>Aratinga solstitialis</i>	<i>Psittacidae</i>			
<i>Eudocimus ruber</i>	<i>Threskiornithidae</i>			
<i>Tauraco persa</i>	<i>Musophagidae</i>			
(Withaningsih <i>et al.</i> , 2024)	<i>Nisaetus bartelsi</i>	<i>Accipitridae</i>	Blood	- 2550F/2718R: 100%
(Akrom <i>et al.</i> , 2020b)	<i>Serinus canaria</i>	<i>Fringillidae</i>	- Blood - Feathers	CHD1F/CHD1R: - Adult males blood and feather: 100%. - Adult females blood and feather: 100%. - Juveniles feather: 100%
(Samad <i>et al.</i> , 2023)	<i>Macrocephalon maleo</i>	<i>Megapodiidae</i>	- Feathers - Eggshell	2550F/2718R: - Feather: 100% - Eggshell: 100%.
(Adelia <i>et al.</i> , 2025)	<i>Columba livia</i>	<i>Columbidae</i>	Feathers	2550F/2718R: 100%
(Wirastika <i>et al.</i> , 2015)	<i>Leucopsar rothschildi</i>	<i>Sturnidae</i>	Feathers	- P2/P8: 90% - 2550F/2718R: 86.7% - 1237L/1272H: 73.3%

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Title	Species	Family	Sample	Sexing Success Rate
(Nugraheni <i>et al.</i> , 2019)	<i>Agapornis roseicollis</i>	<i>Psittaculidae</i>	Feathers	NP + P2 + MP (combined): 100%
(Azzahra <i>et al.</i> , 2025)	<i>Columba livia</i>	<i>Columbidae</i>	Blood	2550F/2718R: 100%
(Oktanella, 2017)	<i>Zosterops flavus</i>	<i>Zosteropidae</i>	Feather	- P2/P8: 100%
	<i>Miraфра javanica</i>	<i>Alaudidae</i>		
	<i>Agapornis sp.</i>	<i>Psittaculidae</i>		
	<i>Nymphicus hollandicus</i>	<i>Cacatuidae</i>		
(Pinayungan <i>et al.</i> , 2024)	<i>Aratinga solstitialis</i>	<i>Psittacidae</i>	Feather	P2/P8: 100%
(Fatona <i>et al.</i> , 2025)	<i>Agapornis fischeri</i>	<i>Psittaculidae</i>	Feathers	CHD1F/CHD1R: 100%
(Vera <i>et al.</i> , 2021)	<i>Nisaetus cirrhatus</i>	<i>Accipitridae</i>	Blood	- InSex-F/InSex-R: 100%
	<i>Haliastur indus</i>	<i>Accipitridae</i>	Blood	
	<i>Passer montanus</i>	<i>Passeridae</i>	Blood	
	<i>Lonchura fuscans</i>	<i>Estrildidae</i>	Blood	
	<i>Pycnonotus zeylanicus</i>	<i>Pycnonotidae</i>	Blood	
	<i>Tyto alba</i>	<i>Tytonidae</i>	Feather	
	<i>Gallus gallus</i>	<i>Phasianidae</i>	Blood	
	<i>Pavo muticus</i>	<i>Phasianidae</i>	Blood	
	<i>Macrocephalon maleo</i>	<i>Megapodiidae</i>	Eggshell membrane	- InSex-F/InSex-R: 84.6%; 2 failed samples likely due to low quality from eggshell
(Pamulang and Haryanto, 2021)	<i>Pycnonotus aurigaster</i>	<i>Pycnonotidae</i>	- Blood	P2/P8:
	<i>Pycnonotus melanicterus</i>	<i>Pycnonotidae</i>	- Feathers	- Blood: 100% - Feather: 50%
(Akrom <i>et al.</i> , 2020a)	<i>Serinus canaria</i>	<i>Fringillidae</i>	Buccal swab	CHD1F/CHD1R:
	<i>Columba livia (Pigeon)</i>	<i>Columbidae</i>		- Adults: 100% - Nestlings: 100%
(Yuda <i>et al.</i> , 2020)	<i>Nisaetus cirrhatus</i>	<i>Accipitridae</i>	- Blood	2550F/2718R: - Blood: 100% - Tracheal swab: 71% - Cloacal swab: 9%
	<i>Spilornis cheela</i>	<i>Accipitridae</i>	-	
	<i>Nisaetus bartelsi</i>	<i>Accipitridae</i>	Tracheal swab	
	<i>Ichthyophaga leucogaster</i>	<i>Accipitridae</i>	- Cloacal swab	
	<i>Ichthyophaga ichtyaetus</i>	<i>Accipitridae</i>		

