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The Major Histocompatibility Complex and Primate Behavioral Ecology: New Tools and Future Questions

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Abstract Since the serendipitous discovery of the effect of the major histocompatibility complex (MHC) on mate choice in laboratory mice nearly 40 yr ago, there has been sustained interest in the role that MHC genes may play in vertebrate sexual behavior. However, the challenges posed by MHC genotyping have long hampered progress in this area. We briefly introduce the documented links between MHC and behavior, before presenting an overview of the genotyping methods that were available before the introduction of new sequencing technologies. We then clarify why next-generation sequencing represents a major breakthrough in MHC genotyping by reviewing the recent successes —and pitfalls— of pioneer studies applying these techniques, before envisioning their revolutionary implications for future MHC studies in evolutionary ecology and primatology. We hope that our practical guidance to the design of MHC-based projects will promote and facilitate the integration of a MHC component into the research agendas of primatologists.

Keywords Kin discrimination · major histocompatibility complex · mate choice · MHC genotyping · next-generation sequencing · primates · sexual behavior

Introduction

The important immune function of genes of the major histocompatibility complex (MHC) was known well before the serendipitous discovery of their potential influence on behavior in 1976 (Yamazaki *et al*. 1976). MHC molecules are cell surface glycoproteins responsible for recognizing foreign peptides (antigens), and presenting

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them to specialized immune cells (such as lymphocytes) to initiate the appropriate immune response. MHC is a large cluster of highly polymorphic genes, and this variability is thought to be selected by the great variety of pathogenic agents (Apanius *et al*. 1997; Doherty and Zinkernagel 1975; Hughes and Yeager 1998). Accumulating evidence suggests that MHC genes have several nonimmune functions, including a role in reproduction and materno–fetal interactions as well as a role in behavior and mate choice thought to be mediated by olfactory communication (Alberts and Ober 1993; Penn and Potts 1999; Potts and Wakeland 1993). It is, however, still unclear whether sexual selection and reproduction play a significant role in maintaining MHC polymorphism. Owing to their influence on a variety of fitness-related traits, such as disease resistance, reproduction, and mate choice, and the complexity of the selective mechanisms that generate and maintain their polymorphism, MHC genes have emerged as a popular model for evolutionary biologists and behavioral ecologists (Apanius *et al*. 1997; Milinski 2006; Piertney and Oliver 2006; Sommer 2005). Here we first outline the potential that studying MHC variability in wild populations offers for understanding animal behavior, before explaining the technical challenges that have thus far restricted rapid progress in this field, and highlight novel approaches that will broaden the use and scope of MHC studies in behavioral ecology and primatology. We provide a glossary to guide nonspecialist readers through molecular biology terminology.

MHC and Behavior

MHC and Mate Choice

Evidence for MHC-dependent mate choice comes from all major vertebrate taxa. Various mating strategies have been described, including choice for partners possessing 1) compatible, 2) diverse, or 3) particular MHC genotypes (Milinski 2006; Penn and Potts 1999; Ruff *et al*. 2012; Yamazaki and Beauchamp 2007).

In the first case, choosing a compatible partner to favor a good combination of genes in the offspring (Trivers 1972) may take the form of choice for maximally or optimally dissimilar partners, or for similar partners. Choice for MHC dissimilar partners may help to limit the deleterious effects of inbreeding or, alternatively, increase offspring diversity at specific MHC genes. A high MHC diversity may help fighting against a greater variety of pathogens (heterozygote advantage: Doherty and Zinkernagel 1975) but may also increase the risk of reacting against self-derived peptides (and thus of autoimmune disease), so that optimal diversity may sometimes be more advantageous (Woelfing *et al*. 2009). Finally, choosing a similar mate may in particular contexts limit potential outbreeding costs.

In the second case, it is less clear why females may choose diverse (or heterozygous) partners, because paternal heterozygosity is not transmitted to offspring. Such a strategy may nevertheless be observed when females choose to mate with healthy partners if heterozygous individuals are more vigorous than homozygotes, or if it favors the transmission of rare MHC alleles to offspring because the possession of a rare allele is generally associated with heterozygosity at the considered locus (Apanius *et al*. 1997). This latter form of mate choice does not differ from MHC-disassortative mate choice.

In the third case, female choice may also target particular MHC genotypes conferring protection against dominant pathogenic pressures in a given environment. As possessing specific MHC genotypes may often be more important than possessing a high MHC diversity for parasite and disease resistance (Apanius *et al*. 1997), this form of MHC-dependent mate choice may be common. However, it is difficult to detect in nature, as testing for the effects of particular MHC alleles requires large sample sizes, and few studies —none in primates— have so far been able to generate these large-scale tests (Eizaguirre *et al*. 2009b).

Primate studies have detected choice for partners possessing dissimilar (Schwensow *et al*. 2008a; Setchell *et al*. 2010), diverse genotypes (Sauermann *et al*. 2001; Schwensow *et al*. 2008a,b) or no mate choice at all (Huchard *et al*. 2010a), suggesting that mate choice may be flexible and context dependent (Setchell and Huchard 2010). For example, mate choice for dissimilarity may be favored in inbred populations, whereas mate choice for particular genotypes may be favored under high pathogenic pressure. Consequently, much remains to be done to understand the evolutionary drivers of MHC-dependent mate choice by measuring its fitness benefits across a range of environmental conditions and in populations exhibiting contrasted genetic structures.

