Resolving Phylogenetic and Morphological Patterns in Poachers (Agonidae): Insights from Mitochondrial and Nuclear DNA, Habitat Type, and Armor Investment

Abstract

Understanding evolutionary relationships in marine fishes offers insights into morphological evolution and ecological adaptation. This study explores how mitochondrial (COI) and nuclear gene phylogenies relate to armor morphology, habitat, and geographic distribution in Agonidae, a family of armored benthic fishes. Using genomic data, phylogenetic trees were generated and compared with morphological and ecological traits. Armor morphology was measured using skeleton-to-armor volume ratios from CT scans. Habitat classifications and geographic distribution was used to compare phylogeographic patterns. Both COI and nuclear phylogenies showed resolved clades for some genera, but others appeared polyphyletic. Discrepancies between mitochondrial and nuclear trees suggest complex evolutionary histories, including incomplete lineage sorting or hybridization. Armor traits were consistent within some clades, but similar traits appeared to be spread out across phylogeny trees, showing possible convergent evolution. Species from similar habitats sometimes clustered, however habitat categories were potentially too broad to determine distinct patterns. Partial geographic structuring was observed, with some clades associated to distinct regions such as the North Pacific and Arctic. These findings show that phylogeny may partially explain variation in morphology and ecology, however environmental factors and evolutionary convergence also influence armor evolution. Incorporating genetic, morphological, and ecological data offers a more complete view of Agonidae diversity.

Introduction

Understanding how marine fish species are related to each other is crucial to identify distinct evolutionary lineages and offers insights into how morphology has varied across lineages and changed overtime. One group that demonstrates the importance of understanding evolutionary relationships is the poachers (Agonidae), a family of benthic fishes that are incredibly diverse and display ecological specialization across species (Sheiko & Mecklenburg, 2004). These unique fish are shielded by bony plates on their body which are arranged in specific rows and groups, and serves a multitude of functions including defense and survival in their harsh environments (Sheiko & Mecklenburg 2004; Vandenberg et al. 2024). Poachers are largely understudied, however, recent studies have combined genomic sequence data and morphological traits such as body armor in order to understand evolutionary processes and distribution of marine fishes (Stevenson et al. 2021).

Despite improvements in phylogenetic analysis by combining mitochondrial DNA and nuclear gene data, discrepancies are common (Toews & Brelsford 2012). Discordance refers to the evolutionary relationships derived from mitochondrial DNA do not match those from nuclear DNA, which suggests that the two genetic markers tell different evolutionary relationships (Morales-Briones et al. 2021). Armor morphology has evolved multiple times across fish lineages, but as a result of conflicting patterns between mitochondrial DNA and nuclear genetic markers, its relationship to phylogeny and geographic distribution in Agonidae is not well understood (Toews & Brelsford 2012; Vandenberg et al. 2024). Additionally, habitat type may influence the evolution of armor trait, however its relationship to phylogenetic patterns remain unclear (Lemopoulos & Montoya-Burgos 2021). This study addresses the gap by testing how

mitochondrial and nuclear phylogenies support patterns in armor morphology (skeleton:armor volume ratio), habitat type, and geographic distribution in Agonidae species.

A genetic approach in this study provides insights into evolutionary history of poachers at the molecular level, which is not possible through morphology alone. Genetic data can identify divergence patterns across species and can be used to confirm if patterns observed in morphological data and ecological traits like habitat type is consistent with genetic data (Smith & Busby 2014). The cytochrome oxidase I (COI) gene is commonly used as a mitochondrial marker for species identification, which is precise and allows for comparisons with existing genetic databases (Hebert et al. 2003). Nuclear genes are also an important data tool that allows for a more complete view of evolutionary history and can be used to interpret phylogenies on a species level (Curto et al. 2012). Incorporating COI and nuclear genetic markers can offer evidence of evolutionary processes such as hybridization or incomplete lineage sorting, which is relevant to understanding the complexity of diverging patterns in Agonidae (Toews & Brelsford, 2012). Additionally, armor morphology is an important trait of Agonidae which plays a large role in their function, protection, and serves as a tool to identify poacher species. Analysis of skeleton to armor volume ratio using CT scans allows for high-resolution morphological data. It can help test whether the evolution of armor is consistent with genetic divergence or environmental pressures. Also, using geographic distribution data provides context for the variation of genetic and morphological data across species. Habitat type is another key ecological factor that may influence morphology, and understanding its relationship to phylogenetic patterns can clarify whether similar armor traits arise from shared ancestry or adaptation to similar environments. Analyzing distribution of poachers shows whether armor morphology is shaped by ancestry or other factors, such as predation (Solomatov et al. 2025).

