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Introduction

With close to 10 million species on Earth according to an estimation (Mora *et al.* 2011), species diversity in nature never ceases to amaze and inspire scientists or nature lovers. So does the question of the origin of species, "that mystery of mysteries" as Darwin called it (Darwin 1859). Reproductive isolation between populations—the centre of the biological species concept formulated by Mayr (1942)—has driven extensive research in sexually reproducing taxa. Barriers to reproduction are identified across the life cycle of an organism: before mating (premating), after mating but before the formation of embryo (postmating prezygotic), and after a hybrid embryo is formed (postzygotic).

Hybrid dysfunction contributes significantly to the accumulation of reproductive isolation as a postzygotic reproductive barrier, and is thus important to speciation. Interpopulation hybrids commonly show reduced fitness compared with their parental species, both in nature and in the laboratory (Burton *et al.* 2013). Such reduction of fitness has extrinsic and/or intrinsic components. The former denotes lower hybrid fitness in specific ecological environments, and the later reflects developmental issues independent of the environment (Servedio & Noor 2003, Rogers & Bernatchez 2006). Loci involved in both parts can be either identical or interacting, linking extrinsic and intrinsic barriers (Kulmuni & Westram 2017). Researchers need to explore both extrinsic and intrinsic sources of reduced hybrid fitness, and both together if possible, to better understand how postzygotic isolation arise and works (Servedio & Noor 2003).

The course of events leading from minor environmentally mismatches affecting hybrid fitness to severe genetic incompatibilities causing sterility or inviability can be seen as a speciation continuum (Hendry *et al.* 2009, Shaw & Mullen 2014, Stankowski & Ravinet 2021). It is crucial to pinpoint the major evolutionary forces at play in species pairs at different pace along the speciation spectrum, so as to reveal when population divergence leads to complete reproductive isolation (i.e. proceed through all stages of the speciation continuum) or instead collapses or is reversed (Gow *et al.* 2006, Taylor *et al.* 2006, Kleindorfer *et al.* 2014, Keagy *et al.* 2016). Climate's contribution to speciation has mostly been explored in setting geographical ranges of populations, linking to historical climate fluctuations such as glacial cycles (Hewitt 1996). However, adaptation to divergent climate itself by separate populations can potentially cause various types of reproductive barriers, and it is surprisingly rarely studied (Qvarnström *et al.* 2015).

Mitonuclear co-adaptation, ensuring normal mitochondria function, is essential to climate adaptation, and has been recognized as a probable source of intrinsic incompatibilities between divergent species and populations (Burton *et al.* 2013, Hill 2017). Mitochondria synthesise ATP to power all cellular activities of a eukaryote organism, through a series of protein complexes on their membrane, named the OXPHOS (oxidative phosphorylation) system (Saraste 1999, Morales *et al.* 2015). Both mitochondrial and nuclear genes together encode for proteins and/or RNAs for mitochondrial activities not limited to OXPHOS, and thus show tight co-adaptation as a population benefits from phenotypes well suited to its surroundings (Brown *et al.* 2004, Hill 2017). When mitochondria and nuclear genome from different parental sources mismatch in hybrids, hybrids may suffer from physiological

dysfunction. More empirical evidence in natural systems is needed to test this theory of mitonuclear incompatibilities, bringing together genes, phenotypes and fitness in speciation.

The passerine birds collared (*Ficedula albicollis*) and pied flycatchers (*Ficedula hypoleuca*) hybridise bidirectionally in secondary contact zones. Hybrids are found sterile, although hybrid adults can still have breeding activities. Previous research demonstrated that in these two species, genes concerning reproductive traits, plumage and species recognition, are Z-linked, and several studies report higher level of species divergence of Z chromosome than autosomes (Sætre *et al.* 2003, Sæther *et al.* 2007, Ellegren *et al.* 2012, Qvarnström *et al.* 2016). There is difference in plasticity of resting metabolic rate between the two species, and elevated metabolic rate in hybrids than either parental species, suggesting hybrid incompatibility in relation to mitochondrial function, and candidate mitonuclear OXPHOS genes were identified (McFarlane *et al.* 2016, McFarlane *et al.* 2018, van der Heijden *et al.* 2019). Thus flycatcher hybridisation is an ideal system to further investigate postzygotic isolation in relation to mitonuclear dysfunction under divergent climate adaptation.

Extrinsic and intrinsic genetic incompatibilities

Both extrinsic and intrinsic sources of hybrid dysfunction are known to act as important postzygotic reproductive barriers, but the build-up of small environmental mismatch to severe genetic incompatibilities along the speciation continuum is generally unknown and tricky to study, when most study systems fall at two ends of the spectrum (Coyne & Orr 2004). In this section I introduce the concept of extrinsic and intrinsic incompatibilities, review the possible evolutionary forces causing them, and establish how divergent climate adaptation can drive speciation, especially linking to the production of extrinsic and intrinsic barriers.