MHC and Social Signaling: A Wider Behavioral Function of MHC?

An obvious challenge in understanding MHC-biased mate choice is identifying its proximate basis, through modes of signaling and perception of genetic information among individuals. MHC contributes to the discrimination of self and nonself at the molecular level and may also contribute to individual and kin discrimination at the organismic level, with behavioral consequences that may extend further than mate choice, by affecting cooperation among kin (Brown and Eklund 1994; Ruff *et al*. 2012), parent–offspring recognition (Yamazaki *et al*. 2000), or even species diversification (Eizaguirre *et al*. 2009a). The role of MHC genes as determinants of sensed genetic individuality through olfactory cues has been highlighted in various vertebrate taxa (Penn 2002; Yamazaki and Beauchamp 2007). A MHC signature in odors has been identified by several studies in rodents (Kwak *et al*. 2008, 2011; Willse *et al*. 2006) as well as in a primate species, the mandrill (*Mandrillus sphinx*: Setchell *et al*. 2011). In rodents, MHC similarity can be perceived beyond overall genomic similarity (Yamazaki and Beauchamp 2007; Younger *et al*. 2001a), and some studies suggest this may be the case in humans, too (Havlicek and Roberts 2009a). Although physiological pathways linking MHC genes and odor production are far from being understood at the organismic level (Kwak *et al*. 2011), the tight genomic linkage between MHC and a large cluster of olfactory receptor genes observed in humans and rodents raises the possibility of a functional connection between both, where the possession of particular MHC genotypes might be associated with particular olfactory abilities or preferences (Ehlers *et al*. 2000; Younger *et al*. 2001a). This functional association is further supported by the activation of vomeronasal receptors by MHC-derived peptides in rodents (Leinders-Zufall *et al*. 2004). More importantly, a recent study has demonstrated that particular HLA alleles directly influence the production of specific volatile organic compounds at the cellular level, leading to a cell-specific odor "fingerprint" (Aksenov *et al*. 2012).

It is, however, important to realize that MHC genes are not the only candidates for the genetic basis of chemical communication. For example, major urinary proteins (MUPs) have been linked to the individual odor-based regulation of species-specific behavior in mice (Cheetham *et al*. 2007; Hurst *et al*. 2001, 2005), and genes coding for MUPs represent an equivalent polymorphic complex but with no implications for immune function. Comparing their respective effect on social and sexual behavior would thus be very interesting (Thom *et al*. 2008). Recent findings of functional MUP genes in non-human primates such as mouse lemurs suggest that the role of MUPs in olfactory signalling is not limited to rodents, though functional MUP genes have not been detected in humans (Logan *et al*. 2008).

Overall, increasing evidence suggests that MHC genes directly influence socio-sexual behavior. However, much of this evidence remains correlative, except for experiments performed on humans and rodents, and the ecological and evolutionary significance of MHC-dependent behavior, as well as its universality, remains to be established.

MHC Genotyping: Historical Challenges and New Alternatives

The single main obstacle to elucidating the role of MHC in behavior, and more generally in population ecology, lies in the challenges of MHC typing. These challenges currently limit the scale of studies, include difficulties associated with low-quality DNA from noninvasive (fecal) samples, and occasionally result in unreliable genotyping. The next section reviews the approaches adopted by molecular ecologists to overcome these problems, before introducing the contribution of new sequencing technologies.

Why Is MHC Notoriously Difficult to Genotype?

Three features of MHC genes pose major challenges to MHC typing (Babik 2010; Bernatchez and Landry 2003; Piertney and Oliver 2006): 1) its extreme allelic polymorphism, 2) the presence of pseudogenes (nonfunctional genes), and 3) the frequent gene duplications that have resulted in variation in the number of loci within and across species. The parts of MHC proteins involved in binding foreign antigenic peptides determine the functional differences between MHC alleles and frequently represent the regions of interest for behavioral ecologists, thus setting the targets for genotyping. They represent the most variable regions of MHC, which can count >8500 alleles in humans (Robinson *et al*. 2011). This polymorphism precludes the application of molecular tools routinely available to type genetic polymorphisms, such as sequence-specific priming, except when extensive background information is available regarding MHC variability in the species of interest, as is the case in humans and some organisms used in biomedical research such as laboratory rodents or rhesus macaques (*Macaca mulatta*). A widespread alternative consists in sequencing short fragments containing the antigen-binding sites but a critical difficulty there consists in designing primers that may amplify the full range of allelic variation (Babik 2010). Failing this step may cause investigators to miss an unknown fraction of the MHC variation in the genotyping process, which will cast doubts on the results

of downstream analyses. Designing efficient primers requires characterization of the primer binding region and may take place as an incremental process consisting in cloning and amplifying the region of interest repeatedly until full characterization (Babik 2010; Sepil *et al*. 2012).

