Are pedigree-based mutation rates the Rosetta Stone of molecular ecology? Promises and pitfalls of whole genome comparisons among closely related individuals.

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"The life of a new mutation is not an easy one."

Phillips, 1997

Abstract

Germline mutations are the raw material for natural selection, driving species evolution and the creation of earth's biodiversity. Life on earth would stagnate without this driver of genetic diversity. Yet, it is a double-edged sword. An excess of mutations can have devastating effects on fitness and population viability. It is therefore one of the great challenges of molecular ecology to determine the rate and spectrum by which these mutations accrue across the tree of life. Advances in high-throughput sequencing are providing new opportunities for characterizing these rates and patterns within species and populations, thus informing essential evolutionary parameters such as the timing of speciation events, the intricacies of historical demography, and the degree to which lineages are subject to the burdens of mutational load. Here, we will focus on the applications and limitations of whole-genome comparisons among closely related individuals in what are typically described as "trio" analyses for the detection of germline mutations as they arise in real time. By sequencing and comparing whole-genomes generated for individuals of known relatedness – typically, parent to offspring – investigators can ideally count and characterize mutations as they appear per generation. The promise for gaining insight into classic hypotheses of molecular evolution is high, though so too is the cost. Namely, the technical challenges are daunting given that pedigree-based studies are essentially

34 searching for needles in a haystack. Even so, the opportunities are so enticing, and the field so

35 young, we can say with confidence that fundamental insights have only just begun to emerge.

1 | INTRODUCTION

Spontaneous *de novo* germline mutations (DNMs) fuel the engine of evolution. Without them, natural selection has no material on which to act. But the vast majority of mutations do not confer a selective advantage and can thus lead to genomes that are burdened with mutational loads that hamper or prevent opportunities for adaptation through negative selection at linked sites (Charlesworth, Morgan, & Charlesworth, 1993). Thus, the mechanisms by which they are generated and transmitted from one generation to the next is of fundamental interest to the field of molecular ecology. The distribution and frequency of mutations across the genome regulate virtually every aspect of an organism's function and fitness. In addition to these direct genetic applications, accurate mutation rate estimates within species and populations can inform essential evolutionary parameters such as the timing of speciation events and key aspects of historical demography such as whether populations are expanding, shrinking, or have experienced population bottlenecks. Indeed, it is difficult to conceive of a biological phenomenon more important to our understanding of speciation, population genetic theory, molecular adaptation, life history strategy – and ultimately, conservation biology – than the accurate measurement of the *de novo* mutation rate across the tree of life.

In this review, we will focus on new insights into the causes and consequences of mutation rate evolution that are being ushered in by the ever-expanding innovations in whole genome sequencing (WGS). The power of next-generation sequencing methods (NGS, a term that now, 15 years in, seems charmingly obsolete) is well-known (Mardis, 2008a, 2008b). With the advent

of massively-parallel sequencing technologies (Rogers & Venter, 2005), and more recently, with accelerating improvements to single-molecule long-read technologies (Miga et al., 2020), we are steadily progressing from an era wherein whole genome analysis was restricted to genetic model organisms (namely, humans) to one wherein WGS applications are available for virtually any organism for which DNA can be obtained. Specifically, we will focus on the applications and limitations of whole-genome comparisons among closely related individuals in what are typically described as "trio" analyses for the detection of germline mutations in a single generation (Howell et al., 2003; Scally & Durbin, 2012). By sequencing and comparing whole-genomes generated for parents and their offspring, as well as other relatives (i.e. pedigrees) – investigators can count and characterize the mutations that occur within a generation of sequencing (Feng, Pettersson, et al., 2017; Koch et al., 2019; Pfeifer, 2017; Scally & Durbin, 2012; Smeds, Qvarnstrom, & Ellegren, 2016; G. W. C. Thomas et al., 2018a). To date, these studies have nearly exclusively examined the appearance of single base-pair substitutions and this review is focused accordingly. Nonetheless, we wish to note from the outset that there is a pressing need to enlarge the focus of pedigree-based studies to capture insertion and deletion events, as well as other mutations effecting structural variation (Course et al., 2020; Harris & Pritchard, 2017; Tatsumoto et al., 2017).