<u>Extrinsic selection against hybrids</u>

Exploration of the extrinsic sources of hybrid dysfunction finds its root in the ecological speciation concept, where reproductive barriers emerge because of ecologically based divergent selection acting on the parental populations (Schluter 2000, Rundle & Nosil 2005, Schluter 2009, Nosil 2012). Hybrids resulting from crossing between individuals belonging to populations residing in different environments often have intermediate phenotypes that are maladaptive in either parental environment, leading to reduced fitness (Rundle & Whitlock 2001, Schluter & Conte 2009). It may be viewed that the divergent parental populations occupy two adaptive peaks in the fitness landscape, while hybrids fall in between in the fitness valleys for lack of suitable niche (Hendry *et al.* 2007, Nosil 2012, Figure 1). Evidence of such ecological mismatches comes from studies of a wide range of emerging species of for examples insects, birds and fish (e.g. Grant & Grant 1993, Grant & Grant 1996, Hatfield & Schluter 1999, Bendall *et al.* 2017, Rajkov *et al.* 2018). However, not many of these studies have directly tested individual hybrid performance in the wild, meaning that the relative importance of various sources of reproductive isolation often remains unknown. In some cases, hybrids can enjoy high fitness as there exists a suitable intermediate ecological environment for them to occupy or they can exceed both parental species in trait value due

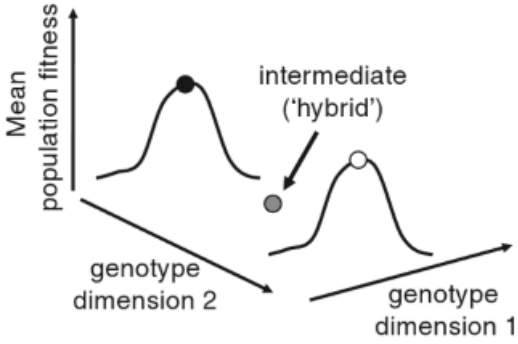

Figure 1. Parental populations are on adaptive peaks under divergent ecological selection, and intermediate hybrids fall in the fitness valley. Reprinted from Nosil 2012.

to transgressive segregation, even resulting in hybrid speciation, though it is outside of the scope of the discussion here (Rasmussen *et al.* 2012, Sætre & Ravinet 2019).

Sources of prezygotic isolation are often considered important in the context of ecological speciation, especially when the speciation progress during ongoing gene flow (Coyne & Orr 1998, Schluter 2001, Via 2001, Ortiz-Barrientos *et al.* 2009), because prezygotic isolation evolves relatively fast and acts early in the lifecycle (Coyne & Orr 2004). However, sources of post-zygotic isolation play an important role in rendering speciation reversal less likely and studies of ecologically based sources of post-zygotic isolation are also important for understanding the bridges between the various stages of the speciation. Ecological selection can generate intrinsic barriers as a by-product and further facilitate speciation, but emerging sources of intrinsic incompatibilities are often overlooked in research on early stages of population divergence (Kulmuni & Westram 2017).

<u>BDMI model and intrinsic selection against hybrids</u>

Research on intrinsic postzygotic isolation focuses on the arise of genetic incompatibilities that persist independent of the ecological environment. The theoretical background lies in the Bateson – Muller- Dobzhansky incompatibilities (BDMIs), named after its initial proponents from the last century (Bateson 1909, Dobzhansky 1934, Dobzhansky 1936, Muller 1942), which describes the molecular origin of intrinsic hybrid dysfunctions and has been since considered a keystone concept in speciation studies (Coyne & Orr 2004). This model theorises that hybrid dysfunction comes from incompatible epistatic interactions between genes that have evolved separately in allopatric populations or consecutively in one population and are never appear together for selection to work on until hybridization, where incompatibilities can occur between at least two genes (between derived genes and ancestral or other derived genes), and hybrid dysfunction arises (Bateson 1909, Dobzhansky 1934, Dobzhansky 1936, Muller 1942, Figure 2). The model also predicts that, at the early stage of the speciation process, incompatibilities accumulate increasingly fast like a snowball (Orr 1995). There is substantial evidence confirming that such genetic incompatibilities do exist in model systems like fruit flies, house mice, marine copepods, yeast and plants, (Lamnissou *et al.* 1996, Orr & Irving 2001, Sweigart *et al.* 2006, Bomblies *et al.* 2007, Ellison & Burton 2008, Lee *et al.* 2008), and the genes involved have been identified in some cases, such as *Nup160*

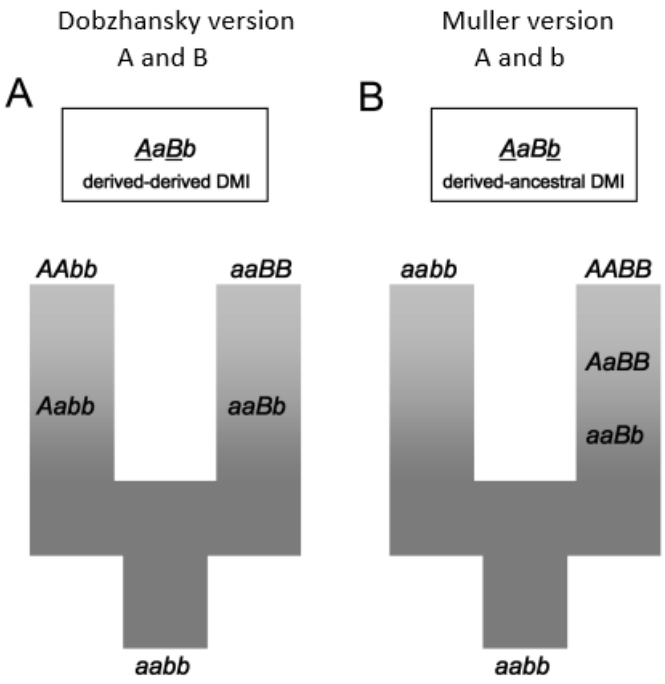

Figure 2. The arise of BDMIs in evolutionary history using a two-locus model. The bottom shows ancestral population where alleles a and b are fixed. The population then splits into two, and across time new alleles are fixed, shown on top of the two scenarios. Reprinted from Presgraves 2010.

