The genetic basis and enigmatic origin of melanic polymorphism in pomarine skuas (*Stercorarius pomarinus*)

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Abstract

A key outstanding issue in adaptive evolution is the relationship between the genetics of intraspecific polymorphism and interspecific evolution. Here we show that the pale/dark ventral plumage polymorphism that occurs in both the pomarine skua (*Stercorarius pomarinus*) and arctic skua (*S. parasiticus*) is the result of convergent evolution at the same locus (*MC1R*), involving some of the same amino acid sites. The dark melanic *MC1R* allele in the pomarine skua is strongly divergent from the pale *MC1R* alleles. Whereas the dark allele is closely related to *MC1R* alleles in three species of great skua (*S. skua*, *S. maccormicki*, *S. lonnbergi*), the pale pomarine skua *MC1R* alleles present a star-like pattern in an intermediate position on the haplotype network, closer to alleles of the long-tailed skua (*S. longicaudus*). Variation at other nuclear loci confirms a close relationship between the pomarine skua and the great skuas. The plumage polymorphism in pomarine skuas might have arisen in the common ancestor of pomarine and great skuas, only being retained in pomarine skuas. Alternatively, the pale and melanic *MC1R* alleles may have evolved independently in different lineages and been brought together in pomarine skuas by hybridization. In this case introgression of a pale *MC1R* allele into the pomarine skua from another skua lineage is most likely. Our current data do not permit us to distinguish between these hypotheses, and assaying genome-wide variation holds much promise in this regard. Nevertheless, we have uncovered an intriguing example of a functionally important allele within one species that is shared across species.

1. Introduction

While there has been substantial progress in defining the genetic basis of adaptive evolution, many uncertainties remain [1]. One key issue is the extent to which conserved genetic mechanisms underlie phenotypic convergence both at the level of loci and individual mutations [2]. A second issue is the relationship between genetic variation underlying adaptive phenotypes within species and evolution between species. While many studies have described adaptive genetic variants within or between populations of the same species, it is rarely known whether this variation is relevant between species (but see [3, 4]). Related to this is the occurrence of adaptive introgression of alleles among species [5, 6].

The skuas (*Stercorarius*), a group of kleptoparasitic seabirds, are an excellent clade to study these questions. Morphologically they form two groups comprising smaller species and larger species ("great skuas", previously placed in the genus *Catharacta*). Two of the smaller species, the arctic skua (*Stercorarius parasiticus*) and pomarine skua (*S. pomarinus*), share a striking plumage polymorphism, in which the ventral plumage in adults of both sexes is either pale, or dark and heavily melanised. In contrast, while some species of great skuas do show plumage colour variation [7, 8], this variation is more continuous and a discrete polymorphism as found in arctic and pomarine skuas is absent. The phylogeny of skuas has previously been a source of contention, but there is considerable evidence that pomarine skuas are the sister group to the great skuas, with the other smaller species - arctic skuas and long-tailed skuas (*S. longicaudus*) - forming a separate clade [9-11].

In arctic skuas it has previously been shown that variation at the melanocortin-1 receptor (*MC1R*) locus is associated with the plumage colour polymorphism [12]. Three linked amino acid variants at *MC1R* are completely associated with melanism in this species [13]. The nature of adaptation on plumage colour in arctic skuas is still being elucidated.

Early work implicated an effect of mate choice [14], but more recent research has favoured apostatic selection in which the success of kleptoparasitism in relation to morph colour is frequency-dependent, as well as sex-specific effects [15-17]. Population genetic data indicate the action of divergent selection on *MC1R* along the north-south cline in morph frequency of arctic skuas [13]. In contrast, the genetic basis of the plumage polymorphism in pomarine skuas has not been studied so far. In addition, little is known about selection on plumage colour in this species, in which melanic plumage is rare and does not appear to vary systematically in frequency across the breeding range [7,8].

