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Tinjauan Ilmiah Pendekatan Molekuler untuk Penentuan Jenis Kelamin Ikan Hias

A Review of Molecular Approaches for Sex Determination in Ornamental Fish

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INTISARI

Industri ikan hias merupakan sektor akuakultur bernilai tinggi secara global, di mana identifikasi jenis kelamin yang akurat sangat penting untuk meningkatkan efisiensi pemuliaan, nilai ekonomi, dan pengelolaan stok. Namun, metode penentuan jenis kelamin secara fenotipik sering kali tidak andal akibat munculnya dimorfisme seksual yang terlambat, pengaruh lingkungan, serta divergensi genetik akibat domestikasi dan seleksi buatan. Tinjauan ini merangkum dasar biologis penentuan jenis kelamin pada ikan hias serta mengevaluasi berbagai pendekatan molekuler yang digunakan untuk identifikasi jenis kelamin dalam konteks genetik dan lingkungan yang beragam. Ikan *teleost* menunjukkan keragaman mekanisme penentuan jenis kelamin yang sangat tinggi, meliputi sistem XX/XY, ZZ/ZW, poligenik, dan epigenetik. Keragaman ini sering disertai seleksi buatan yang menyebabkan pergeseran wilayah gen penentu jenis kelamin. Oleh karena itu, tidak terdapat satu penanda molekuler yang bersifat universal untuk semua ikan hias. Metode berbasis PCR, seperti penanda SCAR, RAPD, AFLP, dan InDel, tetap menjadi pendekatan paling mudah diakses dan efektif pada populasi yang telah divalidasi, sementara teknologi NGS memungkinkan penemuan SNP dan variasi struktural gen regulasi jenis kelamin secara menyeluruh dan reprodusibel. Penanda transkriptomik dan epigenetik memberikan pemahaman mekanistik tentang diferensiasi jenis kelamin spesies yang plastis dipengaruhi lingkungan, meskipun memiliki keterbatasan sebagai alat diagnostik mandiri. Teknologi portabel dan *point-of-care* menunjukkan potensi untuk identifikasi jenis kelamin di lapangan, namun masih memerlukan validasi lebih lanjut pada ikan. Tinjauan ini menegaskan bahwa penentuan jenis kelamin secara molekuler pada ikan hias memerlukan pemilihan penanda yang tepat, validasi multi-strain, serta integrasi data genetik, epigenetik, dan lingkungan untuk menjamin keandalan dan penerapan yang luas.

Kata kunci: ikan hias; penentuan jenis kelamin; penanda molekuler; domestikasi; akuakultur

ABSTRACT

The ornamental fish industry is a globally significant sector of aquaculture in which accurate sex identification is essential for optimizing breeding efficiency, economic value, and stock management. However, traditional phenotypic sexing methods are often unreliable due to late sexual dimorphism, environmental influences, and extensive strain divergence driven by domestication and selective breeding. This review synthesizes current knowledge on the biological basis of sex determination in ornamental fishes and evaluates molecular approaches used for sex identification across diverse genetic and environmental contexts. Teleost fishes exhibit exceptional diversity in sex determination systems, including XX/XY, ZZ/ZW, polygenic, and epigenetic system. Such diversity is often accompanied by selective breeding which may shuffle sex-determining regions. Consequently, no single molecular marker is universally applicable across ornamental taxa. PCR-based methods, such as SCAR, RAPD, AFLP, and InDel markers, remain the most accessible tools and are effective within validated populations, while next-generation sequencing enables genome-wide discovery of sex-linked SNPs and structural variants to provide high-resolution and reproducible diagnostics. Transcriptomic and epigenetic markers offer mechanistic insight into sex differentiation particularly in plastic or temperature-sensitive systems, although they are limited as standalone diagnostic tools. Emerging portable and point-of-care platforms show promise for on-site sex identification but require further validation in fish. This review emphasizes that robust molecular sexing in ornamental fish requires careful marker selection, multi-strain validation, and integration of genetic, epigenetic, and environmental data. Expanding genomic resources and standardized validation frameworks will be critical for translating molecular sexing into reliable, industry-wide applications.