Title	Species	Family	Sample	Sexing Success Rate
	<i>Ictinaetus malaiensis</i>	<i>Accipitridae</i>		
	<i>Milvus migrans</i>	<i>Accipitridae</i>		
	<i>Tachyspiza virgata</i>	<i>Accipitridae</i>		
	<i>Pernis ptilorhyncus</i>	<i>Accipitridae</i>		
(Yuda and Wajjwalku, 2022)	<i>Pycnonotus zeylanicus</i>	<i>Pycnonotidae</i>	Feathers	- 2561-w/2728-w: 100%

Although eggshell, oral swab, anal swab, and casque data are reported here for completeness, each represent fewer than 10% of all records and will not be examined in detail in the discussion. Similarly, family-level comparisons are not pursued further because 14 families were represented by only one or two studies. Emphasis in the discussion is placed on sample type and primer performance, which provide more robust and generalizable insights into molecular sexing accuracy.

### ***Synthesis of Primer Success Rate and Performance Index Results***

Overall, the synthesis revealed that blood samples consistently produced the highest success rates across primer sets, while feathers yielded more variable outcomes and other types showed limited reliability. Primers such as CHD1LF/CHD1LR and NP + P2 + MP primer sets retained strong performance even after normalization, confirming their broad applicability, whereas set like 2561-w/2728-w showed reduced values once PPI was applied, reflecting their limited generalizability. By combining raw success rates with PPI, the analysis provided a balanced view of primer efficiency, highlighting those with reproducible utility across families and sample types while controlling for bias introduced by uneven representation

### ***Primer Performance Index Calculation***

Analysis shown here was restricted to blood and feathers samples, as eggshell, oral swabs, anal swabs, and casque tissue were excluded due to the limited number of records available. For each primer, weighed success rates were calculated alongside the number of families. These values were used to derive the Primer Performance Index (PPI) in Table 2.

**Table 2.** Primer Performance Index Calculation

Primer Type	Blood		Feathers		
	Average Primer Success Rate	Primer Performance Index	Primer Type	Average Primer Success Rate	Primer Performance Index
1237L/1272H	11.1%	0.13	1237L/1272H	73.3%	0.38
2550F/2718R	75.0%	0.47	2550F/2718R	98.3%	0.56
2561/2728	100.0%	0.51	2561/2728	-	-
InSex-F/InSex-R	100.0%	0.54	InSex-F/InSex-R	100.0%	0.52
P2/P8	21.4%	0.20	P2/P8	86.9%	0.57
CHD1F/CHD1R	84.0%	0.50	CHD1F/CHD1R	75.0%	0.44
CHD1LF/CHD1LR	98.8%	0.56	CHD1LF/CHD1LR	-	-
NP + P2 + MP	100.0%	0.51	NP + P2 + MP	97.4%	0.66
AV1237L/AV1272H	60.0%	0.31	AV1237L/AV1272H	-	-
AV2550F/AV2718R	100.0%	0.51	AV2550F/AV2718R	-	-
AVP2/AVP8	60.0%	0.31	AVP2/AVP8	-	-
CHDZ/CHDW	0.0%	0.01	CHDZ/CHDW	-	-
2561-w/2728-w (Direct PCR)	-	-	2561-w/2728-w (Direct PCR)	100.0%	0.52

In blood samples, the highest primer performance was observed for CHD1LF/CHD1LR primer set (PPI 0.56, 98.8% success), followed closely by InSex-F/InSex-R primer set (0.54, 100%) and CHD1F/CHD1R primer set (0.50, 84%). Other primers such as NP + P2 + MP, AV2550F/AV2718R, and 2561/2728 primer sets maintained consistent efficiency (PPI 0.51, 100%). In contrast, P2/P8 (0.20, 21.4%), AVP2/AVP8 (0.31, 60%), AV1237L/AV1272H (0.31, 60%), 1237L/1272H (0.13, 11.1%), and CHDZ/CHDW (0.01, 0%) primer sets performed poorly. In feather samples, NP + P2 + MP primer set achieved the strongest outcome (PPI 0.66, 97.4%), followed by P2/P8 (0.57, 86.9%) and 2550F/2718R (0.56, 98.3%) primer sets. InSex-F/InSex-R and 2561-w/2728-w primer sets (Direct PCR) both reached solid values (0.52, 100%), while CHD1F/CHD1R primer set showed moderate efficiency (0.44, 75%) and 1237L/1272H primer set remained the weakest (0.38, 73.3%). These results provide a clear comparative overview of primer reliability across sample types, reinforcing the value of high-performing primers for consistent molecular sexing.