The similar plumage polymorphism in arctic and pomarine skuas could have arisen by five genetic mechanisms: I: single origin in a common ancestor and maintenance through subsequent speciation events; II: single origin in one lineage and transfer to the other lineage via introgression; III: convergent evolution via different genetic mechanisms; IV: convergent evolution via different mutations at the same locus (*MC1R*); V: convergent evolution via the same mutations at the same locus (*MC1R*).

In this study we determined whether *MC1R* is associated with the plumage polymorphism in the pomarine skua. We investigated the evolutionary history of melanism in the pomarine skua by comparing *MC1R* variation in pomarine skuas with other skuas, and by comparing this to variation at neutral nuclear loci.

2. Results

We found a perfect association between a variant *MC1R* allele (allele “M”) and plumage coloration in pomarine skuas in three independent samples (Fig. 1). First, among adults migrating through the Lena Delta, Siberia, all dark individuals (N = 3) were homozygous or heterozygous for allele M whereas all pale individuals (N = 7) were homozygous for non-M

alleles (Fisher's exact test, $P < 0.008$). Second, among museum specimens of adult birds from several sites, all dark individuals ($N = 4$) were heterozygous for M, whereas all pale individuals were homozygous for non-M alleles (Fisher's exact test, $P < 0.028$). Third, single chicks sampled from broods from one dark morph and one pale morph parent had the M allele in three out of four cases whereas the M allele was absent from chicks with two pale morph parents ($N = 19$ broods; Fisher's exact test, $P < 0.01$; note that future adult coloration cannot be determined from chick colour).

The variant *MC1R* allele (allele M) is highly divergent and has nine fixed differences from all other *MC1R* alleles in pomarine skuas (Fig. 2). These fixed differences include five non-synonymous substitutions, two of which are at identical sites (12, 230) to sites associated with melanic polymorphism in arctic skuas, with one of these (E12K) being an identical mutation in the two species.

On a haplotype network, the melanic *MC1R* alleles in the arctic and pomarine skuas are widely separated, ruling out both a single origin of the plumage polymorphism in a common ancestor or an introgressive origin from either arctic into pomarine lineages or vice versa. While the M allele is distant from other alleles in pomarine skuas, a striking finding is that it is closely related to *MC1R* alleles from three species of great skuas (*S. skua, S. maccormicki, S. lonnbergi*, Fig. 2). Another notable feature of the haplotype network is the star-like pattern of pale *MC1R* alleles in the pomarine skua, which is suggestive of directional selection.

The highly divergent functional *MC1R* alleles in the pomarine skua might reflect an ancestral polymorphism in the common ancestor of pomarine and great skuas, with the pale allele being subsequently lost in the great skua lineage (lineage sorting). Alternatively, either the pale or dark allele may have introgressed from a different skua lineage into the pomarine skua. To investigate this further, we re-sequenced four unlinked neutral loci across the skuas.

For all four loci, the alleles in the pomarine skua cluster with alleles of the great skuas, and for three out of four loci, the pomarine skua even shares alleles with the great skuas (Fig. 3).

3. Discussion

We have uncovered a novel case where convergent evolution at the phenotypic level has been generated by independent molecular evolution at the same locus, and at least in part due to the same mutations. Although based on modest sample sizes, three independent datasets from breeding adult birds, museum skins, and chicks from parents with known plumage morph, all show a significant association between *MC1R* genotype and plumage phenotype. Since dark individuals of the pomarine skua are rare and usually mate with pale individuals, which is confirmed in our dataset from all dark birds in Taimyr being paired with pale birds and almost all individuals with an M allele being heterozygotes, it is very unlikely that the association between the M allele and plumage phenotype in pomarine skuas could be due to hidden population structure.

The wide separation of pomarine skua and arctic skua *MC1R* alleles on the haplotype network allows us to clearly reject shared evolution from a polymorphic ancestor (Hypothesis I above). Recombination or gene conversion among the two functional *MC1R* alleles within each lineage could lead to sequence homogenization and alleles within a species appearing to be closely related. However, this is unlikely in this case because the functional sites are widely separated along the gene, and in addition it cannot explain the high divergence of pale and melanic alleles in the pomarine skua. The wide separation of pomarine and arctic skua *MC1R* alleles also makes introgression between these taxa (Hypothesis II) unlikely. Instead our results combine elements of hypotheses IV and V, with convergent evolution at the same locus, including some of the same mutations, implicated in the evolution of melanism.