The objective of this study is to investigate how mitochondrial and nuclear phylogenies align with morphological, ecological (habitat type), and geographic distribution in Agonidae, and the evolutionary processes that occurred. It compares geographic distribution across COI and nuclear phylogenetic trees, evaluates armor investment using skeleton to armor volume ratios, and determines whether morphological traits, habitat types, and geographic patterns are consistent between both phylogenies. Conflicts between these phylogenies may suggest evolutionary processes such as hybridization, convergence, or incomplete lineage sorting. To accomplish this, armor investment, habitat data, and geographic distribution on phylogenetic trees will be compared, and processes, such as convergence, incomplete lineage sorting, or hybridization will be identified.

Alternative Hypothesis

Phylogenetic patterns (from mitochondrial and nuclear data) are associated with armor morphology, habitat type, and/or geographic distribution in Agonidae. Trait clustering within clades shows shared evolutionary history and discrepancies between gene trees show evolutionary processes such as hybridization, convergence, or incomplete lineage sorting.

Null Hypothesis

There is no significant relationship between phylogenetic patterns (mitochondrial and nuclear) and armor morphology, habitat type, or geographic distribution in Agonidae. Observed similarities in traits across clades are due to random variation or sampling bias.

Methods

DNA Extraction

Fish tissue samples (n=100) preserved in 100% ethanol immediately after collection prevented DNA degradation. Each fish ID number and tissue mass were recorded, and tubes were labelled (fish ID, name, date, section). DNA was extracted using the Qiagen DNeasy Tissue Kit. Samples were placed in a 1.5 mL tube with 180 µL of Buffer ATL. To lyse the tissue, 20 µL of Proteinase K was added, then incubated at 56°C for 1–2 hours with occasional vortexing. Once dissolved, 200 µL of Buffer AL was added then vortexed. Then 200 µL of ethanol was added, then vortexed. The mixture was pipetted into a DNeasy spin column in a collection tube and centrifuged at 6,000 rcf for 1 minute. The column was washed with 500 µL of Buffer AW1 and AW2, then centrifuged at 20,000 rcf. DNA was eluted with 100 µL of Buffer AE and stored at 4°C.

PCR

A ~800 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using two primers at stock concentrations of 10µM: CP-F130-DL (5′-GTCTTTCCATAACTGCAATATCAC-3′) and CP-R289-DL (5′-CATSCATGTATCAATACATAGGTAC-3′). A 120 µL master mix contained 75 µL of Phusion Master 2× Mix with HF Buffer, 7.5 µL of both the forward and reverse primer, and 30 µL of 1X sterile de-ionized water. Each PCR reaction contained 20 µL of master mix and 5 µL of template DNA in reaction strip tubes, a total of 25 µL. A negative control using sterile water in place of DNA was included. Amplifications were performed in a miniPCR thermal cycler (miniPCR bio, Cambridge, MA, U.S.A.) which consisted of an initial denaturation at 98 °C for 30 seconds; 31 PCR cycles of 98 °C for 10 seconds, 52 °C for 30 seconds, and 72 °C for 60 seconds; and completed by a final extension at 72 °C for 10 minutes. Reactions were then held at 4°C to be analyzed in a future lab.