Keywords: ornamental fish; sex determination; molecular markers; domestication; aquaculture

INTRODUCTION

The ornamental fish industry is a vibrant and economically significant sector within global aquaculture, with an estimated annual trade of over US $15 billion each year including 125 countries worldwide (Evers et al., 2019; FAO, 2022). Species such as koi (*Cyprinus carpio*), guppies (*Poecilia reticulata*), cichlids, and bettas (*Betta splendens*) are prized for their diverse coloration, patterns, and unique morphologies. This uniqueness drives demand for efficient breeding and reliable sex identification methods (Alam et al., 2024; Wibisono et al., 2024). Sex determination is a critical factor in ornamental fish breeding and trade, influencing growth rates, coloration, reproductive management, and market preferences (Kitano et al., 2024; Malik et al., 2026). For instance, males are often perceived as more desirable due to more vibrant colors and faster early growth, making them more desirable in commercial operations (Andrian et al., 2024; Chen et al., 2022).

Traditional phenotypic sexing methods, which rely on morphological cues and secondary sexual characteristics, face significant challenges in ornamental fish. Many species display late sexual dimorphism, with external differences only apparent after maturation, and environmental factors such as temperature and social interactions can further obscure sex identification (Endo et al., 2024; Kitano et al., 2024; Novindasari et al., 2024; Quertermous et al., 2025). These limitations may lead to increased production costs, inefficient resource allocation, and opportunity cost due to sexing error.

Molecular approaches to sex determination have emerged as transformative tools, offering accuracy, early detection, and applicability across developmental stages and diverse strains (Novindasari et al., 2024; Yang et al., 2024). The integration of PCR-based assays, next-generation sequencing (NGS), and bioinformatics pipelines has enabled the discovery and validation of sex-linked markers. This integration facilitates selective breeding, conservation, and commercial optimization (Y. Liu et al., 2025a; Novindasari et al., 2024; Yang et al., 2024).

Beyond its economic relevance, the ornamental fish sector represents a unique biological and methodological challenge for sex determination studies. Unlike feedstock aquaculture, ornamental breeding frequently involves intense artificial selection, hybridization across color morphs, and strain-specific reproductive traits, all of which can destabilize classical sex-linked inheritance patterns (Martinez-Bengochea et al., 2022). This review therefore evaluates molecular markers used for sex determination in ornamental fish, with a focus on biological complexity, marker selection, and validation strategies across diverse genetic and environmental contexts.

1. BIOLOGICAL BASIS OF SEX DETERMINATION IN ORNAMENTAL FISH

1.1 Diversity of Sex Determination Systems

Fish exhibit unparalleled diversity in sex determination mechanisms, encompassing genetic sex determination (GSD), environmental sex determination (ESD), and polygenic systems (Shen & Wang, 2018; L. Wang et al., 2022). The most common chromosomal systems are male heterogamety (XX/XY) and female heterogamety (ZZ/ZW) (Heule et al., 2014; L. Wang et al., 2022). Polygenic sex-determination systems involve the combined action of multiple genetic loci where each contributing a small effect to the final sex phenotype, making sex determination a quantitative and context-dependent outcome rather than one controlled by a single master sex-determining gene. Meanwhile, environmental factors such as temperature and social hierarchy, are also widespread. These multisystem mechanisms are particularly widespread among teleosts, the most evolutionarily diverse group of ray-finned fishes (Heule et al., 2014; Shen & Wang, 2018). Sex determination mechanism diversity reflects the evolutionary plasticity of fish reproductive strategies and underscores the importance of species-specific approaches in developing molecular sexing tools. A representation of markers used for sex determination in ornamental fish is summarized in Table 1.

Table 1. Representative Molecular Markers for Sex Determination in Ornamental Fish