Second, identifying whether pseudogenes are present is required if the project goal is to characterize functional variation. It is sometimes possible to identify a pseudogene simply by examining its nucleotide sequence, for example, if it contains a stop codon. However, not all pseudogenes show such features and their identification therefore requires ensuring that all alleles are expressed by comparing genotypes obtained by RNA (cDNA) and genomic DNA amplification. RNA should be extracted from tissues expressing the MHC molecules targeted, because certain MHC genes are present only in blood and lymphoid organs (Janeway *et al*. 2005). Although this step should represent a standard for MHC studies (Knapp 2007), it is not always possible to acquire invasive samples from subjects of field studies.

Finally, MHC loci are frequently duplicated, resulting in multiple co-occurring copies in the genome. This means that DNA amplification for a single individual often yields more than the two sequences expected if this individual is heterozygous. The coamplification of multiple alleles has traditionally represented the single main challenge to MHC genotyping. A polymerase chain reaction (PCR) product containing an allelic mix cannot be sequenced using traditional sequencing methods (Sanger sequencing), as a mix of sequences renders the sequencing chromatogram unreadable (Fig. 1). Sanger sequencing therefore requires a preliminary separation of the different alleles present in the PCR product of a given individual, and a variety of methods have been proposed to overcome this challenge. These are briefly detailed in the sections that follow.

What Are the Methods Commonly Used to Genotype MHC in Evolutionary Ecology?

We aim to provide a brief, nonexhaustive, overview of the most common methods used to genotype MHC in organisms for which extensive background knowledge on MHC variability is unavailable, as is frequently the case for the species studied by

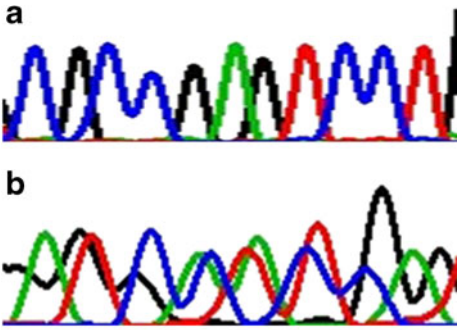

Fig. 1 A concrete example of the difficulties of MHC sequencing: two example chromatograms obtained from Sanger (traditional) sequencing of two MHC PCR products. Each colored peak represents one of the four nucleotides (A, T, G, or C). (**A**) Chromatogram from a pure PCR product containing only one allele. (**B**) Chromatogram from a PCR product containing an allelic mix. This is a problem because Sanger sequencing methods rely on reading a single nucleotide at each time interval.

field primatologists (summarized in Table I). More information can be found elsewhere (Babik 2010). The "gold standard" method of separating the different alleles present in the initial PCR product is cloning the PCR product. Molecular cloning involves inserting a particular fragment of DNA into the purified DNA genome of a self-replicating genetic element —generally a virus or a plasmid— that is then introduced into a bacterial cell and cultivated to generate a large population of bacteria containing identical DNA molecules (Alberts 2002). Consequently, sequencing multiple bacterial colonies for one given PCR product allows the identification of multiple alleles present in the PCR product because each colony "selects" one (and only one) allele at random. In concrete terms, cloning 10–20 PCR products (corresponding to 10–20 focal subjects) may typically require a week of work and the sequencing of 30–50 bacterial colonies for each PCR product to ensure that all alleles present have been identified, which is financially costly.

As a result, alternative methods relying on sensitive electrophoresis have been developed that are substantially quicker and cheaper than cloning. These methods typically occur in two main steps. The first step consists of migrating the different alleles present in the initial PCR product on a gel, in order to separate them according to their differential nucleotide composition using denaturating gradient gel electrophoresis (DGGE; Fig. 2) (Huchard *et al*. 2006; Knapp *et al*. 1997, 2005b; Myers *et al*. 1987) or according to the differential 3D conformation of a single DNA strand using single-strand conformational polymorphism (SSCP; Orita *et al*. 1989; Sommer *et al*.

Table I Summary of the most common MHC genotyping methods used in nonmodel organisms

Genotyping method	Cost per sample	Initial optimization effort	Throughput[a]	High-quality DNA required?	Example study in primates
Cloning	€€€	Low	+	No	(Lukas *et al*. 2004)
Electrophoresis-based methods	€–€€[b]	High	++	Yes	DGGE: (Knapp *et al*. 1997)
					SSCP: (Schad *et al*. 2004)
Microsatellite typing	€	High	+++	No	(Doxiadis *et al*. 2007)
Next-generation sequencing	€–€€[c]	Low	++++[4]	Possibly not	(Huchard *et al*. 2012)

As a very rough indication of the financial costs, each € symbol represents 5–15€, and one sample represents one individual for one gene (so one individual for two genes would cost twice as much). A low optimization effort may represent few days to few weeks (less than a month) of work for one person, while a high optimization effort would likely represent more than that.

[a] Throughput indicates the potential of the method to handle large samples.

[b] These methods may turn out to be more onerous than initially thought when extra separation steps are required.

[c] Costs proportionally decrease as the number of samples increases, but are also conditional on the coverage required, which largely depends on the number of coamplified loci.

[d] Although time spent in the lab is usually much shorter than with other methods, time spent sorting alleles using bioinformatics and statistics tools is substantial, and may typically require a minimum of 3 mo per project.

