Evolutionary origins of germline segregation in Metazoa: evidence for a germ stem cell lineage in the coral *Orbicella faveolata* (Cnidaria, Anthozoa)

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The ability to segregate a committed germ stem cell (GSC) lineage distinct from somatic cell lineages is a characteristic of bilaterian Metazoans. However, the occurrence of GSC lineage specification in basally branching Metazoan phyla, such as Cnidaria, is uncertain. Without an independently segregated GSC lineage, germ cells and their precursors must be specified throughout adulthood from continuously dividing somatic stem cells, generating the risk of propagating somatic mutations within the individual and its gametes. To address the potential for existence of a GSC lineage in Anthozoa, the sister-group to all remaining Cnidaria, we identified moderate- to high-frequency somatic mutations and their potential for gametic transfer in the long-lived coral *Orbicella faveolata* (Anthozoa, Cnidaria) using a 2b-RAD sequencing approach. Our results demonstrate that somatic mutations can drift to high frequencies (up to 50%) and can also generate substantial intracolonial genetic diversity. However, these somatic mutations are not transferable to gametes, signifying the potential for an independently segregated GSC lineage in *O. faveolata*. In conjunction with previous research on germ cell development in other basally branching Metazoan species, our results suggest that the GSC system may be a Eumetazoan characteristic that evolved in association with the emergence of greater complexity in animal body plan organization and greater specificity of stem cell functions.

1. Introduction

The function of the germline, as originally proposed by Weismann in his ‘doctrine of the continuity of the germ plasm’, is to serve as an immortal cell lineage that faithfully transmits hereditary information to future generations [1]. Because the soma has no evolutionary potential, the units of selection are individuals, rather than the cells that compose these individuals. Although Weismann’s doctrine proved useful in the development of the Modern Synthesis, there are many violations of the core tenets of his theory, most notably the assumption of no somatic influence on germ cell lineages [2,3]. Embryological and molecular studies demonstrate that many multicellular plants and fungi do not segregate germ cells from somatic stem cells. In plants, germ cells are specified continuously from meristematic stem cells, which can also differentiate into various somatic cell types. While it is clear that not all cells have access to the germline, still, there is no barrier to prevent somatic mutations of stem cells, with dual functionality as precursors to both somatic and germ cells, from being directly incorporated into gametes [4–6]. In contrast, diverse mechanisms are used to differentiate somatic and germ cell identity across multicellular animals (Metazoa). In bilaterians, the germline may be established through localization of germ-cell-specific molecules in the oocyte (preformation) or through epigenetic signalling during or following embryogenesis (epigenesis) [7]. Mutations that arise between zygote formation and germline specification may be incorporated into gametes. However, the chance of this occurring is greatly reduced after the germline is segregated...
hydrozoan cnidarians [2,3,8]. Although the timing of germ cell specification can vary among bilaterians, once the germ cell fate is determined, somatic cell lineages no longer have the opportunity to access the germline.

In basally branching metazoan phyla, such as Cnidaria, there is inconclusive evidence on the presence of a germ–soma barrier equivalent to that in bilaterian metazoans [9,10]. Among the most well-studied asexual basal metazoans (non-bilaterians), molecular and functional markers of the germline suggest that germ cells arise de novo from multipotent or pluripotent stem cells during asexual budding [7,10–15]. Hence, mutations that proliferate in rapidly dividing somatic stem cell lineages may be inherited by the germline. However, many of these studies rely on expression patterns of ‘germline genes’, such as Piwi, Vasa and Nanos, to identify germ cells, even though such genes might be markers of general pluripotency rather than of germline cells in non-bilaterians [8,16–20]. In addition, typical cellular markers of germline cells in bilaterian metazoans, including nuclear or cytoplasmic germ granules or chromatid bodies, fail to distinguish putative germ cells from somatic stem cells in non-bilaterians [20]. Despite the difficulties in identifying an independent germline cell lineage in basal metazoans, cell cloning experiments in the hydrozoan cnidarians Hydra oligactis and Hydra magnipapillata have demonstrated the presence of separate populations of multipotent stem cells (MPSCs) of the interstitial lineage that are only capable of differentiation into germs cells and are self-renewing. This strongly suggests that there may be a segregated germ stem cell (GSC) lineage present in hydrozoans, similar to the GSC system in bilaterians [21–23]. However, other studies in cnidarians in regard to germline specification have not been able to clearly distinguish somatic stem cell and GSC lineages [17,18].