System Type	Species	Marker Type	Target Gene/Region	Method	Reference
XY (Male heterogamety)	Guppy (*Poecilia reticulata*)	SNP	Y-linked region	NGS, PCR	(Fraser et al., 2020)
	Koi carp (*Cyprinus carpio*)	SCAR	ArS.9-15	PCR	(Novindasari et al., 2024)
	Betta (*Betta splendens*)	RAPD	Unknown	RAPD-PCR	(Mulyani et al., 2024)
	Japanese parrotfish (*Oplegnathus fasciatus*)	InDel	XY-linked genomic regions	NGS, PCR	(Xiao et al., 2024)
ZW (Female heterogamety)	Salangid fish (*Neosalanx brevirostris*)	SNP	W-linked region	NGS, PCR	(Yang et al., 2024)
	Pluang Chomphu fish (*Tor tambra*)	W-derived (SNP)	W-linked region	NGS, PCR	(Surachat et al., 2022)
Polygenic	Paradise fish (*Macropodus opercularis*)	Transcriptomic	*sox9*, *cyp19a1, etc*	RNA-seq	(F. Liu et al., 2023)
	Snakeskin Gourami (*Trichopodus pectoralis*)	Transcriptomic	rdh7, vamp3, etc	RNA-seq	(Jantawongsri et al., 2025)
Environmental	Zebrafish (*Danio rerio*)	Epigenetic	DNA methylation	Bisulfite sequencing PCR	(Hosseini et al., 2022; Valdivieso et al., 2023)
	Blotched Snakehead (*Channa maculata*)	Epigenetic	*cyp19a1a* methylation	Bisulfite sequencing PCR	(Huang et al., 2023)

The comparative overview in Table 1 underscores that no single molecular marker class can universally resolve sex across ornamental fishes. Species with heteromorphic sex chromosomes (e.g., guppy, Salangid Fish) permit relatively straightforward SNP- or InDel-based assays, whereas polygenic and environmentally modulated systems (e.g., paradise fish, gourami, zebrafish) require multilocus or expression-informed approaches. Importantly, the coexistence of XY and ZW loci within closely related taxa (Koi and Zebrafish in Cyprinidae) highlights frequent evolutionary turnover of sex-determining regions. This reinforces the need for marker validation at the population and strain levels rather than reliance on phylogenetic proximity alone.

1.2 Master Regulators and Candidate Genes

Recent research has identified several master sex-determining genes (MKRs) and candidate regulators in fish, including dmrt1, amh, sox9, cyp19a1a, and others. These genes orchestrate the cascade of gonadal differentiation and are frequently targeted for molecular sexing due to their conserved roles and early expression patterns during development (Fan et al., 2025; Herpin & Schartl, 2015; M. Li et al., 2015; Rajendiran et al., 2021).

- **dmrt1**: A conserved DM-domain transcription factor pivotal in testis formation and maintenance. It interacts with sox9 and represses female-promoting genes such as foxl2 and cyp19a1a. Lineage-specific isoforms and alternative splicing of dmrt1 have been observed across vertebrates, including zebrafish (Guo et al., 2005; Herpin & Schartl, 2015; Sharma et al., 2022).
- **amh/amhrII**: The anti-Müllerian hormone (amh) and its receptor (amhrII) are central to male differentiation. In several teleosts, including Nile tilapia and Sebastes rockfish, gene duplications (e.g., amhy) have been identified as sex-determining loci, with strong male-specific expression and functional validation through CRISPR knockouts (M. Li et al., 2015; Nakamoto et al., 2021; Song et al., 2021). It has been showed that high temperature (29 °C) induces expression of amhy as masculinisation signal in pejerrey (Rajendiran et al., 2021).
- **sox9**: sox9 is a critical transcription factor for the differentiation of Sertoli cells and testis development in vertebrates. In mammals as comparison, sox9 is upregulated following sry activity and maintains Sertoli cell identity via feedback loops and cooperation with factors like dmrt1. In teleosts, sox9 expression is prominent during male testicular differentiation. Expression of dmrt1 is found to upregulate sox9, promoting testicular genes transcription (Herpin & Schartl, 2015; Rajendiran et al., 2021).
- **cyp19a1a**: This gene encodes aromatase, the terminal enzyme in estrogen biosynthesis, and is critical for ovarian differentiation. In species with environmental sex determination (ESD), such as temperature-influenced sex differentiation in zebrafish and pejerrey, cyp19a1a is subject to epigenetic regulation, including DNA methylation (Fan et al., 2025; Rajendiran et al., 2021).