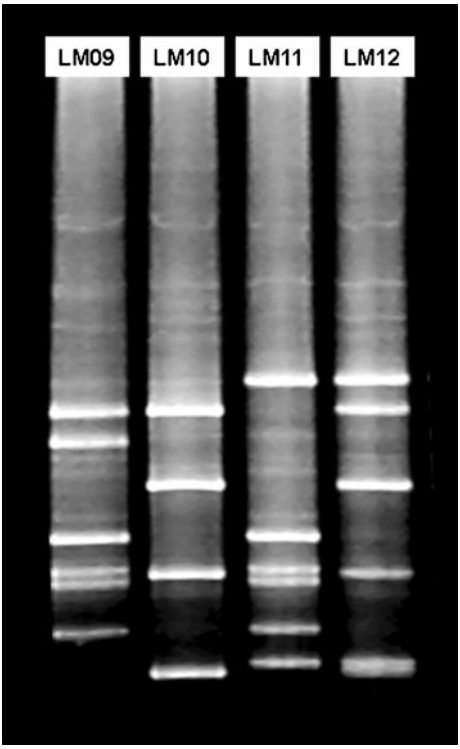

Fig. 2 Example of an electrophoresis gel (DGGE) separating the different alleles of a mixed PCR product. PCR products of a variable fragment of MHC class II DRB are shown for four chacma baboons (*Papio ursinus*) identified by different codes with different MHC genotypes. The thickness of the lowest band of the individual LM12 reveals the limits of the resolution of this gel in separating alleles: two alleles comigrated in this location, and additional separation steps were thus required to separate these sequences.

2002). The second step consists of excising each separate band (which corresponds to one allele) from the gel and reamplifying it to sequence it. A gel may accommodate 10–20 PCR products (typically 10–20 focal subjects) and preparing and running it may take a working day. Although more efficient than cloning, this method is still impractical for processing >100 individuals. Electrophoretic methods are also difficult to optimize to obtain a resolution able to separate alleles differing by only a point mutation (Knapp 2005b), which are common in MHC genes. As a result, they often require extra separation steps, including cloning (Huchard *et al*. 2008).

Finally, a last approach consists of identifying microsatellite polymorphisms tightly linked to a variable MHC region of interest, for example, located in the intron of the MHC gene targeted (de Groot *et al*. 2008; Doxiadis *et al*. 2007). This method can be carried out easily on a large number of samples but requires considerable optimization to identify appropriate microsatellite polymorphisms and link them to the corresponding MHC allele.

What Is the Alternative Offered by New Sequencing Technologies?

The recent introduction of next generation sequencing (NGS) to MHC genotyping shows a great deal of promise to circumvent some of the challenges inherent to the

complexity of MHC structure. It greatly improves the scope of MHC studies by considerably extending the scale of the projects that can be undertaken. 454-technology has now been successfully applied to MHC genotyping in a number of studies (Babik *et al*. 2009; Galan *et al*. 2010; Sepil *et al*. 2012; Wegner 2009; Zagalska-Neubauer *et al*. 2010) including one in primates (Huchard *et al*. 2012). Similar to cloning in principle, but without the need for living recombinant organisms, NGS provides individual reads of each single DNA molecule present in a sample, thus avoiding the delicate step of separating the multiple alleles present in a PCR product (Metzker 2010; Shendure and Ji 2008). The fragment length that can be analyzed using 454-pyrosequencing ranges from *ca*. 100–500 base pairs, which represents the typical length of the most variable MHC exons that are routinely sequenced by MHC studies. We briefly describe in the text that follows the basic principle of 454-sequencing that is useful for MHC genotyping and recommend some well-illustrated reviews for additional information regarding the principle and chemistry of next-generation sequencing (NGS; Metzker 2010; Shendure and Ji 2008). In a first step, the DNA of each individual is amplified with a set of tagged primers, that is, primers that contain a unique label (in the form of 4–10 nucleotides) specific to each individual involved in the experiment. In a second step, PCR products from all individuals can be mixed (as they are identified by their individual tag) and the clonal amplification of each DNA molecule is performed by an "emulsion PCR." During this amplification step, each single DNA strand from the DNA mix is captured by a different bead and the bead–DNA complex is encapsulated into a water droplet in an oil solution. A PCR amplifies each DNA molecule enclosed into the "emulsion bubble," leading to thousand copies of the same DNA template in each bead. In a final step, the beads are deposited in a PicoTiterPlate for pyrosequencing. Each bead binds to one microwell of the PicoTiter Plate (which contains *ca*. 10^5–10^6 wells depending on the sequencer model) and the PCR products enclosed in each bead are sequenced in parallel using a bioluminescence method. A sequencing run takes 4–6 h and provides an output consisting of 10^5–10^6 sequences (commonly referred to as "sequencing reads"), each of which can be attributed to one individual thanks to its primer tag, allowing a list of the reads possessed by each individual to be established. The average number of reads per individual is referred to as the "coverage" and is adjusted at the optimization stage: if a maximum of two alleles per locus is expected, a coverage of 20–40 reads/individual may be sufficient to ensure that both alleles are captured (Galan *et al*. 2010). If the number of gene copies targeted is greater, a higher coverage is required. Given that the total number of sequences per sequencing run is fixed, the coverage determines the number of individuals that can be sequenced in any given run. With a targeted coverage of 100 reads per individual, 10^4 individuals can be genotyped in one run. It is important to keep a comfortable safety margin when establishing a targeted coverage, as there is extensive variation in the number of reads across individuals (and possibly alleles). One sequencing run, from the initial amplification stage to the final sequencing stage, typically takes one full week of work.