Involvement of some of the same amino acid sites, and mutations, is striking, and it is notable that one of these sites (230) has also been implicated in melanism in the rock pocket mouse [18]. There are a few other cases where closely related vertebrate taxa have convergently evolved melanism due to mutations in the *MC1R* gene (e.g. in cats [19] and falcons [20, 21]). Our findings also have parallels with some studies of haemoglobin evolution at high altitude in birds (e.g. [22]). However, functional studies will be required to confirm which mutations contribute to melanism. One difference of note among the polymorphic skuas is that the melanic *MC1R* allele is dominant in pomarine skuas but partially dominant in arctic skuas, in which heterozygous birds show varying degrees of melanism [12]. In contrast to the arctic and pomarine skuas, the south polar skua, *S. maccormicki*, shows ventral plumage colour variation which is more continuous in nature and partly related to sex [7, 8, 23]; there is no association between *MC1R* variation and plumage colour in this species (Janssen & Mundy, in prep). Overall, these results add to the large body of evidence implicating a role of *MC1R* in the evolution of pale/dark coloration in vertebrates [24], with over eight examples in diverse avian taxa [12, 20, 21, 25-28].

A striking result was that the melanic *MC1R* allele in pomarine skuas is more closely related to *MC1R* alleles in the great skuas than to the pale *MC1R* alleles in pomarine skuas. The relationship of functionally important intraspecific variation (microevolution) to interspecific variation (macroevolution) is a key question in evolutionary genetics. There is relatively little information on this issue, partly because demonstration of functional relevance of fixed differences among species is more difficult than for variation segregating within populations (e.g. see [29]). Our results show a clear link between these levels of evolution for the melanic *MC1R* allele present in pomarine skuas, and add to a few other cases in the literature [3, 4, 30, 31].

A major question is the evolutionary origin of the divergent melanic and pale *MC1R* alleles in pomarine skuas. These could have either arisen within the same lineage or by hybridization among lineages. Because of the close relationship of the melanic pomarine *MC1R* allele to *MC1R* in the great skuas, the first explanation requires that the polymorphism arose in the common ancestor of the pomarine and great skuas and the pale allele was subsequently lost in the great skua lineage. This is plausible since balancing selection on plumage morphs could maintain the polymorphism for long time periods and across speciation events. One difficulty with this explanation is that it requires the pale *MC1R* allele to be ancestral to the melanic *MC1R* allele and so it is difficult to explain the star-like pattern of pale *MC1R* alleles that is suggestive of recent directional selection.

Introgression has been inferred among skuas [9, 32] and is quite common in seabirds in general [33]. If the polymorphism did arise by introgression then, given the close relationship between pomarine and great skua alleles at other loci, the most likely direction of introgression is of the pale *MC1R* allele into the pomarine lineage. This hypothesis is supported by the signal of directional selection of pale alleles in the haplotype network. A selective advantage for introgressed pale alleles is plausible since pale birds are found at high frequency in high arctic latitudes in all three skua species that breed there (pale morphs in pomarine and arctic skuas and the monomorphic pale long-tailed skuas). The question then arises of which skua species could be the source of the pale *MC1R* allele that introgressed into the pomarine skua. *MC1R* haplotypes of the long-tailed skua are the most closely related to the pale allele in the pomarine skua, and these skuas have a pale phenotype. However, although the breeding ranges of pomarine and long-tailed skuas overlap, no hybrids or breeding attempts between the species have been reported, and they have substantial ecological and biological differences [7, 8].

Overall therefore, ancestral polymorphism and introgression of a pale *MC1R* allele are both plausible mechanisms for the origin of the *MC1R* polymorphism in pomarine skuas but each have certain weaknesses. Genome-wide data on genetic variation would be very useful in discriminating between these hypotheses and is an exciting prospect for the future.