While master key regulators such as dmrt1, amh, sox9, and cyp19a1a offer biologically intuitive targets for sexing assays, their regulatory roles do not automatically translate into diagnostic reliability. Alternative splicing and epigenetic modulation evident in teleosts can decouple gene presence from phenotypic sex. As a result, markers derived directly from MKRs tend to perform best when integrated with epigenetics, sex-linked structural variants, or SNPs identified through comparative genomics, rather than used in isolation (Chailertrit et al., 2023; Domingos et al., 2018; Tao et al., 2018).

The interplay between these genes and environmental factors (e.g., temperature, pH, social cues) underlies the remarkable plasticity of sex determination in fish (Quertermous et al., 2025; Rajendiran et al., 2021). For instance, Removal the dominant male of a protogynous fish (*Notolabrus celidotus*) species will trigger sex change in the initial female into male (Quertermous et al., 2025). Meanwhile, temperature-dependent sex reversal has been well documented in species such as Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*), where elevated temperatures during early development can induce masculinization (Biswas et al., 2021; Lu et al., 2025). Such phenotypic shift is often mediated by epigenetic modifications, particularly suppression of cyp19a1a and modulation of dmrt1-associated regulatory regions under masculinizing conditions, which will reduce estrogen synthesis and promote testis development (Rajendiran et al., 2021; Valdivieso et al., 2023).

These findings highlight the dynamic crosstalk between genetic regulators and environmental inputs, reinforcing the need for integrated molecular and ecological approaches to sex control in aquaculture.

2. MOLECULAR APPROACHES TO SEXING

2.1 PCR-Based Methods

Polymerase chain reaction (PCR) remains the cornerstone of molecular sexing, enabling amplification of sex-linked sequences with high sensitivity and specificity (Huang et al., 2023). PCR-based assays target candidate gene regions (e.g., dmrt1, amh, cyp19a1a) or sex-specific fragments identified through genomic comparisons (Rajendiran et al., 2021). Among them, RAPD and AFLP markers are low-cost but less reproducible, SCAR markers strike a balance with moderate cost and high reliability, and InDel markers tend to offer high reliability with moderate to higher assay costs during InDel region discovery.

- **Sequence-Characterized Amplified Region (SCAR) Markers:** Sequence-characterized amplified region (SCAR) markers, often developed from sex-specific sequences or sex-linked microsatellite (SSR) loci, enable rapid PCR-based sex identification in fish by targeting genomic regions associated with sex determination. In cases derived from SSR loci, SCAR markers exploit repeat-length polymorphisms that segregate with sex which allows male- and female-specific PCR patterns. These markers function not because they determine sex themselves, but because they are inherited alongside the sex-determining loci. Their reliability has been confirmed across multiple populations and families, making them robust tools for molecular sexing within validated populations, although they are not universal SNP-based sexing assays (H. Liu et al., 2018; Novindasari et al., 2024; Song et al., 2012).
- **RAPD Markers:** Randomly amplified polymorphic DNA (RAPD) markers have been employed to identify sex-associated genomic regions in fish, typically by detecting fragments linked to nearby sex-related genes rather than directly targeting the genes responsible for sex determination. In turbot (*Scophthalmus maximus*), only a small subset of RAPD primers produced sex-linked markers, and the association was observed primarily in a specific family. These findings illustrate that while RAPD markers can serve as an initial tool for identifying candidate regions for sex determination, their effectiveness must be carefully validated in each population before they can be reliably applied in breeding or genetic studies (Lal et al., 2024; Mulyani et al., 2024; Vale et al., 2014).
- **AFLP Markers:** Amplified fragment length polymorphism (AFLP) and markers provide additional resolution because it samples genome-wide structural variation without requiring prior sequence knowledge. AFLP works by digesting genomic DNA with restriction enzymes, ligating universal adaptors, and selectively amplifying a reproducible subset of fragments based on restriction-site context rather than gene identity. In white trevally, AFLP produced reproducible genomic fingerprints that allowed identification and sequencing of a male-specific fragment for locus association (Agawa et al., 2022).
- **InDel Markers**: InDel primers are designed to flank a genomic region containing an insertion or deletion, enabling the amplification of fragments that differ in length depending on the presence or absence of the polymorphism. In *Oplegnathus fasciatus*, InDel markers were developed by designing primers flanking insertion or deletion polymorphisms. These markers allow precise, locus-specific assays for sex identification (Xiao et al., 2025).