in fruit flies and *Prdm9* in house mice (Flachs *et al.* 2012, Tang & Presgraves 2015, reviewed in Johnson 2010, Presgraves 2010). However, many of these studies rely on laboratory crosses of species pairs that have diverged for a long time and do not naturally hybridise (though see Schumer *et al.* 2014, Turner & Harr 2014). While natural systems exist where there is hybrid sterility and/or inviability, individual genes causing the problems remain unknown in most cases, making it difficult to study the potential links between extrinsic and intrinsic sources of selection against hybrids. Research is undergoing to tackle this (such as Powell *et al.* 2020). BDMIs arise regardless of environment and form intrinsic incompatibilities. It stands for irreversible barriers to gene flow, and is thus an important step in speciation (Coyne and Orr 1998).

Evolutionary forces and population patterns

Little is known about the evolutionary forces that drive hybrid incompatibilities in nature, though hypotheses, as presented below, were developed to explain observed patterns in many populations.

Classic drift, even if theoretically possible to build up incompatibilities, is considered too slow as a process compared to selection to facilitate reproductive barriers in a short time on its own (Ehrlich & Raven 1969, Qvarnström *et al.* 2016). Divergent natural selection faced by allopatric populations can possibly shape incompatibilities, as BDMIs are not directly favoured by natural selection, but rather a by-product of genetic divergence due to selection acting on interacting or linked gene units (Coyne & Orr 2004, Johnson 2010). This is in line with the ecological speciation concept.

There are evolutionary processes alternative to natural selection that may contribute to the build-up of hybrid incompatibilities. Often observed is that hybrid sterility evolves before hybrid inviability, suggesting sex-specific evolutionary forces in play, such as sexual selection (Wu 1992, Wu *et al.* 1996, Presgraves 2010a, Turissini *et al.* 2018). It is not hard to reason, as male-female coevolution driven by divergent sexual selection can easily and rapidly take different trajectories among populations (Arnqvist & Rowe 2013), posing another source of incompatibilities. Sexual conflict and genome conflict are two other probable causes (Johnson 2010, Presgraves 2010b, Arnqvist & Rowe 2013, Qvarnström *et al.* 2016). For example, sexual conflict over maternal provisioning of offspring can drive a dynamic arms race between paternally expressed growth promoters and maternally expressed growth suppressors, leading to hybrid inviability (Kondoh & Higashi 2000, Arnqvist & Rowe 2013). Divergence of selfish genetic elements and corresponding suppressors in a genome can also accumulate hybrid incompatibilities, as demonstrated in *Drosophila* and *Mus* (Frank 1991, Presgraves 2010b). These processes can facilitate specific patterns of hybrid fertility or survival, which in turn helps us distinguish the underlying molecular mechanisms.

It is widely observed that the heterogametic sex often suffers more from lethality, inviability and sterility among interspecific F1 hybrids, called Haldane's rule (Haldane 1922). The dominance theory provides an explanation that works in both XY and ZW systems: recessive X- or Z-linked BDMI alleles in heterogametic taxa will be expressed in hemizygous hybrids but masked in the heterozygous (Muller 1942, Turelli & Orr 1995). It doesn't justify, however, the more frequent occurrences and faster evolution of hybrid sterility than inviability (reviewed in Presgraves 2010a). Moreover, asymmetric sterility or inviability of hybrids from reciprocal crosses is usual, termed Darwin's corollary to Haldane's rule (Presgraves 2002, Bolnick & Near 2005, Good *et al.* 2008, Woodruff *et al.* 2010), and highlight the contribution of sex-specific factors. In XY systems, incompatibilities may partially come from the rapid divergence of male-specific genes driven by sexual selection (Wu 1992, Wu *et al.* 1996, Presgraves 2010a). However, more generally, there are implications of uniparentally transmitted BDMI factors that are small in number but have large effects (Turelli & Moyle 2007, Presgraves 2010a). For instance, the drive theory suggests that genetic conflict over transmission of sex chromosomes leads to coevolving meiotic drive systems, causing divergence of genes related to gametogenesis (Frank 1991, Hurst & Pomiankowski 1991, Presgraves 2010a). Maternally inherited mitochondria can also have genetic clashes with paternal inherited genome in hybrids (Burton *et al.* 2013, Hill 2017). For instance, the "mother's curse" theory describes build-up of mutations deleterious to males on maternally inherited mitochondria, due to the lack of transmission of mitochondria genes from males to their offspring (Gemmell *et al.* 2004). Suppressors or compensatory alleles are selected on the nuclear genome to restore male fitness but can be mismatched in hybrids when parental mitonuclear system differ (Gemmel *et al.* 2004, Hermansen *et al.* 2004). If incompatibility genes lie both on the nuclear genome and mitochondria, their interaction can create even more curious patterns, depending on the location of the nuclear genes. If they are autosomal or X-linked, dysfunction will increase across hybrid generations in both sexes, but if they are Z-linked, dysfunction will be strong in F1 females and weak in F1 males, but then decrease in F2 females and increase in F2 males, all due to changing proportion of compatible alleles after meiosis and recombination (Hill 2018).