In conclusion, we have revealed a novel case of convergent phenotypic evolution due to the same locus, and at least in part to the same mutations. Moreover, we have uncovered a link between polymorphic and fixed functionally relevant variation in a bird. Our results emphasise the unique biological insights that are possible when the genetic basis of traits is understood [34], and the continuing utility of colour variation as a model system in evolutionary genetics [24, 35, 36].

4. Material and methods

(a) Sampling

Blood, feather and tissue samples from pomarine skuas were collected between 2005 and 2008, from chicks with parents of known plumage colour morph at the Pyasina delta, Taimyr, Russia (74.97 N, 86.50 E; one chick per brood, N = 23 broods) and from migrating adults at the Lena delta, Russia (72.2 N, 128.5 E; N = 10). Toe pad samples from adult museum specimens from the collections at the Tromsø University Museum, Norway (N = 5) and Natural History Museum of Denmark (N = 2) were also used (see ESM Table 1 for details). Plumage colour of adults was classified as "pale", including individuals with or without dark breast band, or "dark". The following blood and feather samples from other skua species were collected between 2001 and 2009: great skuas (*S. skua*) from Bear Island, Norway (74.26 N, 19.10 E; N = 4) and Loppa, Finnmark, Norway (70.35 N, 21.43 E; N = 8), brown skuas (*S. lonnbergii*) from Bird Island, Antarctica (54.00 S, 38.05 W; N = 10) and south polar

skuas (*S. maccormicki*) from Svarthamaren, Dronning Maud Land, Antarctica (71,88 S, 05.17 E; N = 25). For sampling details for arctic skuas (*S. parasiticus*), long-tailed skuas (*S. longicaudus*) and kittiwakes (*Rissa tridactyla*) see [13]. Samples of the common guillemot (*Uria aalge*) used as an outgroup for intron analyses were obtained from Latrabjarg, Iceland (N = 1) and Hornøya, Norway (N = 1). A subsample of all skua species (N = 5 to 6 per species) was used for intron analyses.

(b) Laboratory methods

MC1R

We sequenced a 1131bp segment of the *MC1R* gene (846bp of coding sequence and 285bp of upstream non-coding sequence), including all of the sites known to be involved in colour variation in other vertebrates, using primers skua 5' utr [13] and MSHR25 [37] and following methods described previously [Supplementary Online Material, 13]. Because there was only one adult dark *S. pomarinus* in the dataset that was homozygous for the melanic *MC1R* allele (M allele), we confirmed for five more heterozygous dark individuals that they have one copy of the melanic *MC1R* allele by TOPO-TA cloning (Life Technologies). These included two dark adults and three chicks with one dark and one pale parents.

Because the DNA from *S. pomarinus* museum specimens was degraded, only short fragments of the *MC1R* were amplified and sequenced. These were a 165 bp-fragment including codon 12 and a 51 bp-fragment including codon 230, chosen to confirm that substitutions at these sites are associated with plumage colour as in the other *S. pomarinus* in this study and because variation in both of these codons has previously been shown to be associated with plumage colour in *S. parasiticus* [13]. See ESM for further details.

Neutral loci

We sequenced introns from four different nuclear genes: a 492-bp fragment of *GHR* intron 5 (primers GHR-5.2F and GHR-5.2R from [38]), a 767-bp fragment of the *myoglobin* intron 2 (primers Myo2 and Myo3 from [39]), a 908-bp fragment of the *fibrinogen* intron 7 (primers FIB-BI7U and FIB-BI7L from [40]) and a 1391-bp fragment of *TYRP1* intron 5, using primers TYR1332F 5'-GAATGGAACAGGAGGGCAAAC and TYR1332R 5'-TCCAATAGGGGCATTCTCCAG, that were designed from chicken mRNA. *GHR*, *myoglobin* and *fibrinogen* are autosomal whereas *TYRP1* is Z-linked. See ESM for further details.