Although pedigree-based studies are only recently emerging for non-model organisms, they are already refining our views of long-standing hypotheses. It is worth noting here, however, that though the output and cost of Illumina sequencing typically used for discovering mutations is improving, the required sequencing depth and number of genomes to be generated can make

costs significant for studies of even modest scale. Given that confidence in calling mutations is entirely dependent upon a high-quality reference genome, which until very recently have been available for humans and other primates, pedigree-based DNM studies are correspndingly biased. Namely, the preponderance of our understanding comes from human studies (Guy Amster, Murphy, Milligan, & Sella, 2019; Gao et al., 2019; Genomes Project et al., 2010; Goldmann, Veltman, & Gilissen, 2019; Harris & Pritchard, 2017; Jónsson et al., 2017; Kessler et al., 2020; Kong et al., 2012; Rahbari et al., 2016; Roach et al., 2010; Rodriguez-Galindo, Casillas, Weghorn, & Barbadilla, 2020; Scally & Durbin, 2012; Seoighe & Scally, 2017; Smith, Arndt, & Eyre-Walker, 2018; Turner et al., 2019), though increasingly from non-human primates (Bergeron et al., 2020; Besenbacher, Hvilsom, Marques-Bonet, Mailund, & Schierup, 2019; Langergraber et al., 2012; Moorjani, Amorim, Arndt, & Przeworski, 2016; Pfeifer, 2017; G.W.C. Thomas, 2019; Gregg W. C. Thomas et al., 2019; G. W. C. Thomas et al., 2018b; Venn et al., 2014; Wu et al., 2020) and other mammals (Koch et al., 2019; Lindsay, Rahbari, Kaplanis, Keane, & Hurles, 2019; H. C. Martin et al., 2018; Ohno, 2019).

Though the *de novo* rate of mutation would ideally be accomplished by sequencing the germline itself, in practice this is rarely feasible outside of a model organism such as lab mice. Instead, investigators must take advantage of available tissues such as blood, skin, or in postmortem cases, organ tissue. While this causes some concern for DNM studies actually characterizing a mosaic of germline and somatic mutations (Li 2014), with our understanding of somatic mutation rates still developing (Muyas et al. 2020), pedigree-based studies have nonetheless characterized many features of the mutational spectrum such as the type of base

pair changes, their frequency, biases in genomic regions, and parental origin. Measuring the spontaneous mutation rate has subsequently led to an increased understanding of the biological, ecological, and life-history causalities of rate variation among organisms (Figure 1). These are heady propositions for the field of molecular genetics and ecology, though as is true of any emerging field that attempts to apply new technologies to old questions, there are as many pitfalls as promises.

2 | THE PROMISES

2.1 Reconciling substitution and mutation rates: application to divergence time estimation

It is understandable that biologists often use the terms "substitution rate" and "mutation rate" interchangeably. In both instances, the term is meant to describe the amount of genetic change over time in species and populations across the Tree of Life. Such rate estimates are fundamental to a multitude of applications, most especially to the practice of divergence time estimation. If branch lengths can be measured in units of evolutionary change and calibrated to absolute time, speciation events can be placed in geological and climatological context (Zuckerkandl & Pauling, 1965). Aside from this commonality, however, the two measures differ manifestly. Whereas mutations are the spontaneous result of the DNA replication machinery (Ohno, 2019; Seoighe & Scally, 2017), appearing abruptly and with an unknown evolutionary fate, substitutions are the manifestation of that fate, wherein selection has either purged deleterious, tolerated neutral, or fixed adaptive mutations within a population (J. B. S. Haldane,

1927; Phillips, 1997) – a theorem accepted long before the structure of DNA was even known. And though it has historically been assumed that substitution rates based on putatively neutral sites such as third codon positions represent the neutral rate of molecular evolution and approximately the mutation rate, the disagreement between estimates of *de novo* mutation rates and phylogenetic substitution rates measures suggests that selection is more pervasive than has been previously appreciated (Lynch, 2010).