To address current uncertainties in the mechanisms of germ cell development in basally branching metazoans and to further clarify evolutionary origins of germ cell developmental modes in Metazoa, we assessed the potential for the existence of an independently segregated GSC lineage in corals, which are members of Anthozoa, the sister-group to all remaining Cnidaria [24–26]. We employ a novel methodology for studying germline development, which uses a 2b-RAD sequencing approach [27] to distinguish between continual germ cell specification, in which germ cells are differentiated from the MPSC lineage throughout adulthood, and deterministic germline segregation, in which GSCs are differentiated from MPSCs early in development but thereafter function independently of MPSCs. In a continual mode of germ cell development, somatic mutations within MPSCs can proliferate in both somatic and germ cells, whereas deterministic germline segregation (GSC lineage specification) prevents the vast majority of mutations from incorporation into germ cells, since only GSCs are able to differentiate into germ cells [2,3]. Our 2b-RAD approach allowed us to determine the frequency of somatic mutations within a long-lived, colonial Metazoa.

Our experimental system is the Caribbean coral species Orbicella faveolata (Scleractinia: Anthozoa), which grows through asexual polyp budding and reproduces sexually once a year by broadcast spawning [28]. Colonies of this species grow indeterminately and can reach massive sizes, potentially passing through millions of cell divisions between zygote formation and adulthood [29]. Thus, there is a significant probability that somatic mutations that arise sufficiently early in the process of colony growth can drift to high frequencies within a colony (up to 50% since O. faveolata is diploid), thereby creating a mosaic genotypic composition across the colony surface (figure 1). Furthermore, colonies of O. faveolata accumulate biomass through outward, radial growth; thus, polyps on opposite sides of a colony can be separated by many decades or even centuries of growth, depending on the size of the colony [30]. Polyp lineages on opposite sides of a colony, then, potentially harbour many unique somatic mutations.

Our goal was to test the aforementioned competing hypotheses of germline development by identifying somatic mutations that differentiate opposite sides of exceptionally large O. faveolata colonies and assessing their potential for gametic transfer. Our results demonstrate that somatic mutations are not transferable to germ cells, which indicates, contrary to prevailing expectations [31], that corals may in fact possess a segregated GSC lineage.

2. Experimental procedures

(a) Sampling

Two massive O. faveolata colonies were sampled from the Flower Garden Banks National Marine Sanctuary, referred to throughout as colony A and colony B (figure 1a). Two fragments (approx. 20 cm²) were collected from opposite sides of each colony. The fragments were kept in an on-board flow-through system until spawning night, when they were isolated in individual bowls to collect sperm–egg bundles. Replicate sperm were retained for genotyping (figure 1b). Right after collection of sperm samples, two samples of neighbouring groups of three to five polyps from the same fragments were taken for genotyping (\( n = 4 \) adult somatic samples per colony). All samples were placed in 95% ethanol and stored at \(-80°C\) until DNA isolation.

(b) 2bRAD Library preparation and sequencing

Each of the somatic and gamete samples was processed in technical duplicate, resulting in a maximum of 16 samples (eight adult tissue and eight sperm) per colony (electronic supplementary material, figure S1). DNA was isolated using phenol–chlorophorm method following Davies et al. [29]. Genotyping was performed using the 2b-RAD method of Wang et al. [27]. This protocol uses type IIb restriction enzymes that excise 36 bp fragments around the recognition site. Two different restriction enzymes (BcgI and AlfI) were used to construct 2b-RAD libraries for each sample to increase the number of sites sampled from the genome. Final library preparations were combined and sequenced on two lanes of the Illumina HiSeq 2500 platform. Some of the replicate libraries failed due to various reasons: one of the replicate adult tissue samples from colony A, two replicate sperm libraries from colony B and one replicate sperm library from colony A (electronic supplementary material, table S1). Raw sequence data can be found on NCBI-SRA, accession no. PRJNA294592.