Gel Electrophoresis

A 1% agarose gel was prepared using 0.2 g of agarose in 20 mL of 1X TBE buffer. Two strips of 8 tubes (rows A and B) contained: ladder, DNA or PCR, and controls. Row A contained two wells of 4 µl of ladder, DNA extracts, a gel control, with 6X dye in each sample. Row B contained two tubes of 4 µl of ladder, PCR products, a positive control, and negative control, with 6X dye in each sample. Samples were run on the gel tray for 45 minutes at 48 DV.5A, immersed in 30 ml of 1X TBE buffer. DNA ladder provided reference bands of known sizes. Higher weight DNA bands appeared near the top, which showed successful extraction. Lower, PCR product bands at near the bottom showed successful amplification. Missing bands suggested failed PCR. Positive controls confirm reactions, negative controls confirm no contamination.

Phylogenetic Trees
Phylogenetic trees were generated using MEGA 12. The mitochondrial COI dataset included 98 sequences ranging from 533 to 686 bp, and the nuclear dataset included 94 sequences totaling 13,536 bp. The Standard Bootstrap method was used with 100 replications Maximum Likelihood was used as the statistical method. Additionally, the "General Time Reversible model" was used and gaps and missing data were treated using the "use all sites" option. Codon positions 1st, 2nd, 3rd, and noncoding sites were selected.

Habitat Data
Habitat type data for each Agonidae species included in the analysis was retrieved from FishBase, an online database of fish species. Information such as depth range and general habitat classification (soft bottom, demersal, benthic, intertidal, rocky area, coastal) was recorded for each species to categorize their habitat type. This data was used to compare ecological traits across the phylogenetic trees and find the relationship between habitat type, armor morphology, and geographic distribution.

Results

DNA Sample Sequencing
A total of 95 poacher fish samples were collected and preserved in 100% ethanol to prevent DNA degradation. From these, 24 samples were successfully sequenced for both mitochondrial COI and nuclear genes. These samples were used for phylogenic analysis.

Gel Electrophoresis
Gel electrophoresis demonstrated successful or unsuccessful DNA extraction and PCR amplification (Fig. 1). In Row A of the gel, high molecular weight smears near the top confirmed successful DNA extraction in multiple fish tissue samples. In Row B, clear bands in the lower-middle region of the gel (~800 bp) display successful amplification of the COI gene using PCR. The presence of bands in the positive PCR control and no bands in the negative control support the reliability of the reactions and ensured there was no contamination. However, during the reaction, the ladder stayed compressed near the wells, suggesting that the gel may not have run long enough to reach full separation. Running the gel for a longer period or at a higher voltage could improve resolution and allow more accurate size estimation.