With the above included, many explanations point to BDMIs being often located on sex chromosomes, cytoplasmic organelles like mitochondria, or simply uniparentally inherited epigenetic factors. This is further confirmed by existing research that identified hybrid incompatibility genes, as half of them reviewed by Presgraves (2010a) are located on sex chromosomes or mitochondria, contributing to either sterility or inviability.

Divergent climate adaptation leading to speciation

Adaptation of populations, to divergent climate, can build up different types of reproductive barriers, and may be particularly relevant for speciation studies under ongoing climate change. This remains however a field insufficiently explored (Keller & Seehausen 2012, Qvarnström *et al.* 2015), as the empirical work on ecological speciation mostly concentrates on resource use in young species pairs (Nosil 2012).

The environment individual organisms are exposed to depend on a number of abiotic factors such as climate and biotic factors such competitors, predators and parasites (Keller & Seehausen 2012). Adaptation to specific environments and/or diets results in well-adjusted biochemical reaction on a molecular level (Kingsolver 2009), which in turn influence growth, development, reproductive timing and life-history trade-off on the individual level (Angilletta Jr 2009). Limited physiological tolerance to factors including temperature, moisture, elevation and pH can then contribute to habitat isolation of populations doing badly in interchanging environment, while fine-tuned phenology can bring on temporal isolation. These pre-mating barriers have received relatively more attention, especially in plants, and some empirical evidence can be found in systems such as phlox and monkeyflowers (Campbell *et al.* 2005, Lowry & Willis 2010, Keller & Seehausen 2012).

Climate adaptation possibly facilitates speciation through interplay with sexual selection and assortative mating theoretically, though empirical tests are lacking. Expression of sexual display traits and its relationship with fitness can be dependent on the abiotic environment (e.g. Snell-Rood & Badyaev 2008, Reudink *et al.* 2015), and pleiotropic genes can regulate sexual signals and physiological traits at the same time, such as the ones regulating melanin (Wiley *et al.* 2005, Ducrest *et al.* 2008, Roulin *et al.* 2008, Qvarnström *et al.* 2015).

When there is hybridisation, hybrids may have intermediate phenotypes, not accustomed to either parental habitat and decoupled in terms of time with food peak or seasonal change, selected against without access to intermediate environments. Climate driven adaptation can thus cause extrinsic incompatibilities. When adaptation under different climates have put forward divergent genetic units, especially ones sensitive to environmental factors, upon secondary contact hybrids can suffer from intrinsic incompatibilities, too. The involved alleles may be well-linked to those causing extrinsic incompatibilities. Mitonuclear genes require tight co-adaptation and are temperature sensitive (Dowling *et al.* 2008) meaning that they are good candidates in this context. Intrinsic postzygotic barriers are generally harder to study in ecological speciation as they usually become most obvious at late stage of speciation and their strength are easily masked by pre-mating barriers that occur earlier in the time course of life-history events (Coyne & Orr 2004). Indeed, fewer studies have tested these postzygotic barriers, and there is especially a lack of evidence for divergent climate adaptation leading to intrinsic postzygotic isolation (but see review by Keller & Seehausen 2012 about thermal

adaptation and speciation), despite the theory being intuitive and plausible. In this scenario, genes involved in hybrid dysfunction can evolve quickly as a result of selection for divergent selection.

All in all, divergent climate adaptation is likely to be related to several fast-evolving sources of reproductive isolation. We are in dire need for studies in natural systems having gone through divergent climate adaptation, so as to partition different evolutionary forces and their relative contribution leading to divergence in the incompatible genes, and thereby identify key players in speciation.

Species divergence in mitochondrial function and the arise of reproductive isolation

After establishing the asymmetrical pattern of hybrid dysfunction in Part 1, I naturally zoom in on the role of uniparentally transmitted factors in speciation, and in particular on the role of mitochondrial genes. The cytoplasmic organelle mitochondria are seen as the powerhouse of eukaryotic cells (Burton *et al.* 2013, Stier *et al.* 2017). More than 90% of adenosine triphosphate (ATP), the main energy carrier molecule for cellular activities, are synthesized in mitochondria through the process of oxidative phosphorylation, or OXPHOS (Saraste 1999, Nicholls & Ferguson 2013). In this section I describe the structure of mitochondria in general and of the OXPHOS respiration pathway specifically. Then I point out the critical role of mitonuclear interaction in normal mitochondrial function, and review how mitonuclear coadaptation could be the molecular mechanism bridging divergent climate adaptation. Finally, I discuss how mitonuclear clashes can arise when populations with divergent climate adaptation interbreed.