(c) De novo genotyping and mutation discovery

De novo and genome reference-based analyses were used to identify variants that consistently differentiated opposite sides of a colony. However, only the de novo results are presented
used a custom script, mutationMiner.pl (electronic supplementary material, file S1), which identified high-sequence coverage sites that were consistently heterozygous in all adult replicates from one side of a colony but homozygous in all adult replicates from the other side of the colony. Genotypes of the sperm at the same RAD tags that contained a putative somatic mutation in the adult samples were assessed to determine if the somatic mutation was transferred to gametes. We also searched for sites that were heterozygous in all sperm samples from one side of the colony but were homozygous in sperm samples from the opposite side of the colony to determine if there were any germline-specific mutations. To exclude the possibility of chimerism within colonies, the same method for identifying somatic mutations was used to identify sites that differed between the two genetically unrelated coral individuals.

(d) Assessing false discovery rate
To ensure that the discovered somatic mutations are not the result of genotyping errors (i.e. allelic dropout), we identified variants that were consistently genotyped when the adult replicate samples were paired incorrectly. Thus, we made groups of replicates in which we paired all possible combinations of two adult replicates from one side of a colony with two adult replicates from the other side of the same colony, and identified SNPs that were present in one group of replicates but not the other. If any of the identified SNPs had high-read depth counts (more than 10 reads per RAD tag), this would indicate the potential for false positives among our set of somatic mutations, since we expect only true somatic mutations to be identified consistently and at high-read depths. Conversely, if the identified SNPs have consistently low-read depth counts (less than 10 reads per RAD tag), the somatic mutations are very unlikely to be artefacts.

(e) Validating somatic mutations
For further validation of the candidate somatic mutations, we used the draft genome of *O. faveolata* (M. Medina 2014, personal communication) to design primers for PCR amplification and Sanger sequencing of 300 bp regions surrounding the mutations. Because the *O. faveolata* genome is not yet complete, only seven of the nine RAD tags containing a somatic mutation could be identified in the genome. A double peak in the sequence chromatograms at the appropriate base pair position was taken as evidence for a true somatic mutation rather than an artefact produced during RAD library preparation or data analysis. Furthermore, we performed BLAST searches against the NCBI nucleotide collection database to rule out the possibility that the somatic mutations were sequenced from symbionts or bacterial communities associated with the colonies.

(f) Frequency of somatic mutations
The frequency of somatic mutations was determined by dividing the number of discovered somatic mutations by the total number of bases genotyped. The base pair value genotyped in a diploid organism is twice the product of the length of each RAD tag minus the length of the recognition site (6 bp), since the site itself cannot be variable, and the total number of RAD tags sequenced (2 × 30 bp × no. RAD loci), including both homozygous and polymorphic tags. Estimates of the number of somatic mutations that have accumulated over the lifetime of an individual polyp lineage within one side of a
colony, from zygote formation to adulthood, are half the product of the mutational frequencies and the genome size (estimated at 800 Mb; M. Medina 2014, personal communication).

### 3. Results

**a) Somatic mutations within *Orbicella faveolata***

In order to ensure accurate identification of true somatic mutations, multiple technical replicates were generated for each adult and sperm tissue sample, and only SNPs that could be consistently genotyped in all four replicates of the adult tissue samples from one side of a colony but not the other were considered as candidate somatic mutations (electronic supplementary material, figure S1). In the two *O. faveolata* colonies sequenced (colony A and colony B), a total of nine somatic mutations were identified, including four mutations within colony A and five mutations within colony B (figure 2; electronic supplementary material, S1). Although some somatic mutations had low read depths (less than five sequence reads) (figure 2d/), we still considered these in our dataset, as these mutations probably arose later in the development of the colony and thus have not yet reached an appreciable frequency in somatic cells. Furthermore, these low-frequency somatic mutations were successfully confirmed with Sanger sequencing, in addition to the other five that could be located within the draft genome of *O. faveolata* (electronic supplementary material, figure S3). Identification of somatic mutations is improved at high read depths, which explains why a majority (seven out of nine) of the identified mutations are present at frequencies greater than 25%. However, no somatic mutation exceeded an average allele frequency of 50%, which is the expectation for somatic mutations in diploid organisms. Furthermore, BLAST searches against the NCBI nucleotide collection database resulted in no significant hits to any of the 36 bp RAD tags containing a somatic mutation (electronic supplementary material, table S5). Also, since somatic mutations appear in RAD tags that are shared by adult and sperm samples, it is unlikely that these variants are due to adult-specific DNA contaminations, such as bacteria or symbiotic algae. Chimerism can be excluded as a possible explanation for the mutational differences within colonies, as the number of sites that distinguish two genetically unrelated individuals that possibly formed the chimera is dramatically greater than the number of somatic mutations identified. Indeed, depending on the restriction enzyme used (BcgI or Alf), the number of sites in which one individual is heterozygous and the other is homozygous (same criteria used to identify somatic mutations) is 3398 and 3425 sites, respectively.