(c) Statistical analyses

Sequences were edited with Chromas Lite version 2.01 (www.technelysium.com.au) and Bioedit version 7.0.5 [41]. Haplotypes were inferred by parsimony or resolved using PHASE version 2.1 [38,39] if a dataset included genotypes with two or more ambiguous sites. For some melanic *S. pomarinus*, haplotypes were additionally confirmed by TA cloning (see above). Median-joining haplotype networks were generated using Network version 4.6.0.0 with standard settings (http://www.fluxus-engineering.com; [44]).

Tests of association between *MC1R* genotype and plumage phenotype were conducted using Fisher's exact tests.

Ethics. All samples were obtained under applicable national and international laws. Access to study sites in Norway was legally approved by the appropriate county authorities. The Norwegian Directorate for Nature Management licensed the capture and sampling of birds (blood and feathers) by a ringing permit to KJ. Collection of samples was conducted

according to Norwegian ethics regulations. Pomarine skua samples from Taimyr were collected with the permission to carry out fieldwork in the Great Arctic State Nature Reserve, including the handling of animals, from Dr. V. L. Chuprov, director. Pomarine skua samples from the Lena delta were collected in agreement with the local nature reserve, within the activities of the Norwegian-Russian International Polar Year program. The permit to collect samples from south polar skuas was granted by the Norwegian Polar Institute in accordance with the regulations relating to the protection of the environment in Antarctica, and the National Animal Health Inspection/Norwegian Food Safety Authority.

Data accessibility. DNA sequences: Genbank accessions XXXXX-XXXXX.

Authors' contributions. K. J. conceived the study, designed the experiments, carried out experiments, analysed the data and drafted the manuscript. N.I.M. conceived the study, designed the experiments, analysed the data and drafted the manuscript. Both authors gave final approval for publication.

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Figure Legends

Figure 1. A: *MC1R* genotype – plumage colour association in adult pomarine skuas. *MC1R* allele M is associated with dark plumage colour in birds from the Lena Delta (N = 10), and museum specimens collected at multiple sites (N = 7) B: Chick *MC1R* genotype – parental plumage colour association for single chicks from broods from different combinations of parental phenotypes at Taimyr (N = 23 broods). All P values are for Fisher's exact tests.

Figure 2. Median-joining *MC1R* haplotype network in six species of skua. Each line segment represents a single substitution in the 1131bp alignment: black line = non-coding (5' UTR), thin blue line = synonymous, thick blue line = non-synonymous, thick red line = fixed non-synonymous differences among pale and melanic alleles in pomarine and in arctic skua. For pomarine (N = 74) and the three species of great skua – great (N = 24), brown (N = 20), south polar (N = 50) - circles are approximately proportional to sample size. Data for arctic skua, long-tailed skua and gull outgroup (*Rissa tridactyla*) are taken from [13].

Figure 3. Median-joining haplotype networks for four nuclear loci in six species of skua. Outgroup species are the gull *Rissa tridactyla* and the guillemot *Uria aalge*. No outgroup data are available for *GHR*. Colour coding by species is the same as Figure 2. Ovals highlight clusters containing haplotypes of the pomarine and three great skua species.