By explicitly considering these differential processes, one that occurs nearly instantaneously, and the other that occurs over evolutionary time scales, we may be able to reconcile the early observations that *de novo* rates can be substantially higher than phylogenetically derived substitution rates (Denver, Morris, Lynch, Vassilieva, & Thomas, 2000), which in certain cases has been observed to be as much as an order of magnitude higher (Ho et al., 2011; Howell et al., 2003). Surprisingly, however, the opposite has been observed in humans with *de novo* rates substantially lower than phylogenetic rate estimates, when rescaling per-generation mutation rates to per-year based on a generation time of approximately 30 years (Moorjani, Gao, & Przeworski, 2016; Scally & Durbin, 2012; Ségurel, Wyman, & Przeworski, 2014). In the former case, the issue of time dependence has been evoked to explain the discrepancy, with molecular evolutionary rate estimates depending upon the time since genomes have diverged (Ho et al., 2011; Moller, du Plessis, & Stadler, 2018; Soubrier et al., 2012). In the latter case, spontaneous rate slowdowns have been evoked to explain the differential (Besenbacher et al., 2019; Moorjani, Gao, et al., 2016) as befits the hominid rate slowdown hypothesis (Goodman, 1985). The lower per-year mutation rate compared to the per-year substitution rate is often attributed

to an increase in generation time along the human branch. And indeed, recent comparisons of baboon and human rates suggest that the slowdown may have occurred much earlier in the anthropoid lineage than has been previously appreciated (Wu et al., 2020).

These observed discrepancies contradict the theoretical expectation that the neutral substitution rate approximates the spontaneous per-generation mutation rate (Kimura, 1983). Thus, the unbiased measurement of *de novo* mutation rates should allow investigators to avoid the known obstacles to divergence time estimation incumbent to phylogenetic methods, notably the requirement of an external calibration necessary for converting measures of sequence divergence to a per-year rate. Theoretically, per-generation *de novo* rates can overcome this constraint by rescaling branch lengths measured in substitutions per-site, with the caveat that generation times in years are known (Langergraber et al., 2012). In such a case, we simply divide the *de novo* rate by generation time to get a per-year rate that is not dependent on external calibration (G. Amster & Sella, 2016). Although simplistic, this approach, may be more appropriate when estimating divergence times of recent speciation events where incomplete lineage sorting is a concern, compared to concatenated fossil-calibrated methods (G. Amster & Sella, 2016; dos Reis et al., 2018; C. H. Martin & Hohna, 2018; Poelstra et al., 2020). The difference between models such as the multispecies coalescent (MSC) and concatenation that consider incomplete lineage sorting are reviewed by Tiley et al. (2020) and the utility of mutation rates for divergence time estimation are increasingly noted in accumulating empirical examples (Angelis & dos Reis, 2015; C. H. Martin & Hohna, 2018; Poelstra et al., 2020; Yoder et al., 2016). The age of introgression events could be similarly

dated when using models that jointly account for incomplete lineage sorting and episodic gene flow (Flouri, Jiao, Rannala, & Yang, 2020).

2.2. Connecting the dots between generation time and *de novo* mutation rates

The section above is meant to emphasize the distinction between substitution rates and *de novo* mutation rates, though in this section we will use the term mutation rate more generally to indicate any measure of genomic differentiation between two lineages. This is a convenience that allows us to review what is an extensive literature on the correlations between certain life-history traits within lineages and the rate at which their genomes evolve and diverge. In a seminal paper that examined differential rates of mtDNA evolution, Martin and Palumbi ((A. P. Martin & Palumbi, 1993)) noted that rates tend to scale with body size, with large mammals (like whales) having slow rates, medium-sized mammals (like primates) having intermediate rates, and small mammals (like rodents) showing the fastest rates. They also noted a potentially confounding pattern wherein poikilotherms, with their relatively slow metabolic rates, tend to have slower rates than similarly sized homeotherms with their higher metabolic rates. They acknowledged, however, that body size, generation time, and metabolic rate are not entirely independent traits given that large-bodied organisms tend to have longer generation times, and often, slower metabolisms. They concluded by synthesizing a "nucleotide generation time" model, thereby emphasizing the impact of metabolic rate and the mutagenic effects of oxygen radicals over absolute generation time as measured in years.

The generation time hypothesis has experienced many modifications in subsequent years with assertions that, because males experience more germline cell divisions than females (more about this to follow), there will be a much stronger male mutation bias in organisms with long generation times than those with shorter generation times (Goetting-Minesky & Makova, 2006). Conversely, in short-lived species the relative contribution of spermatogenesis will be much smaller, and therefore the rate per generation will be roughly constant (G. Amster & Sella, 2016). Or, as another possible explanation, longer generation times will correlate with higher mutation rates simply as a byproduct of increased lifespan (G. W. C. Thomas et al., 2018b). Though a quick survey of these varying hypotheses might suggest that they are in conflict, one common assumption binds them: each works from the assumption that mutation rates are inextricably linked to the phenomenon of cell division, with more cell divisions yielding higher rates.