Physiological structure of mitochondria and the OXPHOS pathway

The mitochondrion has two layers of phospholipid membranes that hold in between them an intermembrane space, and enclosed by the inner membrane is the mitochondrial matrix containing mtDNA and ribosome (Stier *et al.* 2017, Figure 3). The mitochondrial genome is highly reduced compared to nuclear genomes. In animals it typically has a size of 14-18 kb, harbours 37 genes in total (13 protein coding genes, 2 rRNAs and 22 tRNAs), and doesn't include introns, but it is very much functional and essential (though inadequate) for cellular respiration (Burton *et al.* 2013). The mitochondrial genome is replicated and expressed within mitochondria (Burton *et al.* 2013). The mitochondrial ribosome is formed by rRNAs encoded by the mt genome and ribosomal proteins encoded by the nuclear genome (Burton *et al.* 2013).

OXPHOS is carried out by a series of five multi-subunit respiratory enzyme complexes located in the inner membrane of the mitochondria (Saraste 1999, Morales *et al.* 2015, Figure 3). Complexes I-IV, arranged in a specific orientation, form an electron transport chain (ETC) and perform substrate oxidation, while complex V carries out ATP synthase (Saraste 1999, Figure 3). OXPHOS is only completed through the coupling of respiration and ATP synthesis (Mitchell 1961, Saraste 1999). More precisely, complexes I-IV transfer electrons (e^-) from different substrates including NADH (nicotinamide adenine dinucleotide + hydrogen), succinate and

FAD-linked substrates to oxygen molecules, and meanwhile all except for complex II use the released energy to pump protons (H^+) across the inner membrane, from the mitochondrial matrix into the inter-membrane space (Saraste 1999, Stier *et al.* 2017). This directional transport of protons builds up an electrochemical gradient that stores energy across two sides of the inner membrane, and through complex V protons flows back, releasing energy for phosphorylation of ADP into ATP (Saraste 1999, Stier *et al.* 2017).

During this process, some electrons can escape the transport into oxygen through protein complexes, producing reactive oxygen species (ROS) that can cause oxidative damage and different degenerative disorders and is partly connected to the aging process (Beckman & Ames 1998, Divakaruni & Brand 2011, Speakman *et al.* 2015). Some protons can also backflow without passing through complex V, releasing energy in the form of heat, a process called proton leak, which indicates incomplete coupling of OXPHOS (Divakaruni & Brand 2011). Proton leak allows for adjustment of coupling efficiency, or the proportion of mitochondrial respiration rate used for ATP synthesis, and thus regulates metabolic homeostasis and helps maintain body function (Divakaruni & Brand 2011). Coupling efficiency varies between tissues of the same individual, within individual because of temperature, food intake and diet, and also within and between species (Brand 2005, Salin *et al.* 2015). Traits such as growth rate, number of eggs produced, resistance against hunger and life span are shown to depend on the coupling efficiency (Salin *et al.* 2015). The 'uncoupling to survive' hypothesis postulates