To confirm that these somatic mutational patterns are not false positives, we identified all heterozygous sites (SNPs) that were specific to one group of incorrectly paired adult replicates and not the other. In other words, we made pairings of replicates from the left side of one colony with replicates from the right side of the same colony and searched for SNPs that were genotyped in only one set of pairings. This test was completed for all possible incorrect combinations of replicates. In all cases in which we identified such SNPs, the mean read depth of the minor allele did not exceed 7, whereas the mean read depth of the putative set of somatic mutations is 26, nearly four times greater. This indicates that we are unlikely to identify SNPs that are genotyped consistently and at a high mean read depth due to chance or random errors, such as allelic dropout (figure 3).

The somatic mutational frequency was similar for colony A and colony B, as well as the restriction enzymes used for library preparation (either BcgI or Alf), and was on the order of $3.89 \times 10^{-7}$ to $6.03 \times 10^{-7}$ mutations per base pair (table 1). The estimated genome size of *O. faveolata* is 800 Mbp (M. Medina 2014, personal communication). The total number of somatic mutations per polyp lineage is in the range of 300–500, estimated as the product of the mutation rate and genome size. Precise somatic mutation rates could not be calculated due to uncertainty of the ages of the coral colonies. However, based on the most accurate estimates of growth rates for *Orbicella* species (approx. 1 cm per year) [33,34], the *O. faveolata* colonies that we sampled are approximately 200–400 years old. Somatic mutation rates should therefore be between $2.0 \times 10^{-9}$ and $6.0 \times 10^{-9}$ mutations per year.

**b) Somatic mutations in soma and gametes**

None of the nine mutations identified in the somatic samples could be identified in the gametes (sperm) produced by the same coral fragment, despite consistently high read depth counts for most of the sperm replicates at these loci (figure 2). Since the sperm was only collected from polyps that were within the same fragment that was sampled for adult tissue, it is very unlikely that lack of identification of the mutations in the sperm was due to dilution with sperm genuinely lacking such mutations (figure 1b). Additionally, no sperm-specific (germline) mutations were identified.

### 4. Discussion

**a) Patterns of inheritance of somatic mutations point to existence of a GSC lineage in corals**

We sought evidence that the anthozoan cnidarian *O. faveolata* possesses an independent GSC lineage in order to better
Figure 2. Read depth of major alleles (white bars) and somatic variants (black bars) in somatic and gamete samples from (a–d) colony A and (e–i) colony B. Left and right indicate the sides of the colony. Each quadrant in plots (a–i) shows read depth values of four replicates. Missing bars indicate the replicate was not sequenced or the genotype information is missing for that particular RAD tag. (a) Locus 2840, (b) locus 5591, (c) locus 10 380, (d) locus 1315, (e) locus 4475, (f) locus 9012, (g) locus 24 667, (h) locus 27 644 and (i) locus 1248.
Figure 3. Distribution of read depths of the true set of somatic mutations (n = 31) and read counts of minor alleles (false somatic mutations, n = 72) that differed between somatic samples paired incorrectly. Red ticks indicate the average read depth of the somatic mutations at the two loci (10380 and 1248, respectively) that were not found in the draft genome and therefore could not be validated by Sanger sequencing. (Online version in colour.)

understand the evolutionary origins of germ cell developmental modes in Metazoa. We chose to study the Caribbean coral *O. faveolata* because it can grow to massive sizes, potentially accumulating numerous somatic mutations. We identified nine SNPs that are present at moderate to high frequencies across all replicates on one side of a colony, but are absent on the other side. However, we did not find these variants in the gametes, which indicates that *O. faveolata* possesses a GSC lineage that functions independently of an MPSC lineage [2,3]. A somatic mutation that is near its maximum possible frequency (50%) should be present in the majority of somatic stem cells. Thus, if germ cells are differentiated from this pool of somatic stem cells (continuous germ cell specification), there is a high probability that the mutation will be present in gametes. A segregated GSC lineage, however, can prevent incorporation of these high-frequency somatic mutations into gametes.