Figure 1

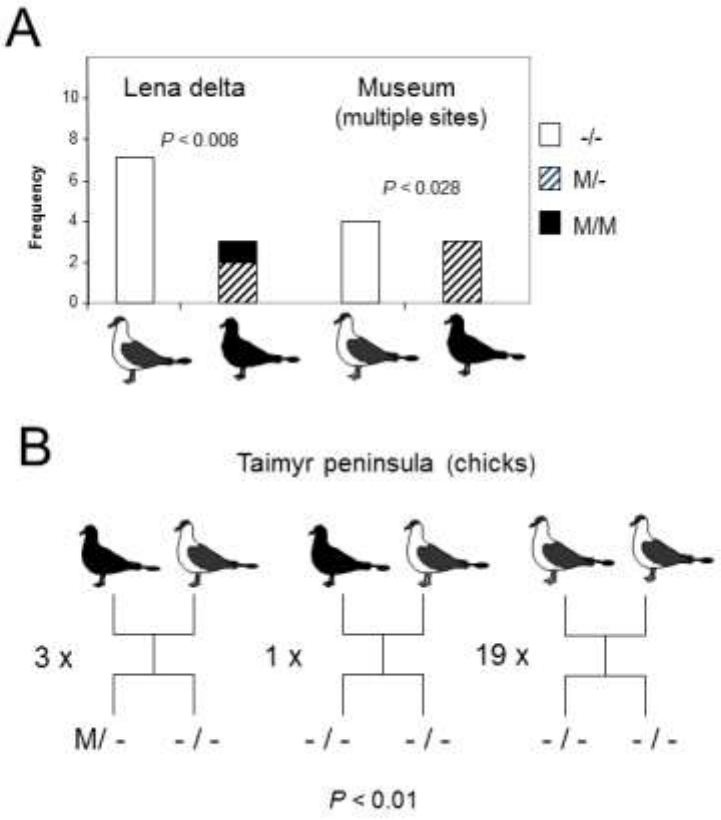

A
Lena delta
Museum
(multiple sites)
P < 0.008
P < 0.028
Frequency
10
8
6
4
2
0
-/-
M/-
M/M
B
Taimyr peninsula (chicks)
3 x
1 x
19 x
M/ -
- / -
- / -
- / -
- / -
- / -
P < 0.01