The utility of NGS technology comes at the cost of frequent genotyping errors. Pioneering studies have suggested that this difficulty may be overcome by stringent quality control, allowing true and false alleles to be sorted (Babik *et al*. 2009; Galan *et al*. 2010; Huchard *et al*. 2012; Sepil *et al*. 2012; Wegner 2009; Zagalska-Neubauer *et al*. 2010). The high frequency of false alleles is, in fact, not specific to NGS and is

common in any MHC genotyping method that relies on DNA amplification; it is very frequent in cloning (Huchard *et al*. 2012) and present in electrophoretic methods (Knapp 2005b). However, the high throughput of NGS precludes manual allele sorting, and requires bioinformatics to automate this process. Although several authors have provided valuable guidance on how to proceed (Babik *et al*. 2009; Galan *et al*. 2010; Sepil *et al*. 2012; Wegner 2009; Zagalska-Neubauer *et al*. 2010), this task should not be underestimated and may take 3 mo (or more) of work for a given project. It represents the main drawback of 454-sequencing applied to MHC genotyping, which otherwise has the key advantages of relatively low costs per sample, high throughput, and rapid optimization (once primers are identified). It may also allow relatively smooth genotyping of the most complex multilocus systems with extensive allelic polymorphism (Sepil *et al*. 2012; Zagalska-Neubauer *et al*. 2010).

Designing an MHC Project: A Practical Guide

This section provides some practical advice to the design of a MHC project in behavioral ecology. It is broken into three sections focusing on identifying the most suitable study system, the best genotyping approach, and the most appropriate scale of projects in the time frame and funding conditions of a typical research project.

What Is the Ideal Study System?

It is a species for which 1) prior information on MHC variability and suitable primers are available for several MHC loci; 2) high-quality DNA can be obtained from a large number of recognizable individuals in the wild or in free-ranging populations; 3) high-resolution behavioral and life-history data can be obtained from the same individuals as well as, if possible, physiological and parasitological data (although the need for the latter obviously depends on the research question to be addressed); 4) multigenerational data are available as well as a molecular-based pedigree, which will allow validation of MHC genotyping and the investigation of multiple questions relating to MHC's influence on mate choice and patterns of parentage; and 5) a captive population of the same species is accessible so that some questions can be addressed using an experimental approach, allowing further mechanistic exploration of the processes under scrutiny. Although not many primate models score all these points, some of them do. The phylogenetic proximity of anthropoid primates to humans may facilitate the use of molecular tools developed on HLA, the human equivalent of the MHC, in many of these species (de Groot *et al*. 2002; Doxiadis *et al*. 2007). In addition, primatology counts a number of long-term studies documenting the behavior of many individuals throughout their life (Kappeler and Watts 2012). There is certainly no need to have all these advantages to start a comprehensive MHC study, but combining a couple of them may considerably increase the scope of the questions that can be addressed.

Which Genotyping Approach Should Be Adopted?

The constraints and the opportunities of the study species should guide the approach used (see Table I). In brief, and regardless of the method employed,

difficulties in optimizing the genotyping are to be expected if only low-quality DNA (from noninvasive samples) is available and if appropriate primers have to be designed. Low-quality DNA will also require that most or all samples are genotyped in duplicate or triplicate to ensure reliability (Knapp 2005a; Lukas *et al*. 2004). The standards of the field are progressively moving away from studies that investigate variation in a single short DNA fragment, so future projects should plan to analyse variation in more than one MHC gene, especially because sequencing technologies now provide this possibility. Sequencing a higher number of variable loci will increase the resolution of measures of MHC similarity among partners, or of MHC diversity within individuals, and may help to identify the most important MHC regions for mate choice or social signals (Huchard *et al*. 2013). In studies in which DNA is available from a large number of individuals, there may be a trade-off between the number of individuals and of loci to be sequenced, the optimal value of which will depend on the research question, the ease with which many loci can be sequenced, and the extent of phenotypic data available for some or all individuals. Previous knowledge regarding the relative variability of different MHC loci in the target species will be helpful to identify the best genes to be studied. The topic of the project will also guide this decision. The MHC is divided into several main clusters, including the class I region, which is typically involved in resistance against intracellular pathogens such as viruses, and the class II region, which is typically involved in resistance against extracellular parasites such as macroparasites and bacteria (Janeway *et al*. 2005). Whereas many primate studies have focused on a few variable genes in MHC class II (typically *DRB* and *DQB*), which are known to be important in parasite resistance (Setchell and Huchard 2010), less is known regarding the ecological and evolutionary consequences of MHC class I variation, which is known to influence a number of reproductive functions including materno–fetal interactions (Alberts and Ober 1993), but notoriously more difficult to genotype in Old World Monkeys owing to the complexity of its organization (Otting *et al*. 2005).