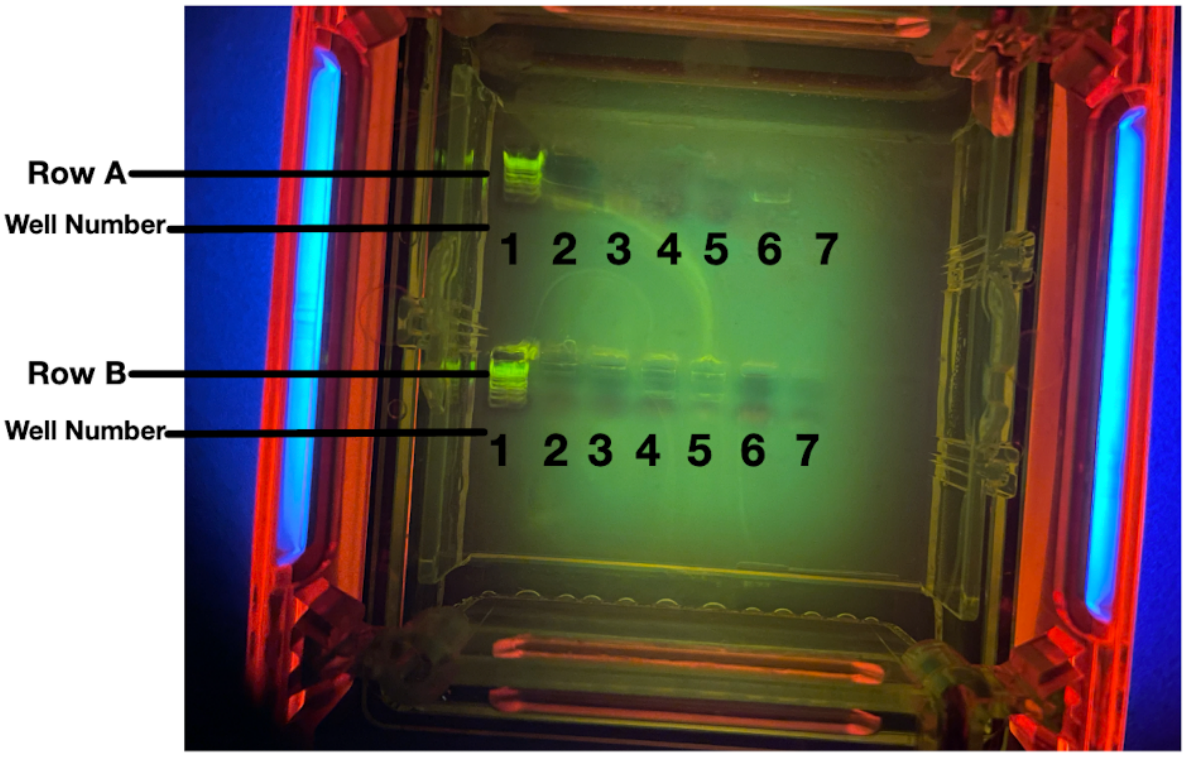


Figure 1. Agarose gel electrophoresis of DNA extracts (Row A) and PCR products (Row B). Row A: ladder in well 1. Wells 2–5 contained DNA samples. Well 6 contained a gel control, well 7 was empty. Row B: ladder in well 1. Wells 2–5 contained PCR samples. Well 6 contained a positive PCR control. Well 7 contained a negative PCR control. Samples were run on a 1% agarose gel at 48 V for 45 minutes in 1X TBE buffer. The DNA ladder is designed for sizing double-stranded DNA.

Phylogenetic Tree Resolution

Phylogenetic trees were constructed using Maximum Likelihood in MEGA 12 with 100 bootstrap replicates, applying the General Time Reversible substitution model. The mitochondrial COI dataset included 98 sequences (533–686 bp), while the nuclear dataset contained 94 sequences (13,536 bp). The COI and nuclear phylogenetic trees display consistent clade configuration for genera such as *Podothecus*, *Bathyagonus*, and *Agonopsis* (Figs. 2 and 3). Overall, both trees exhibit high support, with most values above 90%. A few nodes in the nuclear tree fall below 50% bootstrap support, particularly near deeper branches. *Agonus cataphractus* form well-supported clades in the mitochondrial tree (Fig. 2) but is less resolved in the nuclear tree (Fig. 3). On the other hand, *Sarritor leptorhynchus* shows stronger support in the nuclear tree, while it showed a weaker resolution in the mitochondrial tree. *Aspidophoroides* is monophyletic in both COI and nuclear trees. *Sarritor* and *Hypsagonus* is paraphyletic, with other genera within the clade. *Leptagonus* appears polyphyletic, dispersed across unrelated clades in both trees.