2.3. Male mutation bias and the paternal age effect

The assumption that the number of cell divisions correlates with rate differential is perhaps most associated with the phenomenon of male mutation bias. Well before the advent of NGS technologies, investigators noted the propensity for mutation accumulation to be higher in males than females (Crow, 2000; Ellegren & Fridolfsson, 1997; Hurst & Ellegren, 1998; Shimmin, Chang, & Li, 1993). The evidence for this has come both from comparisons of differential mutation accumulation on sex chromosomes (Axelsson, Smith, Sundstrom, Berlin, & Ellegren, 2004; Bartosch-Harlid, Berlin, Smith, Moller, & Ellegren, 2003; Ellegren & Fridolfsson, 1997; H.

212 B. S. Haldane, 1947; Shimmin et al., 1993) as well as from direct measurements of the paternal
213 contribution of novel mutations in descendent offspring (see (Crow, 2000) for a detailed review,
214 relevant to human disease phenotypes). It was also noted early on that rates accelerate as
215 males age, with older fathers contributing more mutations than younger fathers, a
216 phenomenon with the potential to contribute to disease risks in humans (Crow, 1997; Kong et
217 al., 2012).
218
219 The explanation for both phenomena, male bias and the paternal age effect, appeared to be
220 immediately self-evident: because of the many more cell divisions in spermatogenesis than in
221 oogenesis – and thus the increased likelihood of introduced errors in the replication process –
222 the male germline will accumulate more DNA replication errors throughout life, accelerating
223 with both age and lifespan (Ellegren, 2007; Goetting-Minesky & Makova, 2006). Despite the
224 fact that spermatogonial stem cells are characterized by highly efficient DNA repair and one of
225 the lowest spontaneous mutation rates in the human body (Aitken, De Iuliis, & Nixon, 2020),
226 this idea has dominated the mutation-rate literature for years. And now, with the majority of
227 pedigree-based studies confirming the paternal origin of most DNMs, also finding that their
228 accumulation increases with age (Bergeron et al., 2020; Jónsson et al., 2017; Lindsay et al.,
229 2019; Rahbari et al., 2016; Venn et al., 2014; Wang et al., 2020), the cell-division hypothesis
230 seemed secure. Beyond these generalities, however, the story gets murkier.
231
232 First, the preponderance of studies showing a strong male bias has been skewed towards
233 primates, though with at least one new primate study, focused on mouse lemurs, showing a

very weak male bias (Campbell et al., 2020). That study suffers, however, from being quite small in terms of genomes compared, and also, given that it is the first and only pedigree-based analysis of a strepsirrhine primate, it cannot yet be known if the results are representative of the strepsirrhine clade, or merely an artifact of study design. Second, though they are as yet rare, *do novo* studies of other vertebrates are also beginning to contribute conflicting results. Though earlier phylogenetic-based analyses in birds tended to support the male-bias hypothesis (Axelsson et al., 2004; Ellegren & Fridolfsson, 1997), a more recent study that applies a pedigree approach finds only a modest male bias in birds (Smeds et al., 2016). Similar uncertainties have been reported for monotremes, with comparative genomic (though not pedigree-based) studies failing to reveal a strong male mutation bias (Cortez et al., 2014). Subsequent analysis by the same group has reported nuances that could result from the action of purifying selection on the Y-chromosome in monotremes and other mammals (Link, Aguilar-Gomez, Ramirez-Suastegui, Hurst, & Cortez, 2017). To briefly summarize the above, the taxonomic breadth of pedigree-based studies is understandably, though woefully, inadequate for making general statements about the phylogenetic generality of the male mutation bias.