Any large-scale project —e.g. >100 individuals— should turn to NGS. The number of genotyping platforms increases rapidly, and access to sequencing facilities should never be a limiting factor given that a full MHC project may take only 6 h of sequencing time. Access to help for the bioinformatics following NGS may be more limiting. Regardless of the genotyping method, it is essential to evaluate the genotyping quality by running duplicates for a large subset of samples, or by comparing genotypes among parent–offspring dyads to estimate error rates. Baseline levels of MHC variability observed in the study population should be published along with a demonstration of the genotyping reliability, even if the primary interest of the principal investigator is in behavioral ecology (Huchard *et al*. 2008, 2012; Sepil *et al*. 2012). This is facilitated by the fact that standards in the field of immunogenetics encourage investigators to describe new MHC alleles (for any given species) in publications detailing their characteristics, rather than simply depositing them in public databases. Unreliable MHC genotyping may cast doubts on downstream analyses, and the robustness of this field of research critically depends on the use of rigorous and transparent procedures.

What Scale Can Be Envisaged in the Realm of a Typical Research Project?

MHC genotyping, as already emphasized, can be time consuming and care should be taken to avoid underestimating the optimization stage —typically the most time-consuming part of the whole process— especially in conditions where difficulties are to be expected. Projects should use reasonably large sample sizes, and starting a MHC project with <50 individuals is risky, as most analyses may fall short of power, except perhaps through an experimental approach. Genotyping 500–1000 samples in the context of a typical 3-yr project is realistic using NGS, although handling a high number of samples may require extra time at other stages of the project, such as genotyping these for neutral markers to establish pedigrees or to infer demographic influences on genetic variation. It is good practice to take the time necessary to validate and publish genotyping quality before moving on to the next stages, which should also factor into planned time schedules. Practically, although a large-scale MHC project can be planned in the context of a 3-yr project, it may be incompatible with extensive fieldwork, and it may thus be safer to use behavioral data that are already available. Enough time should be reserved for data analyses, which may typically require tools from adjacent fields including population genetics and molecular evolution, as well as sophisticated statistics. Acquiring a basic understanding of these disciplines is necessary and may be time consuming.

What Is the Future of MHC Studies in Primatology?

New sequencing technologies dramatically increase the scale and scope of studies aimed at elucidating the mechanistic bases and ultimate causes of MHC-biased behavior, and promises rapid progress in the near future. The broad socio-ecological diversity found in the primate order (Kappeler and van Schaik 2002; Mitani *et al*. 2012) and the richness of primate socio-sexual behavior and signaling (Dixson 1998; Kappeler 2002) offer opportunities to address important questions regarding the role of MHC genes in sexual and kin selection. In addition, resources available to primatologists, including some key molecular resources designed for human research and transferable to closely related species (Tung *et al*. 2010), a wealth of comparative data across primate species and populations, as well as a number of multigenerational individually based field studies (Kappeler and Watts 2012) place primates at the forefront of future research. Here, we highlight some specific questions and topics to which the study of primates may provide valuable contributions.

Test the Links Between MHC and Behavior at the Genomic Scale.

The sequencing of the full genome of several primate species has been achieved (Tung *et al*. 2010) and is ongoing for several others. This information will be very helpful for the design of densely spaced markers throughout the MHC region and the whole genome. As a result, primate studies may stand at the forefront of the number of MHC genes and markers examined. Marker design will offer insights into whether specific MHC loci exert an influence on behavior, and may, in turn, help to identify

the pathway linking genotype and phenotype, as specific MHC genes may have specific functions, for example, in reproductive physiology. In addition, these influential MHC loci may be linked to other functional genes causing the observed patterns given the high level of linkage disequilibrium observed in the MHC region (Horton *et al*. 2004), and genomic scans may help to detect effects of this kind. For example, the tight genomic linkage observed between MHC and olfactory receptor genes in humans and rodents (Younger *et al*. 2001b) may suggest that genetic diversity or dissimilarity at olfactory receptor genes may generate observed behavioral biases attributed to MHC genes.

Moreover, it is important to contrast MHC variation measured over an extensive region with wider genomic diversity using markers of the same nature, e.g., single-nucleotide polymorphisms, to determine whether observed behavioral effects are MHC specific or not (see Chaix *et al*. 2008; Laurent and Chaix 2012 for an example of this design in humans). This remains an outstanding question in MHC evolutionary ecology, as most studies have examined the links between behavior and a small MHC fragment (Huchard *et al*. 2010a) without necessarily controlling for wider genomic diversity. When they have controlled for it, they have often used crude estimates in the form of multilocus heterozygosity measured over a handful of microsatellite loci (Balloux *et al*. 2004). As a result, it is often impossible to exclude the possibility that MHC-disassortative mate choice is a simple by-product of inbreeding avoidance based on MHC-independent cues. Genome-wide studies are costly and may be applicable only to a subsample of individuals, so documenting the effect size of the MHC-dependent behavioral bias of interest using a traditional genotyping approach on the mastersample may provide a good starting point to calculate the minimum number of individuals to be included in the genome-wide study.

Understand How Social and Mating Systems Drive MHC-Associated Mate Choice.