## Discussion

### *Influence of Sample Type*

Blood samples comprise 50% of records. Among all biological materials used for avian molecular sexing, blood remains the most consistently reliable source of DNA. It shows near-universal amplification success across taxa, primer sets, and laboratory conditions, with exception in low performing primers such as 1237L/1272H primer set (Fitriana *et al.*, 2023a). Studies covering both captive and wild birds repeatedly report clear CHD-band separation with minimal PCR failure (Zein *et al.*, 2017; Yuda *et al.*, 2020). The presence of nucleated erythrocytes in birds allows DNA extracted from blood to retain high integrity, producing strong template yield with little need for repeat sampling or nested PCR (Turcu *et al.*, 2023). For these reasons, blood continues to be regarded as the benchmark sample type for molecular sex determination in birds. However, its invasive nature raises ethical and logistical concerns. Repeated venipuncture in endangered species and juveniles can cause stress, requires trained personnel, and is difficult to scale in field conditions (Marc *et al.*, 2025).

Feathers, representing 30% of records, illustrate the trade-off between accessibility and reliability. Success ranged from 0% (CHD1F/CHD1R primer set in *Agapornis fischeri* (Fitriana *et al.*, 2023a) to 100% (NP + P2 + MP primer set in *Psittaculidae* and *Cacatuidae* (Purwaningrum *et al.*, 2019; Nugraheni *et al.*, 2019). DNA extracted from *Columbidae* feathers were amplified at 100% with 2550F/2718R primer set but only 40% with CHD1F/CHD1R and 20% with P2/P8 primer sets (Fitriana *et al.*, 2022; Azzahra *et al.*, 2025). This heterogeneity underscores that molecular sexing analysis based on DNA extracted from feathers is not inherently unreliable but contingent on primer choice and DNA integrity. DNA from degraded calamus tissue often reduces amplification, yet when paired with robust primers such as NP + P2 + MP primer set with overnight lysis buffer incubation, feathers can rival blood in accuracy (Argarini *et al.*, 2020; El Islami *et al.*, 2021). Direct PCR is also a great boost for feather samples. It achieved 100% success in *Pycnonotidae* (Yuda and Wajjwalku, 2022). This means feathers are a viable substitute if protocols explicitly address DNA degradation risk by standardizing feather type, storage, and extraction methods.

### *Influence of Represented Families, Primer Type, and Primer Performance Index*

Figure 2 confirms that raptors (*Accipitridae*, 16%) and cockatoos (*Cacatuidae*, 14%) dominate Indonesian molecular sexing research, followed by *Columbidae*, *Psittaculidae*, and *Psittacidae* (each 9%), and *Phasianidae* (7%). This concentration

reflects both conservation priorities and socio-economic drivers: raptors are frequently confiscated and require sexing for rehabilitation, while cockatoos and parrots are central to aviculture and trade (Zein *et al.*, 2017; Yuda *et al.*, 2020). The consequence is methodological bias. Primers validated in these families, such as 2550F/2718R and NP + P2 + MP, appear favorable, but their reliability is partly a product of repeated testing in taxa with suitable CHD intron structures (Yuda *et al.*, 2020; Savitri *et al.*, 2021; Hidayat *et al.*, 2021; Ratri *et al.*, 2022). To address this imbalance, we propose the Primer Performance Index (PPI) as a descriptive normalization metric that combines primer success rate with the breadth of taxonomic testing. The index is intended to reduce overinterpretation of high success rates obtained from narrowly tested primers and to provide a more balanced comparison across primer sets.

Primer performance differed markedly between blood and feathers. CHD1LF/CHD1LR primer set was the most reliable, achieving 100% success in DNA extracted from blood across *Phasianidae*, *Anatidae*, *Muscicapidae*, *Chloropseidae*, *Psittacidae*, *Estrildidae*, and *Passeridae*, and maintaining values above 90% overall (Fitriana *et al.*, 2022; Fitriana *et al.*, 2023a; Fitriana *et al.*, 2023b; Fuadah *et al.*, 2025). NP + P2 + MP primer set achieved 100% in DNA extracted from feathers across *Psittaculidae*, *Cacatuidae*, *Psittichasidae*, *Pelecanidae*, *Anatidae*, *Phoenicopteridae*, *Psittacidae*, *Spheniscidae*, *Threskiornithidae*, and *Musophagidae*, though values dropped to 20-25% in some cockatoo species (Purwaningrum *et al.*, 2019; Nugraheni *et al.*, 2019; Hidayat *et al.*, 2021, Ratri *et al.*, 2022). By contrast, P2/P8 was highly inconsistent. It achieved 0% in *Psittacidae* and 100% in *Pellorneidae* and *Zosteropidae* (Oktanella, 2017; Ó Marcaigh *et al.*, 2021; Fitriana *et al.*, 2023c). The performance of 1237L/1272H primer set remained weak, with mostly between 0-50% depending on family (Wirastika *et al.*, 2015; Fitriana *et al.*, 2023c).