PCR-based assays remain the most accessible tools for molecular sexing. However, their diagnostic accuracy depends strongly on primer design, the genomic context, and the specific strain. SCAR and microsatellite (SSR) markers offer high reliability within well-characterized genetic backgrounds but may show reduced transferability and specificity when applied to genetically divergent ornamental strains. RAPD markers, although historically valuable for exploratory analyses, are limited by poor reproducibility and inter-laboratory consistency, which restricts their long-term utility in commercial breeding programs.

2.2 Next-Generation Sequencing (NGS) and SNP Discovery

NGS technologies have revolutionized marker discovery, enabling genome-wide identification of sex-linked single nucleotide polymorphisms (SNPs) and structural variants (Chailertrit et al., 2023). Approaches such as RAD-seq and whole-genome resequencing may facilitate comparative analysis of male and female genomes, pinpointing sex-specific loci (Feron et al., 2021; Karci & Kafkas, 2022). RAD-seq typically requires ~20–50 individuals per sex with moderate per-locus coverage, whereas whole-genome resequencing needs fewer individuals but higher depth (~10-30x per genome) to reliably detect sex-linked variants.

Sex-linked SNPs are typically identified through structured discovery pipelines that integrate differential subtraction, association mapping, and linkage analysis. For example, whole-genome resequencing of nine populations of *Megalobrama amblycephala* revealed three male-specific markers that were subsequently validated across related species (Fu et al., 2024). Bioinformatic frameworks including Stacks and RADSex are then used to filter candidate loci based on read depth, allele frequency, and sex association, ensuring statistical robustness and reproducibility (Feron et al., 2021; Rochette et al., 2019). Marker reliability is assessed using standard quality control metrics such as genotype call rate and reproducibility, with markers showing excessive missing data or low concordance excluded from further analysis (Turner et al., 2011).

NGS-based approaches therefore provide unparalleled resolution and scalability for molecular sexing, enabling population-level replication, functional annotation of sex-linked regions, and the development of robust sexing panels for non-model ornamental species. Importantly, these pipelines shift molecular sexing from phenotype-correlated proxy markers toward genotype-resolved diagnostics. This transition is particularly critical in ornamental fishes where environmental sex reversal, hybridization, and artificial selection frequently obscure phenotypic sex expression.

2.3 Transcriptomic and Epigenetic Markers

Transcriptomic profiling (RNA-seq) and epigenetic analyses (e.g., DNA methylation) provide insights into sex-biased gene expression and regulatory mechanisms (Huang et al., 2023; Sharma et al., 2022; Valdivieso et al., 2023). Transcriptomic analyses using RNA-seq have shown strong male-biased expression of dmrt1, amh, and sox9 during early and adult testicular development, whereas foxl2 and cyp19a1a are upregulated in developing and mature ovaries. These expression patterns are routinely validated through quantitative PCR and corroborated by histological examination of gonadal structure. In species with environmental or plastic sex determination systems, differential expression of these genes often precedes visible gonadal differentiation which provide early indicators of sex trajectory (Fan et al., 2025; Hosseini et al., 2022; M. Li et al., 2015; Rajendiran et al., 2021; Si et al., 2016).

Promoter methylation of dmrt1 and cyp19a1a plays a regulatory role in temperature-dependent sex determination and environmentally induced sex reversal in many teleost fishes. Elevated temperatures during critical developmental windows are associated with altered DNA methylation patterns at these promoters, leading to repression of cyp19a1a and modulation of dmrt1. These epigenetic modifications directly influence downstream steroidogenic pathways by reducing estrogen synthesis and biasing gonadal differentiation toward the male phenotype. Because methylation states can precede visible gonadal differentiation, they provide early, mechanistically informative indicators of sex trajectory (Rajendiran et al., 2021; L. Wang et al., 2022).

2.4 Portable and Point-of-Care Diagnostics

Emerging technologies such as loop-mediated isothermal amplification (LAMP) and portable qPCR kits are transforming field diagnostics, enabling rapid, on-site sex identification in hatcheries and farms.