The social diversity of primates, including solitary, pair-living, and group-living taxa, and the corresponding diversity in mating systems, with monogamous, polyandrous, polygynous, and polygynandrous mating patterns (Kappeler and van Schaik 2002) offers an opportunity to examine the universality and context dependence of mate choice rules (Setchell and Kappeler 2003). Individual mating strategies appear to be constrained by group size and social structure. For example, alpha males may largely monopolize reproduction in mixed-sex groups of polygynous species (Cowlishaw and Dunbar 1991; Port and Kappeler 2010) and compromise the expression of mating preferences of subordinate males and females. In addition to the social system, demographic effects on population genetic structure may influence the expression of individual mating strategies. For example, research in humans suggests that MHC-disassortative mate choice operates only in close, inbred populations (Chaix *et al*. 2008), and further efforts are needed to understand the links between population genetic structure and the evolution and plasticity of mate choice. Primatologists can play a key role in addressing these questions, because primate societies have been comparatively well studied and provide a wealth of detailed data on behavior and ecology (Setchell and Kappeler 2003).

Investigate Variations in MHC-Dependent Mate Choice Within and Across Individuals, and Their Consequences.

Detailed individually based longitudinal datasets developed by primatologists over decades can be used to measure intraindividual consistency in MHC-dependent mate choice, and to identify factors driving variation, such as fluctuations in social context (Gowaty 2004), e.g., the pool of available partners, or reproductive state (Havlicek and Roberts 2009b). Some large-scale studies can be used to examine the extent and causes of interindividual variation in mating decisions, as well as the fitness consequences of MHC-dependent mate choice, which are far from being established, and even further from being measured (Setchell and Huchard 2010). Primatologists can also pinpoint the mechanisms mediating MHC-biased mate choice. Whereas most published studies of MHC-dependent mate choice rely on indirect measures of mate choice based on biased paternities, it is now important to understand whether these biases occur prior or after copulation and detailed records of mating behavior available in many primate studies can shed light on this question. In addition, the primate order counts some of the brightest and most colorful sexual signals found in mammals, and recent work has highlighted potential connections between the possession of particular MHC genotypes, individual condition, and the intensity of these signals in primates (Huchard *et al*. 2010b; Setchell *et al*. 2009). Future studies may attempt to confirm these intriguing findings using larger sample sizes.

Test the Role of MHC in Kin Discrimination.

Whereas kin recognition has long been thought to be mediated by familiarity through stable bonds created during early development (Waldman 1988), recent studies suggest that individuals may recognize and preferentially associate with unfamiliar relatives such as paternal kin in promiscuous societies where paternity is uncertain (Widdig 2007). These observations suggest alternative mechanisms of kin discrimination, potentially through self-referent phenotype matching, the comparison between own and other's phenotypes (Widdig 2007). Many cues reflect relatedness in nonhuman primates, including visual appearance (Alvergne *et al*. 2009; Kazem and Widdig 2013; Parr *et al*. 2010), vocalizations (Kessler *et al*. 2012; Rendall *et al*. 1996), and odors (Célerier *et al*. 2010; Charpentier *et al*. 2008). Despite a long held belief that monkeys and apes are microsmatic and that their main communicatory channels rely on visual rather than olfactory cues (Heymann 2006), recent developments in the study of primate olfactory communication have revealed that scents reflect genome-wide diversity and genetic relatedness in ring-tailed lemurs (*Lemur catta*: Charpentier *et al*. 2008, 2010) and MHC diversity and dissimilarity in mandrills (Setchell *et al*. 2011). Testing whether the MHC plays a role in kin discrimination is thus an important goal for future research, in primates and other animals. The best evidence so far comes from a recent study in *Xenopus laevis* showing that within sibships, tadpoles associate preferentially with MHC-similar full-sibs, with a positive linear relationship between MHC similarity and the intensity of association among partners (Villinger and Waldman 2012). In addition, new findings indicate that humans can discriminate the odor of self from nonself MHC peptide ligands (Milinski *et al*. 2013). Establishing the role of MHC in kin discrimination in

nonhuman primates will nonetheless represent a challenging task, because it requires showing that affiliation or association among unfamiliar individuals depends on their MHC similarity, independently of their degree of kinship (which may be reflected in other phenotypic cues). To reach that goal, studies will have to compare the social interactions of unfamiliar individuals that exhibit a similar level of kinship but vary in MHC similarity. Experimental designs in captive colonies or comparisons of interactions among paternal kin in long-term field studies of polygynandrous primates —in which familiarity is an unreliable paternity cue— may be able to tackle this challenge.

Elucidate a Potential Role of MHC in Species Diversification.