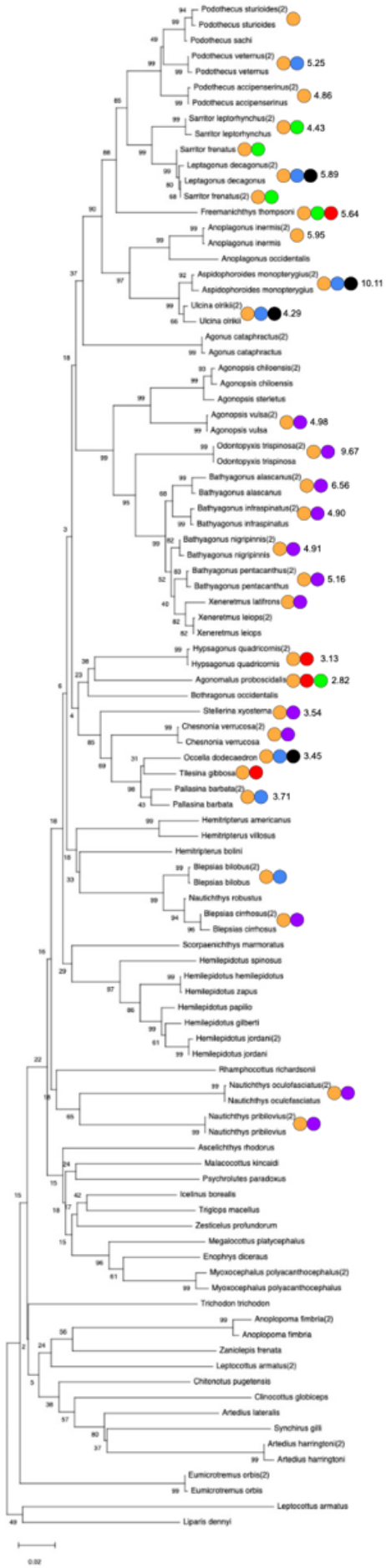
Habitat Type

Species occupying similar habitats often clustered together phylogenetically. For example, soft-bottom (mud, sand) associated demersal species within *Podothecus* and *Occella* formed supported clades in both trees. Similarly, rocky-area associated *Bathyagonus* formed well

supported clades. In contrast, intertidal and soft-bottom habitat types were scattered across clades in both trees, such as within *Blepsias*. It is important to note that habitat classifications from FishBase were often broad, and only limited to categories such as demersal, soft-bottom-associated, intertidal, rocky areas, and bathydemersal. As a result, the depth of ecological patterns in this analysis may be constrained by available habitat data.

Armor Investment Patterns

Skeleton-to-armor volume ratios were mapped onto the branches of both the mitochondrial and nuclear phylogenetic trees for species with available morphological data. Overall, only a limited of sampled species had complete skeleton:armor data. Where data was available, patterns of armor investment varied in consistency. In several cases, closely related species displayed similar armor-to-skeleton ratios. For example, *Agonomalus* and *Hypsagonus* displayed relatively low armor investment levels within their clades in both trees. However, this was not consistent across all taxa. In several genera, species with similar armor-to-skeleton ratios were distributed across phylogenetically distant clades, seen in *Bathygonus* and *Podotheucs*. Additionally, species with lower armor investment were found primarily in the northern Pacific to Arctic Ocean, including *Occella* and *Pallasina*.



Region	Color
Arctic	Blue
Okhotsk Sea	Red
Japan	Green
North Pacific	Orange
Temperate Pacific	Purple
Amphiboreal	Brown

Figure 2. Maximum Likelihood phylogenetic tree of Agonidae based on COI sequences (n = 24). Bootstrap values are indicated at nodes. Colored circles represent sampling regions per specimen, and numbered branches represent the skeleton:armor volume ratio. Habitat type, obtained from FishBase, is included as an additional trait for each species. Tree generated using MEGA 12, GTR model, all codon and noncoding sites included.

Discussion

This research examines how mitochondrial and nuclear phylogenies support or explain patterns observed in armor morphology, habitat type, and the geographic distribution of Agonidae. It compares geographic distribution and habitat type across COI and nuclear phylogenetic trees, evaluates armor investment using skeleton to armor volume ratios, and determines whether morphological traits and geographic patterns are consistent between both phylogenies.

Both agreeing and discordant relationships were observed between mitochondrial (COI) and nuclear phylogenetic trees among Agonidae species. Genera such as *Podothecus*, *Bathyagonus*, and *Agonopsis* were consistently resolved as clades in both trees, and *Agonus* appeared monophyletic across trees. However, there were conflicts between the gene trees, such as *Agonus cataphractus*, which was well-resolved in the mitochondrial tree but poorly supported in the nuclear tree. Also, *Leptagonus* appeared polyphyletic in both trees, displaying potential unresolved relationships. Relationships that do not match could be attributed to including incomplete lineage sorting, hybridization events, or limited sampling (Toews & Brelsford, 2012). These findings demonstrate the importance of using both mitochondrial and nuclear markers to resolve phylogenetic relationships, especially in recently diverged taxa where using mitochondrial data on its own may be misleading (Smith & Busby 2014).