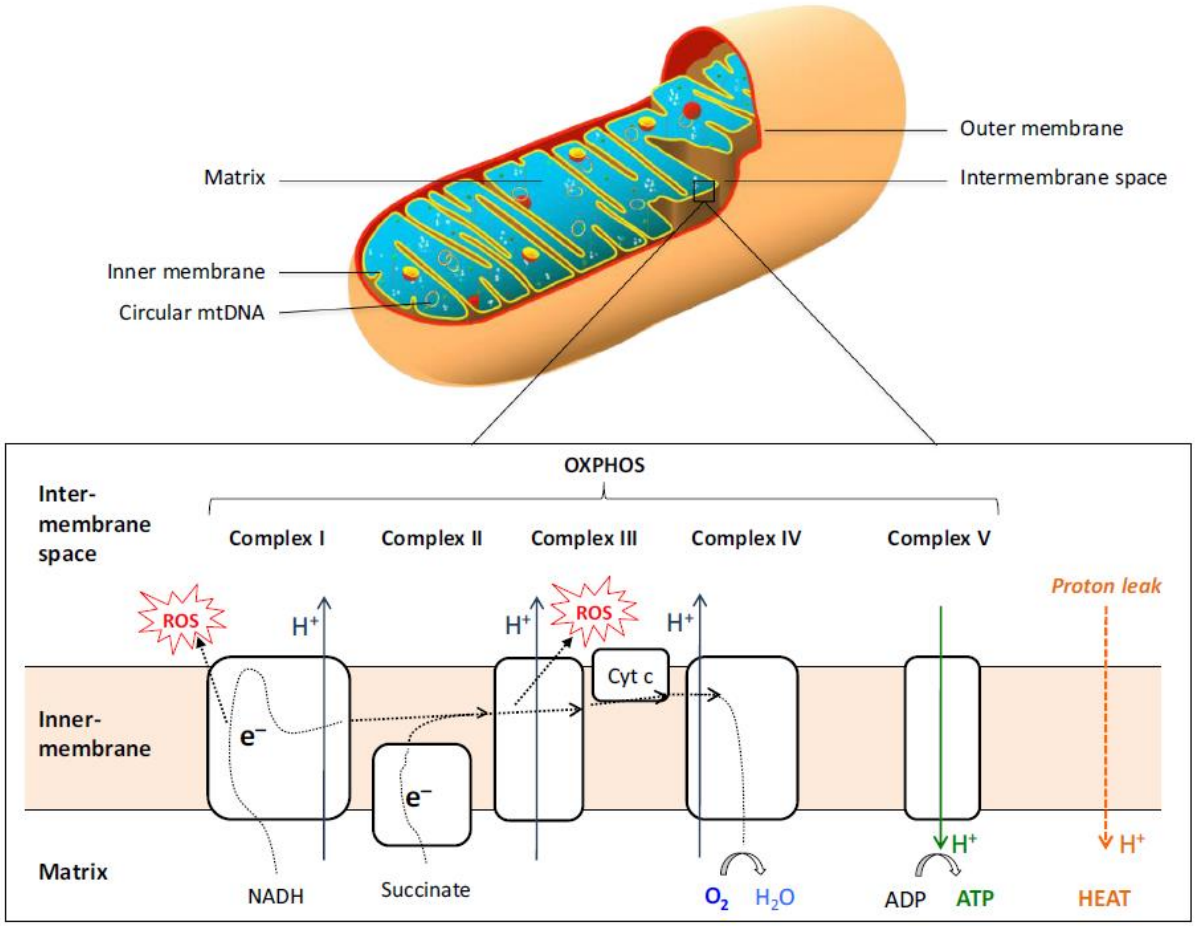

Figure 3. Structure of mitochondrion and the OXPHOS system. Reprinted from Stier *et al.* 2017.

the trade-off between coupling efficiency and damage of corresponding ROS production is the reason why the uncoupling persists (Brand 2000, Amara *et al.* 2007, Keipert *et al.* 2011).

Mitochondria function relies on tight mitonuclear interaction

Cell respiration depends collectively on mitochondrial (mt) and nuclear (N) genomes and their encoded proteins. 1% of proteins used by mitochondria are encoded locally, and 99% are imported (Burton *et al.* 2013). Both genomes encode subunits of metabolic enzyme complexes that are assembled to carry out OXPHOS activities. Mitonuclear interaction for normal mitochondria function can be divided into three parts: protein-protein interaction, protein-RNA interaction, and protein-DNA interaction. The five enzyme complexes carrying out metabolic functions consist of 13 mitochondrial proteins and ~75 nuclear proteins (protein-protein interaction) (Saraste 1999, Hill 2017). Both are featured in Complex I, III, IV and V, while Complex II is solely encoded by nuclear genes (Saraste 1999). These proteins are essential to the OXPHOS process. Additionally, the normal operation of mitochondria also needs replication, transcription and translation, for which purpose about 105 nuclear encoded proteins are imported to complement the 2 rRNAs and 22 tRNAs coded by mt genome (protein-DNA and protein-RNA interactions) (Hill 2017). More N genes are involved in functions such as lipogenesis and heme synthesis (Hill 2017). Therefore, normal mitochondrial function depends on the interaction of 37 mt genes (13 of which encode OXPHOS proteins) and 1500 nuclear proteins (around 180 involved in OXPHOS) (Hill 2017).

Adaptation, selection, evolution, and mitonuclear incompatibilities

As normal OXPHOS structure requires tight mitonuclear interaction, coadaptation and coevolution of N-mt genes are essential to mitochondrial performance. There is usually strong selection for optimal mitochondrial function, and given the special relationship between mt-DNA and N-DNA, there is room for development of genetic incompatibilities involving mitonuclear genes.

Appropriate metabolic function is vital to an organism's life, and are at the heart of adaptation to different local environments (Brown *et al.* 2004). Naturally, compatibility of co-working mitonuclear units and high OXPHOS function are under strong selection (Hill 2017). There can be even greater selection for OXPHOS efficiency and fitness cost for organisms with energy demanding life history, for instance birds due to high body temperature (baseline metabolic rage) and activities such as flying, migration and winter breeding (Hill 2017, Tobler *et al.* 2019).

Mitochondria self-replicates and are maternally inherited as an intact organelle in sexually reproducing eukaryotes (Burton *et al.* 2013). The mitochondrial genome is rather conservative with no recombination and is only altered by point mutations and indels (Burton *et al.* 2013, Hill 2017). Nevertheless, the mitochondrial genome typically has multiple-fold more rapid evolution compared to nuclear genome (Osada & Akashi 2012, Burton *et al.* 2013). Uniparental inheritance also means smaller effective population size of mt genome than nuclear genome (Osada & Akashi 2012). These factors make means that slightly deleterious mutations more easily go to fixation in the mitochondrial genome and then can give rise to compensatory nuclear adaptation due to close co-adaptation of mitonuclear genomes (Osada

& Akashi 2012, Burton *et al.* 2013). Since every mt gene is used for mitochondrial function, after random mutation on mt the coevolution is unique (Hill 2017). The fast evolution of mitochondrial genome can therefore lead to rapid divergence regardless of the external environment.

However, different levels of metabolism in various environments provide ample opportunities for adaptive divergence of mitochondrial function (Burton *et al.* 2013, Arnqvist *et al.* 2010, Obler *et al.* 2019). For examples, altitude, environmental stress, and temperature can greatly influence specific metabolic rates and plasticity (Tobler *et al.* 2019). Ecological environments will select for specific mitochondrial functions, and therefore different mitonuclear units. Distinct mitonuclear evolutionary trajectories can thus be a by-product resulting from adaptive divergence (Tobler *et al.* 2019).