The Primer Performance Index (PPI) confirmed these trends. CHD1LF/CHD1LR and NP + P2 + MP primer sets retained strong values after normalization, underscoring their robustness. 2561-w/2728-w showed inflated raw success in DNA extracted from feathers (up to 100%) but dropped after normalization, revealing limited generalizability. 2561-w/2728-w primer set's initial apparent efficiency was inflated by success in limited taxa, while PPI corrected this inflation by taking into account its narrow taxa. 1237L/1272H primer set performance remained consistently poor, confirming its restricted applicability.

These results highlight the importance of considering PPI validation, not just raw primer efficiency. A primer tested across many families with moderate success may be more reliable in practice than one showing perfect success in a single species (Marc *et al.*, 2025). Raw success for context or taxa specific application is a strength, but PPI is essential for cross-taxa analysis to avoid overinterpretation.

### ***Implications for the Bali Starling (*Leucopsar rothschildi*) Breeding***

Two studies provide complementary insights into primer performance. The 2015 study reported sexing success rates of 90% with P2/P8, 86.7% with 2550F/2718R, and 73.3% with 1237L/1272H primer sets. Meanwhile, a later study demonstrated 100% success with CHD1F/CHD1R and 2550F/2718R primer sets in both blood and feather samples, but only 40.9% in blood and 0% in feathers with P2/P8 (Fitriana *et al.*, 2020; Wirastika *et al.*, 2015). None of those primers top their peers in the sample type they were used. The best primers 2550F/2718R and CHD1F/CHD1R only earned moderate PPI, meaning they are not as universal as the primer with highest PPI. Bali Starling conservation programs should prioritize primers with the highest PPI first before trying taxa specific primer. It will also benefit from technical refinements such as overnight incubation or direct PCR to improve feather DNA yield (Argarini *et al.*, 2020; El Islami *et al.*, 2021; Yuda and Wajjwalku, 2022).

Early and accurate sexing is not a technical luxury but a conservation necessity. Mis-sexed pairs waste breeding seasons and compromise genetic management. By integrating robust primers into Bali Starling workflows, managers can minimize pairing errors, safeguard genetic diversity, and accelerate recovery efforts. As a flagship species, the Bali Starling's adoption of modern molecular sexing sets a precedent for other monomorphic birds in Indonesia, bridging the gap between research and practice.

### ***Limitations***

This review is limited by uneven representation across taxa and sample types, with raptors and cockatoos dominating while many families were represented by only one or two studies, restricting generalizability beyond well-sampled groups. Blood and feathers, which accounted for 80% of records, provide the most reliable basis for comparison, whereas eggshell membranes, swabs, and casques were excluded from discussion due to insufficient family and individual numbers that make amplification trends unstable. Primer Performance Index (PPI), although valuable for normalizing success rates across taxa and sample sizes, has inherent constraints. It can inflate reliability when primers are tested broadly but only moderately successful, or undervalue primers with strong raw success in limited taxa. Nuanced methodological differences across studies such as DNA extraction protocols further introduce variability that aggregated metrics cannot capture.

## Conclusions

This review shows that molecular sexing using CHD-based PCR is a reliable tool for sexually monomorphic birds in Indonesia, but its success depends on primer selection, sample type, and taxonomic coverage. Blood and feathers remain the most effective samples, while other materials require further validation. Primer performance varied widely across families, indicating that raw success rates alone are insufficient for broad comparison. The Primer Performance Index (PPI) introduced here integrates success rate with taxonomic breadth, identifying CHD1LF/CHD1LR and NP + P2 + MP as the most robust and generalizable primers. Applying these primers can improve breeding management of the critically endangered Bali Starling (*Leucopsar rothschildi*) by reducing sexing errors and optimizing pair formation, supporting stronger integration of molecular sexing into conservation practice.

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