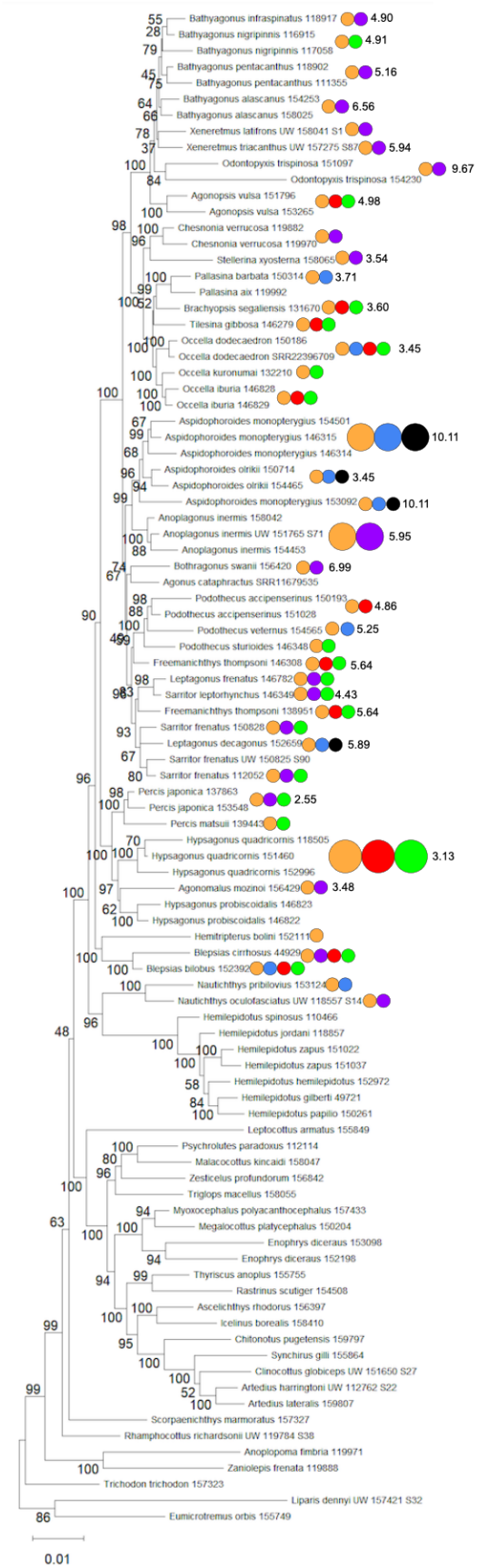
Armor investment, measured as the skeleton-to-armor volume ratio, showed mostly mixed alignment with the phylogeny trees. In some clades, closely related species displayed similar armor investment, suggesting potential evolutionary conservation. However, in many cases, species with similar armor traits were in genetically distant lineages, indicating potential convergent evolution. For example, *Agonomalus* and *Hypsagonus* both displayed relatively low armor investment within their clades in both trees. Several other species with similar armor-to-skeleton ratios were distributed across distant clades, demonstrating convergent evolution of armor morphology potentially in response to similar environmental pressures. Because armor data was only available for a limited number of species, some observed patterns may reflect sampling bias rather than evolutionary trends. However, the mapped traits provide a base for evaluating the evolutionary and ecological significance of armor morphology in Agonidae. This ambiguity shows that while evolutionary relationships may influence armor investment, other ecological pressures such as predation may play a significant role (Price et al. 2015).

Moreover, some trends in armor investment appear to correlate with geographic distribution. For example, several low skeleton-to-armor ratio species such as *Agonomalus* and *Hypsagonus* are mainly distributed in the northern Pacific, where colder, high-latitude environments might select for reduced armor to enhance mobility or energy efficiency in less predator-dense environments (Reynolds et al. 2018). Species with higher armor investment such as certain *Podothecus* tend to occur in shallower temperate areas, where predation pressure may be greater. Although there was no definitive armor ratio pattern based on geography for every species, these trends suggest that

while genetic lineage may show some geographic structure, the evolution and distribution of armor traits in Agonidae are likely shaped by a combination of genetic and environmental factors.