Even so, new research is shedding light on this classic hypothesis, adding nuance to the mechanisms proposed to explain the pattern. Namely, the simple "cell-division" mechanism is increasingly being called into question. As early as 1998, Hurst and Ellegren ((Hurst & Ellegren, 1998)) asserted that the germ-line cell division model "is unlikely to be the whole truth" with regard to male mutation bias (p. 451). Rather, they argued that a multitude of other mutagenic mechanisms might apply, including patterns of methylation, exposure to oxygen free radicals,

temperature, and possible metabolites might act differentially on sperm. New analyses appear to be bearing them out. And indeed, DNA damage due to the hardships of aging is taking on strength as a contributor to the phenomenon (Ohno, 2019). Arguments for the effects of cellular aging are being marshalled from a variety of empirical perspectives including cellular function (Monaghan & Metcalfe, 2019), mutagenesis (Link et al., 2017), maternal aging (Gao et al., 2019), and the timing of puberty (Ségurel et al., 2014; G.W.C. Thomas, 2019; Wang et al., 2020; Wu et al., 2020). The full resolution of these conflicts and complexities will benefit enormously from expanded pedigree-based studies across a more comprehensive phylogenetic sample. These studies have the unique power to determine not only the parental source of DNMs, but also their genomic and developmental context (Jónsson et al., 2017; Narang & Wilson Sayres, 2016).

2.4. Effective population size (N_e), selection, and mutator loci

It has been widely noted that the *de novo* mutation rate varies widely across phylogenetic scales with what appears to be a relatively tight correlation with effective population size (Ne): when N_e is small, rates tend to be high, and conversely, when N_e is large, rates tend to be low. Further, there appears to be a similar correlation with genome size with organisms with large genomes showing higher rates than those with smaller genomes. These relationships were first noted in microbes wherein rates per base pair were observed to vary by approximately 16,000-fold though rates per genome varied only by 2.5 fold (Drake, 1991). Given the "largely mysterious" patterns observed on a site-by-site basis, Drake (1991) supposed that any

underlying rules were likely to be observed in comparisons of the mutation rate per genome per round of DNA replication. Moreover, he mused that the overall rate must have evolved under general evolutionary forces.

Here lie the underpinnings of the drift-barrier hypothesis (Lynch, 2010). Though there are various complexities relating to genome size and to the differential phylogenetic characteristics of eukaryotes and prokaryotes (Sung, Ackerman, Miller, Doak, & Lynch, 2012), the drift-barrier hypothesis states that effective population size (N_e) can explain variation in mutation rates across species due to a larger efficiency of selection acting on DNA replication fidelity in larger populations, especially across large phylogenetic distances. Rather than being a balance between the usually deleterious effects of mutation and the genetic machinery built to reduce those costs, the lower limits of the genome-wide *de novo* mutation rate must be set by the barriers imposed by genetic drift – a parameter set by effective population size. In such a model, the mutation rate will scale negatively with N_e up to the point where further reductions in rate cannot overcome the selective disadvantage of even the weakest "mutator allele."

Pedigree-based and mutation accumulation measurements of *de novo* rates appear to largely bear out these predictions (Figure 2; Table 1) (Bergeron et al., 2020; Besenbacher et al., 2019; Campbell et al., 2020; Feng, Pattersson, et al., 2017; Harland et al., 2017; Jónsson et al., 2017; Keightley, Ness, Halligan, & Haddrill, 2014; Keightley et al., 2015; Koch et al., 2019; Long et al., 2016; H. C. Martin et al., 2018; Pfeifer, 2017; Smeds et al., 2016; Uchimura et al., 2015; Yang et al., 2015). Even so, other explanations have been offered. For example, it has been argued

that drift becomes less effective as a barrier, or is even irrelevant, when mutations are either deleterious or strongly advantageous. And further, that these effects will have differential impacts across the expanse of a given genome (Lanfear, Kokko, & Eyre-Walker, 2014; Martincorena & Luscombe, 2013). This latter nuance in particular has been noted to have relevance to endangered species and subsequent conservation strategies given that both N_e and mutation rate can also vary across the genome (Zeng, Jackson, & Barton, 2019). And of considerable note, as sequencing technologies are becoming ever more precise, the relationship among lineages, their individual histories, and the idiosyncratic interactions of genotype and environment are coming into focus relative to the fixation of various hypermutator and antimutator alleles (Maddamsetti & Grant, 2020). Thus, an exciting frontier is within reach wherein investigators will have the analytical tools to explore the capacity for adaptive evolution as it relates to the interaction of mutation rate, genetic diversity, life history strategies, and environmental conditions (Rousselle et al., 2020).