Figure 2

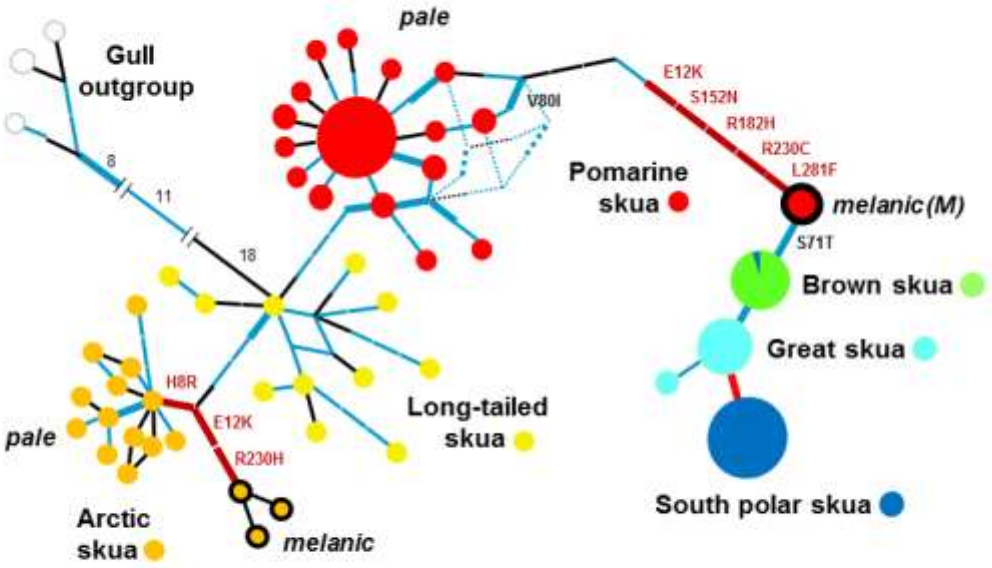
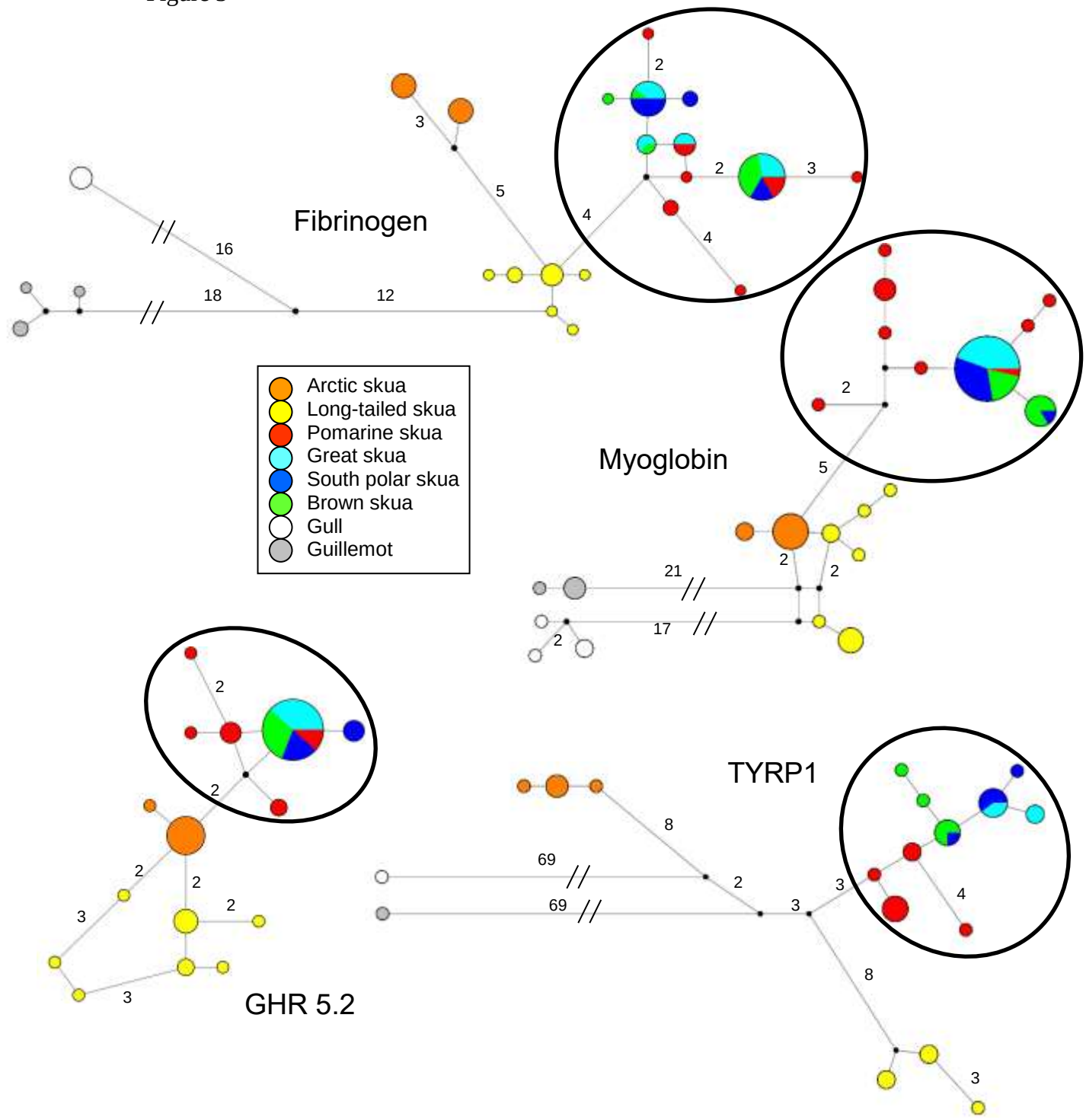

pale
Gull
outgroup
8
11
18
V80I
E12K
S152N
R182H
R230C
L281F
Pomarine
skua
melanic (M)
S71T
Brown skua
Great skua
South polar skua
Long-tailed
skua
H8R
E12K
R230H
pale
Arctic
skua
melanic

Figure 3


Fibrinogen
16
18
12
3
5
4
2
2
3
4
Arctic skua
Long-tailed skua
Pomarine skua
Great skua
South polar skua
Brown skua
Gull
Guillemot
Myoglobin
5
2
2
21
17
2
2
TYRP1
8
69
69
2
3
3
4
8
3
GHR 5.2
2
2
2
2
3
3