Species occupying similar habitats often clustered together phylogenetically, suggesting a potential link between habitat and evolutionary lineage. For example, rocky-area-associated *Bathyagonus* species grouped into well-supported clades, indicating possible divergence due to habitat. However, this pattern was less consistent for soft-bottom species, which were more dispersed across clades, seen in *Blepsias*. This discrepancy may be a result of habitat flexibility within certain lineages or limitations in habitat categorization. It is important to note that habitat classifications used from FishBase were broad, and while some phylogenetic clustering by habitat is evident, the depth of ecological patterns in this study are likely constrained by available habitat data.

This study, while informative, faced some limitations that hinder the depth of analysis. The small sample size of only 24 individuals successfully sequenced for both mitochondrial and nuclear genes, reduces statistical estimation accuracy and may have contributed to conflicting clades. Also, incomplete morphological and geographic metadata for some species limited the ability to fully identify correlations between armor investment, habitat type, phylogeny, and distribution. There were several low-bootstrap nodes in the COI tree (<70% support) and shows uncertainty in deeper evolutionary relationships (Hillis & Bull 1993). Future research would benefit from incorporating additional nuclear loci to enhance resolution of phylogenetic relationships. Also, integrating environmental variables like predation would offer further context for the observed variations in morphology and test whether conflicts between phylogeny trees are a result of evolution or geographic processes. This research reveals that the relationship between phylogenetic divergence and armor morphology in Agonidae is complex, displaying both agreeing and conflicting patterns across mitochondrial and nuclear phylogenies. These findings demonstrate the significance of using an integrative approach by combining genetic, morphological, and geographic data to gain a greater understanding of the forces shaping armor morphology and to identify patterns of trait evolution and geographic divergence in marine fishes.



Region	Color
Arctic	Blue
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Figure 3. Maximum Likelihood phylogenetic tree of Agonidae based on nuclear DNA sequences (n = 24). Bootstrap support values are displayed at nodes. Colored circles represent sampling regions per specimen, and numbered branches represent the skeleton:armor volume ratio. Habitat type, obtained from FishBase, is included as an additional trait for each species. Tree generated using MEGA 12, GTR model, all codon and noncoding sites included.

Statement Response to TA and Peer Feedback for Each Section

Introduction: I fixed some grammar mistakes and added that I used habitat data from FishBase to explain the traits better. My peer also said I needed to explain "discordance" more clearly, so I expanded on the sentence: "Despite improvements in phylogenetic analysis by combining mitochondrial DNA and nuclear gene data, discrepancies are common."

Methods: I clarified the issues with the gel electrophoresis in my methods and results. In the methods section it was mentioned in the feedback that I actually had a few results listed, which should have gone to the results section. Also, I did not originally mention that the gel didn't run long enough, which caused the ladder to be compressed. I also corrected my earlier mistake by specifying that wells 6 and 7 contained PCR controls, not gel controls.

Results: I changed the results section to avoid starting sentences with numbers by rephrasing those sentences. I also adjusted figure labels to ensure they don't block important details like the well numbers or DNA bands, which was recommended. Unfortunately, the gel electrophoresis photo was the best of the photos that our group gathered, so I was unable to more clearly show those results.

Discussion: I changed the discussion by expanding on what I meant by "discordance" between mitochondrial and nuclear trees, and that is essentially conflicts between the gene trees. I also added connections between geographic distribution and skeleton-to-armor ratio, suggesting that environmental pressures may shape armor traits. I also clarified what I meant by "low" bootstrap values by finding a cutoff value.

AI Acknowledgement

AI was used to support specific aspects of this paper. It helped me locate very difficult to find sources, such as scientific papers on what qualifies as a low bootstrap value. This was information I was unable to find on my own. I also used AI to identify appropriate synonyms, which made my writing more concise and clearer. Lastly, when I was unsure how to interpret certain phylogenetic relationships (such as whether a group was polyphyletic or paraphyletic), I described the patterns I observed and used AI to help find the correct terminology. I then looked in other online websites to confirm if the relationships were correct.

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