It is reasonable to hypothesize that in allopatric populations such processes could produce highly coadapted mitonuclear genetic units that are incompatible when brought together through hybridization (Burton *et al.* 2013). Moreover, in ZW system, there can be coadaptation of maternally inherited mt-DNA and Z-linked mitonuclear genes, leading to stronger clashes when mitochondria are brought together with an unfamiliar paternal Z chromosome (Hill 2017, Hill 2018). Consequently, this means that the genetic basis of mitochondrial function can act as the bridge between ecological adaptation and speciation (McKenzie *et al.* 2019, Tobler *et al.* 2019).

Some evidence for mitonuclear incompatibility has been observed, though nothing conclusive has been discovered and very few studies tested it in different ecological backgrounds. There is usually greater introgression of autosomal nuclear genes than sex-linked or mitochondrial genes, though in some cases the opposite was reported (Burton *et al.* 2013, Hill 2017). Still, high mutation load of mitochondria could be the universal explanation no matter which part has higher introgression (Sloan *et al.* 2017). This theory needs to be tested with more investigation and the direct links between genes involved in incompatibilities and/or mitonuclear interaction, mitochondria function (phenotype) and fitness should be established.

Exploration in the flycatcher study system

Naturally hybridizing collared and pied flycatchers are suitable birds to study extrinsic and intrinsic postzygotic isolation in the wild, due to fairly high levels of mixed-species pairing combined with the possibility to follow the fate of individual birds from hatching and throughout their lives. The latter is because they tend to return to the same breeding sites where they have been successful and prefer artificial next boxes to breed (Pärt 1995, Sanz 2001, Qvarnström *et al.* 2010). The two species are closely related, diverged less than one million years ago (Backström *et al.* 2013, Nadachowska-Brzyska *et al.* 2013) and share similar breeding biology (Qvarnström *et al.* 2010). Historically, the two species were probably isolated periodically in separate glacial refuges during Pleistocene (Sætre *et al.* 2001), thus adapted to different climates allopatrically. Currently, the breeding range of pied flycatchers

covers most of Europe and that of collared flycatchers mainly Central and Eastern Europe (Qvarnström *et al.* 2010, Sirkiä & Qvarnström 2021, Figure 4). The two species exist in sympatry in Central and Eastern Europe and on the Swedish islands Öland and Gotland in the Baltic Sea, where they hybridize bidirectionally (Qvarnström *et al.* 2010, Figure 4). There is evidence suggesting asymmetric gene flow from pied flycatchers to collared flycatchers in the recent past (Ellegren *et al.* 2012) but there appears presently to be a high level of reproductive isolation between the two species based on monitoring data.

In the studied hybrid zone, several prezygotic and post-mating prezygotic reproductive barriers between the two species have been revealed. Where both species co-occur, the less competitive pied flycatchers are displaced into poorer habitats and breed relatively later in the season matching with the peak of food abundance in the different habitats (Vallin *et al.* 2012). Ongoing habitat segregation quickly result in a corresponding built up of habitat isolation (Rybinsky *et al.* 2016). The two species have also been shown to respond differently to global climate change with collared flycatchers advancing their onset of breeding more in response to earlier spring (also when differences in habitat use are taken into account, Sirkiä