3 | THE PITFALLS

3.1 Finding needles in a haystack

We hope that the section above adequately describes the myriad insights and applications for accurate measurement of the spontaneous germling mutation rate. An understanding of these rates, both within and among phylogenetic lineages, can inform practical applications, such as divergence time estimation and the underpinnings of genetic load in threatened species, as well

as illuminate the genetic mechanisms that dictate the characteristics and distribution of genomic variation. These insights into speciation, senescence, disease risk, historical demography, environmental impacts (and more) are powerfully enticing. Yet, the cruel reality is that we are looking for needles in a haystack. The technical challenges are enormous and largely relate to the difficulty of identifying true mutations against a background of sequencing errors. Even with 99.99% sequencing accuracy, this will result in ~280,000 errors in a typical 2.8 Gb primate genome. Thus, mutation rates are orders of magnitude lower than the sequencing error rate, even for the most accurate sequencing methods (Figure 3).

3.2 False positives

In addition to sequencing errors, and depending upon the tissue type, there may be a significant number of somatic mutations that can be mistaken for DNMs. Given that new mutations can occur at any stage of embryonic development post-fertilization – especially during the earliest cell divisions when mutagenesis is highly likely – post-zygotic mutations (PZMs) can affect both somatic and germline cells in the developing embryo. The distinction between DNMs and somatic mutations can thus be ambiguous and it is only via large-scale studies that mutational classes and their consequent mutational spectra can be understood (Goldmann et al., 2019).It is also difficult to differentiate between *de novo* and somatic mutation rates because de novo mutations can occur at any stage of embryonic development post-fertilization (especially during the earliest cell divisions when mutagenesis is highly likely), and thus can affect both somatic and germline cells in the developing embryo. The mistaken

identification of somatic mutations for *de novo* germline mutations can happen at substantial rates (Li, 2014; Muryas, Zapata, Guigo, & Ossowski, 2020), and in many cases are a consequence of the tissues sampled for pedigree sequencing. Consequently, the number of *de novo* mutations produced in a single generation can be difficult to differentiate from erroneous variant calls as well as from somatic mutations, and accordingly, stringent variant filtering is commonly applied (Campbell et al., 2020; Garimella et al., 2020; Lindsay et al., 2019; Pfeifer, 2017; Smeds et al., 2016; Winter et al., 2018). Once detected, there are additional options for verifying the biological reality of a given DNM, including validation with PCR-directed Sanger sequencing (e.g., (Koch et al., 2019), treating one or more samples as a technical replicates via sequence duplication (e.g., (Campbell et al., 2020; Kessler et al., 2020), sequencing monozygotic twins (e.g., (Kessler et al., 2020), and/or, as is now becoming standard, testing for ~50% heritability in a third generation transmission (e.g., (Bergeron et al., 2020; Jónsson et al., 2017; Kong et al., 2012; Pfeifer, 2017; G. W. C. Thomas et al., 2018b; Venn et al., 2014).

3.3 False negatives

Prior to validating a DNM, one has to observe it in the first place. With the high levels of computational filtering stringency that are typically applied, however, true *de novo* mutations may be missed and the mutation rate may actually be under- rather than overestimated. Thus, *de novo* rate estimates must deal with a high probability of false negatives as well as false positives (Scally, 2016; Ségurel et al., 2014). In other words, it is highly probable that true DNMs will be missed for largely technical reasons than can range from poor mapping to an

366 inappropriate reference genome (Garimella et al., 2020; H. C. Martin et al., 2018), applying
367 computational filters with too much vigor (Ségurel et al., 2014), or simply poor sequencing
368 quality at specific regions of the genome (Keightley et al., 2015; Pfeifer, 2017; G.W.C. Thomas,
369 2019). This aspect of calculating the spontaneous germline mutation rate is critical given that in
370 the rate calculation equation, the denominator must accurately reflect the callable proportion
371 of the genome. One of the gold standard approaches for calculating the callable fraction of the
372 genome, and accordingly, generating some idea of the false positive rate, has been to generate
373 synthetic mutations in the sequencing reads and then circle back to determine the proportion
374 of those mutations that are recovered using the identical computational pipeline initially
375 employed (Keightley et al., 2014). Yet another approach compares the proportion of false
376 negatives that would be estimated for each possible sequencing depth in each possible
377 offspring. These are then multiplied by the fraction of sites in the offspring with each depth of
378 coverage with the assumption that the overall false negative rate will be the sum of these
379 values (Koch et al., 2019). In summary, the careful evaluation of callable sites is a critical and
380 technically non-trivial aspect of *de novo* rate estimation.
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382 **4 | THE PATH FORWARD**
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384 Among the most pressing need going forward for pedigree-based studies is for comparable and
385 standardized methods wherein all studies confirm to a shared set of practices and procedures.
386 These include, though are not limited to, agreed-upon standards for contiguity and
387 phylogenetic proximity of reference genomes, the read depths utilized for pedigree genomes –