One of the major riddles in our understanding of the evolution of socio-ecological diversity represents the identification of traits responsible for the regulation of gene flow across populations. Sexual selection, along with selection against hybridization or ecological selection, has been considered to be of major importance in this process (reviewed in Hoskin and Higgie 2010). Adaptive traits linking mate choice to environmental changes, sometimes referred to as "magic traits" (Gavrilets 2004), may play a crucial role in the speciation process if they contribute to the translation of adaptation to a new environment into a shift in mating preference. MHC genes have been proposed to represent "magic genes" (Eizaguirre *et al*. 2009a). Several studies have suggested that pools of MHC genes are shaped by local pathogen communities that may vary across populations (Blais *et al*. 2007; Eizaguirre *et al*. 2009a; Wegner *et al*. 2003) and that mate choice favoring locally adapted MHC genotypes might consequently accelerate reproductive separation among populations (Eizaguirre *et al*. 2009b, 2012). This framework represents a new angle linking MHC to biodiversity at an evolutionary scale. Several primate radiations, featuring several cryptic species, incomplete lineage sorting, or hybridization *in situ* (Weisrock *et al*. 2010) provide an excellent opportunity to unravel the tangled influence of genetic and ecological factors in leading to reproductive isolation among closely related species.

Conclusions

MHC figures among the first set of functional genes that have been identified as influencing behavior in laboratory rodents. Sustained research efforts invested in understanding the mechanisms and evolutionary function of this intriguing connection have long been hampered by the challenges posed by MHC genotyping. The first studies to apply NGS to MHC genotyping successfully have thus opened a major technological lock. NGS dramatically expands the potential scope of studies, which can now be undertaken at the genomic and population scale. This opens exciting opportunities to resolve old outstanding questions, such as understanding whether, and to what extent, MHC causes behavioral variation, and whether, and to what extent, sexual selection contributes to the generation and maintenance of MHC polymorphism. NGS will also help to exploit the full potential of longitudinal individually-based primate field studies that have run over decades, and represent the historical strength of primatology. Primatologists thus have key assets at hand to pioneer the next generation of studies addressing the links between MHC and behavior.

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Glossary

454-pyrosequencing (Roche diagnostics)	A method of DNA sequencing by synthesis, using a large-scale parallel pyrosequencing system capable of sequencing roughly 400–600 megabases of DNA every 10 h.
ABS (antigen binding site)	The physical location of MHC molecules where antigens bind and are presented to T cells.
Antigen	Any molecule recognized by an antibody or T-cell receptor.
cDNA (complementary DNA)	Single-stranded DNA produced from an RNA template, lacking introns (introns are the noncoding parts of genes).
DGGE (denaturing gradient gel electrophoresis)	A technique used for separation of DNA fragments according to their mobility under increasingly denaturing conditions.
Electrophoresis	A technique used to separate DNA, RNA, or protein molecules based on their size and electrical charge.
Exon	The sections of a gene that are translated into a protein.
Haplotype	Set of alleles, which are found in adjacent locations (loci) on a single chromosome and inherited as a physically linked set from each parent.
Heterozygosity	Two different alleles at a locus in diploid organisms, resulting from inheritance of different alleles from each parent.
HLA (human leucocyte antigen)	The human major histocompatibility complex.
MHC (major histocompatibility complex)	A complex of tightly linked genes coding for molecules involved in the detection of nonself antigens and their presentation to specialized immune cells such as lymphocytes.
Molecular cloning	involves inserting a particular fragment of DNA into the purified DNA genome of a self-replicating genetic element, generally a virus or a plasmid, which is then introduced in a bacterial cell and cultivated to generate a large population of bacteria containing identical DNA molecules.

MUPs (major urinary proteins)	A subfamily of a larger protein family called lipocalins. MUPs are found in abundance in the urine and other secretions of many animals. They are encoded by a cluster of genes, located adjacent to each other on a single stretch of DNA, varying in number between species: from about 21 functional genes in mice to none in humans.
NGS (next-generation sequencing)	DNA sequencing method, extending basic principles of Sanger sequencing across millions of reactions in a massively parallel fashion. Rather than being limited to a single or a few DNA fragments as in Sanger sequencing, NGS generates hundreds of gigabases of data in a single sequencing run.
PCR (polymerase chain reaction)	Technique used to amplify DNA sequences using specific primers.
Polymorphism (in genetics)	Presence of different alleles at a given locus.
Primer	Short DNA sequences used to target the part of the genome to be amplified in PCR.
Primer binding region	A region of DNA where a single-stranded primer binds to start replication; or a duplication of a complementary DNA sequence during PCR.
Pseudogenes	Gene or allele that is not coding for a functional protein because it contains mutations that disrupt the sense of the sequence.
RNA (ribonucleic acid)	A single-stranded molecule similar to DNA, often resulting from DNA transcription and performing multiple vital roles in the coding, decoding, regulation, and expression of genes.
Sanger sequencing	A method of DNA sequencing, based on a selective incorporation of chain-terminating radioactively or fluorescently labeled dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. The resulting DNA fragments are heat denatured and separated by size using gel electrophoresis.
SNP (single-nucleotide polymorphism)	A type of polymorphism involving variation of a single base pair.
SSCP (single-strand chain polymorphism)	A conformational difference of single-stranded nucleotide sequences of identical length, a characteristic that allows distinguishing the sequences by means of gel electrophoresis (separating different conformations).
SSP (sequence-specific priming)	Also known as allele-specific PCR, a molecular typing method consisting in designing PCR primers that amplify one (and only one) specific allele of a polymorphic gene. Genotyping relies on using all specific-primers designed for each known allele to identify which allele(s) is/are possessed by the individual.

Stop codons	A trinucleotide sequence within a messenger RNA (mRNA) molecule signaling a halt to protein synthesis.

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