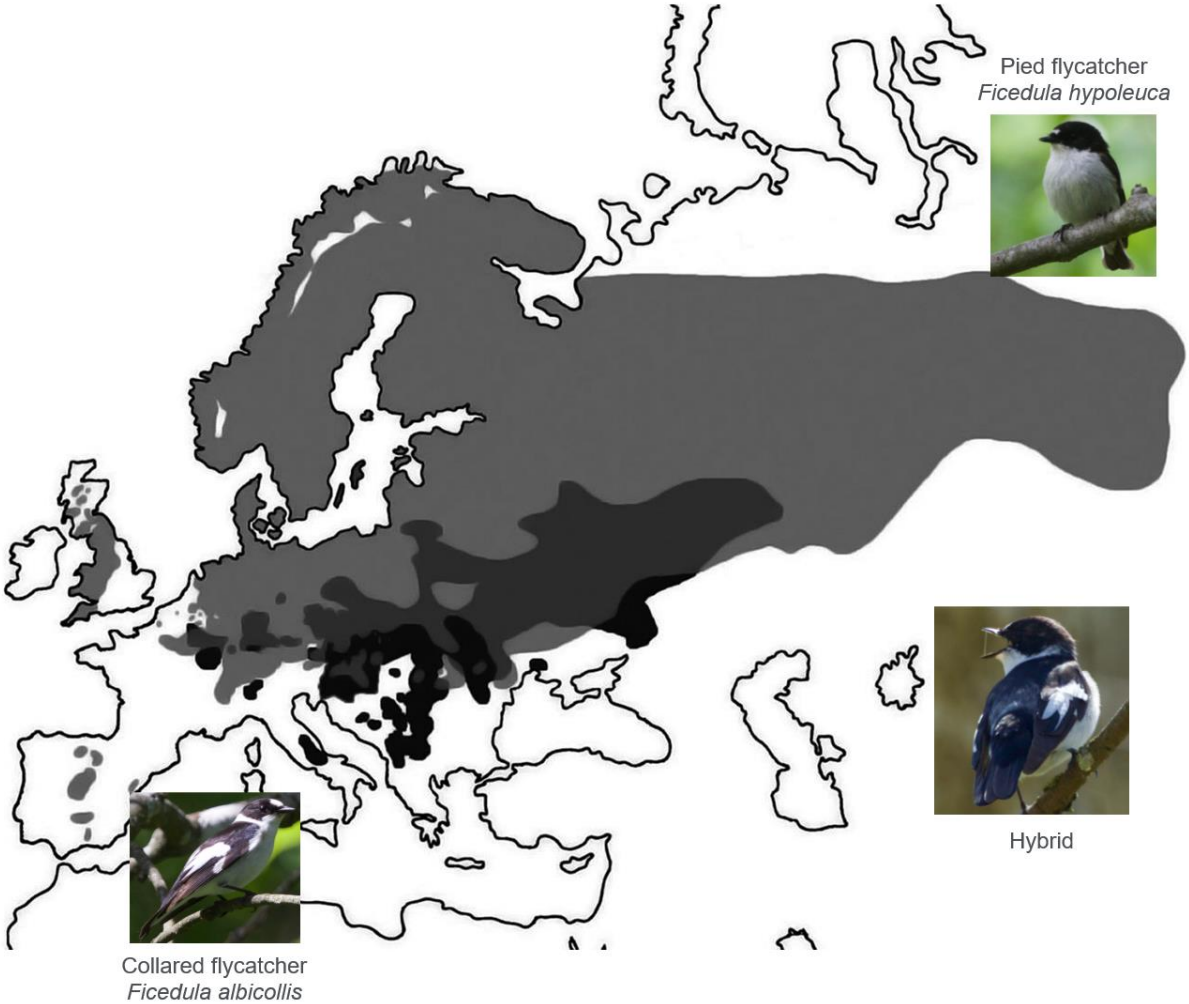

Figure 4. Current breeding ranges of pied (top right; grey colour on map) and collared flycatchers (bottom left; black colour on map) and their hybrids (bottom right; dark grey colour on map). Modified from Sirkiä & Qvarnström 2021.

et al. 2018). This difference in onset of breeding results in temporal isolation (Sirkiä *et al.* 2018). Furthermore, there is also species assortative mate choice based on male song and plumage characteristics (Wiley *et al.* 2005, Qvarnström *et al.* 2006, Sæther *et al.* 2007).

Moreover, research conducted on Öland showed a high degree of postzygotic isolation in this system despite short divergence time. Hybrid nestlings have intermediate growth strategy and environment-dependent survival (Vallin *et al.* 2013), intermediate malaria prevalence (Wiley *et al.* 2009), and hybrid males are sexually selected against (Svedin *et al.* 2018), indicating the role of extrinsic factors in postzygotic isolation. Considering intrinsic aspect, the hybrids experience complete sterility with evidence of impaired sperm morphology, lack of paternity in attended nests, and malfunctioning spermatogenesis (Ålund *et al.* 2013, Segami 2022). Hybrid physiological dysfunction expressed as high metabolic rate is also starting to arise (McFarlane *et al.* 2016). This may result from mitonuclear incompatibilities disturbing the OXPHOS pathway responsible for mitochondrial respiration, as species divergence and signals of positive selection are found in OXPHOS genes of the two pure species (van der Heijden *et al.* 2019).

Taken together, pied- and collared flycatchers can be considered to have reached an advanced stage on the speciation continuum with a large number of sources of reproductive barriers. The spatial and temporal premating barriers are closely related to divergent climate adaptation of the two species, the plumage may have environmental dependence, while the hybrids show emerging signs of intrinsic hybrid dysfunction not only in terms of sterility but perhaps also in terms of reduced viability, relating to mitonuclear activity that is crucial for climate adaptation. This is a brilliant system for continuing investigation in postzygotic isolation through mitonuclear dysfunction after divergent climate adaptation.

Concluding remarks

Speciation research has come a long way in describing various reproductive barriers in different systems along the gradient of the speciation continuum. However, the origin of reproductive isolation, the process of its building up and its genetic basis remain key questions in the quest, a ‘core’ in speciation research. Pre-mating barriers and extrinsic postzygotic isolation typically receive more attention in studies of ecological speciation, but the divergent populations can easily collapse upon change of external conditions, emphasizing the importance of intrinsic postzygotic isolation. The arise of intrinsic incompatibilities is particularly important in keeping the divergence stable and non-reversable, pulling the speciation process into completion. Intrinsic and extrinsic incompatibilities may very well evolve hand in hand, as the genes involved in both can be interacting or simply overlapping.

One possible scenario of speciation is population divergence in allopatric regions with different climate, producing genetic units in each population that work well by themselves but cause hybrids to have maladaptive intermediate phenotypes (extrinsic) or to become sterile and inviable (intrinsic). Mitonuclear incompatibilities are plausible due to high

requirement for physiological specificity in climate adaptation, which is only achievable through co-working mitonuclear genes.

In the future, it is exceptionally important to pursue answers using suitable study systems in nature, where it is possible to disentangle the different evolutionary forces at play as it happens. Naturally hybridising pied and collared flycatchers, with extensive records on the breeding population and identified reproductive barriers, will be my choice for next step research, in hope of approaching the 'core' of speciation question.

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