- **LAMP Assays:** LAMP require minimal equipment, operate at a single temperature, and provide visual readouts. Successful application in avian sexing suggests potential adaptation to fish, pending primer optimization and validation (Elnomrosy et al., 2022).
- **Point-of-Care Kits:** Commercial platforms integrating sample preparation, amplification, and detection are under development. They are currently underutilized because there are still only a few commercially validated, affordable, and user-friendly products tailored to the diverse species and markers used in aquaculture. It aims to democratize molecular sexing for small-scale aquaculture operators and reduce reliance on centralized laboratories (Khodaparast et al., 2024).

Summary of advantage and disadvantage of each molecular sexing approach in this paper is shown in Table 2.

Table 2 Advantages and Disadvantages of Molecular Sexing Approaches

Molecular approach	Advantages	Limitations	Key references
PCR-Based Methods	- Widely accessible and cost-effective - High sensitivity and specificity - Compatible with standard laboratory infrastructure - Rapid implementation - Effective within validated populations	- Diagnostic performance strongly dependent on primer design, genomic context, and strain - Reduced transferability across genetically divergent or ornamental strains - Not genome-wide or SNP-resolved	(Agawa et al., 2022; Huang et al., 2023; Lal et al., 2024; H. Liu et al., 2018; Mulyani et al., 2024; Novindasari et al., 2024; Rajendiran et al., 2021; Song et al., 2012; Vale et al., 2014; Xiao et al., 2025)
Next-Generation Sequencing (NGS) and SNP Discovery	- Genome-wide resolution - Enables discovery of sex-linked SNPs and structural variants - High scalability and reproducibility - Supports population-level validation and cross-species testing - Enables genotype-resolved diagnostics	- Higher cost and infrastructure requirements - Requires bioinformatic expertise and computational resources - Less accessible for routine or small-scale operations	(Chailertrit et al., 2023; Feron et al., 2021; Fu et al., 2024; Karci & Kafkas, 2022; Rochette et al., 2019; Turner et al., 2011)
Transcriptomic and Epigenetic Markers	- Provide mechanistic insight into sex determination and differentiation - Detect sex-biased gene expression prior to visible gonadal differentiation - Informative for environmental and plastic sex determination systems	- Indirect proxies for genetic sex - Expression and methylation patterns can be stage- and environment-dependent - Limited standalone diagnostic reliability	(Fan et al., 2025; Hosseini et al., 2022; Huang et al., 2023; M. Li et al., 2015; Rajendiran et al., 2021; Sharma et al., 2022; Si et al., 2016; Valdivieso et al., 2023; L. Wang et al., 2022)
Portable and Point-of-Care Diagnostics	- Rapid, on-site sex identification; minimal equipment - Suitable for field, hatchery, and farm applications - Potential to democratize molecular sexing	- Limited validation in fish - Primer optimization required - Commercial platforms still under development	(Elnomrosy et al., 2022; Khodaparast et al., 2024)

3. MARKER VALIDATION AND RELIABILITY

Cross-species primers target conserved regions of MKRs (e.g., dmrt1, amh), enabling broad applicability across related species. Useful for initial screening and comparative studies, but may suffer from reduced specificity in divergent strains (Curzon et al., 2023). Meanwhile, species-specific primers are designed from unique sex-linked sequences identified via NGS or comparative genomics. Offer higher accuracy and reliability, especially in hybridized or polygenic systems (Fu et al., 2024; X. Li et al., 2025; Zhao et al., 2025).

The trade-off between cross-species and species-specific primers reflects a broader tension between scalability and precision. While conserved MKR-based primers offer rapid deployment across related taxa, their reduced specificity in hybridized or polygenic systems limits commercial reliability. In contrast, species- and strain-specific primers, although resource-intensive to develop, provide the diagnostic resolution required for ornamental breeding programs where misclassification carries economic and opportunistic costs.

Robust marker validation requires testing across diverse strains, populations, and generations to ensure reliability and minimize false positives/negatives. For example, sex-specific markers in *Megalobrama amblycephala* were validated in wild and domesticated species, enhancing universality (Fu et al., 2024). Marker performance is commonly evaluated using sensitivity which is defined as the proportion of true positives correctly identified, specificity which is defined as the proportion of true negatives correctly identified, and reproducibility which is the consistency of results across replicates. These are critical criteria for assessing the robustness and practical applicability of molecular sexing assays (Turner et al., 2011; K. Wang et al., 2019).