especially given the concern that sites with too many reads may represent repetitive genomics regions rather than DNMs (Wang et al., 2020), shared methodologies for determining false positive and false negative rates, and a standard set of computational filters to achieve aspects of the latter. Thankfully, leaders in the field are coordinating efforts to this end (L. Bergeron, S. Besenbacher, M. Schierup, and G. Zhang, pers. comm.), thus highlighting the future promise of pedigree-based mutation-rate studies.

With each new rate estimate we gain knowledge, particularly as we expand our inquiry across broader phylogenetic scales. Given the pace at which sequencing technologies are improving in accuracy and contiguity, pedigree-based studies should become increasingly more discerning, and with the associated costs continuing to drop, study designs of the breadth and depth previously available only for genetic model organisms will become more common for non-model species. And here, it is worth noting the perhaps unappreciated value of zoos and other living-stock collections for enabling these leaps into new organismal systems. These collections offer precious opportunities for incorporating deep pedigrees of known relatedness, as well as comprehensive databases of individual life history records including age, sex, number of offspring over lifespan, longitudinal health records, cause of death and other fundamental aspects of a given organism's biology (McCluskey et al., 2017). Thus, with a combination of technical, computational, and existing biological resources, we can be confident that classic hypotheses of molecular evolution will be increasingly refined with biological and functional sophistication.

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FIGURE LEGENDS

Figure 1 – Applications of Mutation Rate Estimates. Per-generation mutation rate estimates have been a powerful tool for understanding the demographic history of populations and species. The timing of species divergences and introgression events can be calibrated to absolute time from coalescent models that jointly account for incomplete lineage sorting and cross-species gene flow. Popular methods for studying change in population size over time such as PSMC require a mutation rate, which is often assumed to be 1×10^{-8} for many animal studies. The mutation spectrum can also be utilized when studying structured populations to identify mutational biases between them. There are nine mutational categories (when considering mutations at CpG and non-CpG) that can be revealing about changes in methylation over time. When rate estimates are available for multiple species, the evolution of the mutation rate itself can be studied. Comparisons between mutation rates and substitution rates can reveal life-history changes such as increased generation times, when mutation rates are much slower than expected from substitution rates.

Figure 2 – Relationship between Effective Population Size and Mutation Rate in Animals. Effective population size estimates are plotted on a $\log_{10}$ scale. Mutation rates were estimated per-generation from pedigrees with the exception of mouse, which was a mutation accumulation experiment. A negative relationship seems evident when considering all animals as expected by the drift-barrier hypothesis, but this is not observed when looking within groups where multiple species are available for comparison such as primates.

Figure 3 – Estimating Per-Generation Mutation Rates from Pedigrees. Genomes are sequenced to at least 30x for *n* pedigrees. At a minimum, both parents and an offspring need to be sequenced, but pedigrees that include a third generation are the emerging recommendation. Variants are called jointly across pedigrees. Because genotyping is often sensitive as to not miss variants a number of filtering steps are required to identify putative mutations. Mutations should be heterozygous in the offspring, but homozygous for the reference allele in both parents as well as all other individuals available across pedigrees or other population-level data. A mutation at a site needed to be callable at both parents and the offspring, which is typically determined by sufficient depth of unambiguously aligned reads. Variants are also filtered for allele balance, where between 30% and 70% of the reads should have the alternate allele. Less or more is suggestive of deviating from the expected 50:50 (at least for diploids) and that a mutation is a false positive. The number of mutations passing these filters are then divided by the number of callable sites as opposed to the reference genome length multiplied by the ploidy level. A number of analyses can be used to validate a mutation rate estimate. Where third generation pedigrees are available, approximately 50% of putative mutations should be observed in the third generation. An over-representation of mutations in the second generation would suggest a high number of false positives. Paternal age effects on mutation rate should also be observable. It is now well established that fathers contribute more mutations over time, and linear modeling can be used to estimate the mutation rate before puberty. It is also well accepted that certain types of mutations are more

common than others, such C-to-T transitions, and mutation spectra can be useful for evaluating pipelines used for estimating mutation rates.