4. CHALLENGES IN MOLECULAR SEXING OF ORNAMENTAL FISH

Non-specific amplification remains a common technical challenge in molecular sexing assays for ornamental fish. This issue typically arises from high sequence similarity between sex-linked targets and autosomal regions, the presence of paralogous gene copies, or suboptimal primer design. Such non-specific amplification can lead to false positives or ambiguous banding patterns, particularly in PCR-based assays. To mitigate this problem, rigorous in silico primer evaluation using BLAST-based specificity checks, combined with empirical optimization and validation across known-sex individuals, is essential (Ye et al., 2012).

4.1 Technical Challenge

- **Reproducibility issues** further complicate the deployment of molecular sexing assays. Factors such as primer–dimer formation, secondary structures in template DNA, and variability in PCR conditions across laboratories can significantly affect assay performance. These technical inconsistencies can undermine confidence in marker reliability. Careful optimization of reaction conditions, standardized protocols, and the routine inclusion of internal positive controls such as housekeeping genes (e.g., β-actin), are strongly recommended to improve reproducibility (K. Wang et al., 2019; Ye et al., 2012).

4.2 Biological Challenges

- **Strain variability** represents a major biological constraint on the reliability of molecular sex markers in ornamental fish. Selective breeding for coloration, morphology, and behavior has generated substantial genetic divergence among strains within the same species, which can disrupt primer binding sites and reduce amplification consistency. Consequently, markers that perform well in a single strain may fail when applied more broadly. Multi-strain and population-level validation is therefore critical to ensure marker robustness and commercial applicability (Fu et al., 2024; Kajino et al., 2025). Extensive domestication and selective breeding in ornamental fish can substantially restructure genetic diversity, and given the known lability of sex-determination systems in teleosts, sex-linked markers may not always be transferable across populations or breeding lines (Kwon et al., 2022; Y. Liu et al., 2025b; Prasertlux et al., 2023). For example, Betta splendens is often described as following an XY-like system. However, domestication and strain divergence suggest a more complex, potentially polygenic architecture (Panthum et al., 2022).
- **Environmental sex reversal** further complicates marker interpretation by introducing discordance between genetic sex and phenotypic sex. Environmental factors such as temperature and social interactions can override genetic predispositions during critical developmental windows, resulting in

sex-reversed individuals. In such cases, molecular markers that accurately reflect genetic sex may conflict with gonadal phenotype, challenging their use in breeding and management decisions. This phenomenon is well documented in several teleosts and underscores the need to integrate molecular data with phenotypic and environmental information (Huang et al., 2023; Lu et al., 2025; Rajendiran et al., 2021).

- **Lack of genomic resources for many ornamental species** continues to limit progress in molecular sexing. Many non-model ornamentals lack high-quality reference genomes, annotated transcriptomes, or population-scale sequencing data, constraining marker discovery and primer design. Strategic investment in genome sequencing, public database development, and comparative genomics initiatives is therefore essential to expand molecular sexing capabilities and support standardized assay development across the ornamental fish sector (Kwon et al., 2022; Taslima et al., 2024).

5. CONCLUSION

Molecular sex determination has become an indispensable tool for the ornamental fish industry, offering solutions to the biological complexity that limit traditional phenotypic sexing. This review highlights that the extraordinary diversity and plasticity of sex determination systems in ornamental fishes preclude the use of universal markers, and instead demand species-, strain-, and context-specific strategies. PCR-based assays remain the most accessible and practical tools for routine applications, while NGS-driven SNP discovery provides the resolution required to develop robust, genotype-resolved markers in genetically complex or hybridized stocks. Transcriptomic and epigenetic approaches deepen mechanistic understanding and are particularly valuable in environmentally sensitive systems, although they require integration with genetic markers for reliable diagnostics. Emerging portable technologies promise to bridge laboratory and field applications but require further validation in fish. Ultimately, the reliability of molecular sexing in ornamental fish hinges on rigorous multi-strain validation, standardized protocols, also the integration of genetic, epigenetic, and environmental information. Continued investment in genomic resources and comparative frameworks will be critical to translating molecular sexing from experimental studies into dependable industrial applications that support sustainable breeding, economic efficiency, and biodiversity conservation.

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