The conclusion concerning the presence of a GSC lineage rests on the fact that in our experiment it was extremely unlikely to overlook transmission of these mutations to gametes. For each gamete sample, we performed four sequencing trials, each with an average heterozygote discovery rate (fraction of heterozygous sites with two alleles correctly identified) exceeding 95% (table 1). Thus, the probability of missing a heterozygote, or missing a somatic mutation transmitted to gametes, was less than 5% for all sequencing trials. Hence, the probability of overlooking the transmission of all nine mutations was less than $0.05^9 \approx 1.4 \times 10^{-47}$. Conservatively, even if the heterozygote discovery rate were as low as 0.5, since somatic mutations are typically under-represented as alternative alleles, the probability of missing all nine mutations in all sequencing trials still remains nearly zero, $1.5 \times 10^{-11}$. Thus, we can be quite confident that the cell lineage producing the gametes does not contain somatic mutations. Furthermore, we found no gamete-specific (germ-line) mutations, indicating that the GSC mutation rate is at least an order of magnitude less than the somatic mutation rate in *O. faveolata*. This result is expected, as GSCs should only divide during polyp fission and once a year during gametogenesis.

Although we only identified a total of nine somatic mutations in the two adult colonies, scaling the mutational frequency against the size of the genome in *O. faveolata* demonstrates that the total number of somatic mutations within an adult coral colony is considerable. On the order of 300–500 new mutations can accumulate in a polyp lineage over the lifetime of a colony. Furthermore, this estimate is likely to be a substantial underestimate of the true number of somatic mutations, since our method can only reliably detect moderate to high-frequency mutations that therefore must have appeared early in the lifetime of the colony. The mean is 26, which means that, on average, we can only detect mutations that occur at frequencies greater than 4%. Thus, somatic mutations do have the potential to generate substantial intracolonial genetic diversity in corals. This has also been recently demonstrated by Schweinsberg et al. for *Acropora*, *Pocillopora* and *Porites* coral species [35].

A recent review article examining the capacity for somatic mutations to generate genetic diversity in corals proposes that as many as 100 million mutations can arise in a small colony (30 cm). Furthermore, the authors contend that these mutations may serve as a key source of genetic variation necessary for adaptation to rising ocean temperatures, one of the most prevalent stressors contributing to coral decline worldwide [31]. While we recognize somatic mutations as a source of genetic diversity within coral colonies, our results demonstrate that this type of variation is not heritable, at least not in *O. faveolata*. Asexual fragmentation [36], however, could act as a mechanism to propagate somatic genetic variation in *O. faveolata*.

The possibility of heritable somatic variation, however, has been suggested by another recent article in which adults and gametes of mature colonies of the acroporid coral *Acropora hyacinthus* were genotyped using microsatellite markers. In four colonies, the authors found novel alleles specific to the gametes. A few of the variants identified could be considered potential somatic mutations, both of which were found in the parental colony and gametes [37]. However, these results are not inconsistent with our findings. In the event that GSCs are destroyed, as could happen due to natural fragmentation or predation of *A. hyacinthus* colonies, MPSCs can replace GSCs, and thus any mutations carried within the somatic stem cell lineage will become part of the germ line. In fact, both *Hydra* and planaria can regenerate GSCs from toti/multipotent somatic stem cells [38]. The ability to regenerate GSCs should be an especially important trait for organisms capable of asexual propagation, as missegregation of GSCs during budding would inhibit the sexual reproductive capabilities of the asexual clone. Additionally, the colony morphology of *O. faveolata* makes it much less prone to fragmentation or predation. Thus, regeneration of GSCs may not occur as often, potentially leading to lower germline mutation rates in *O. faveolata* compared with corals with branching morphologies. Ultimately, this suggests that GSCs may not form an entirely independent cell lineage, as they might be regenerated by somatic stem cells under certain circumstances. Alternatively, it is possible that there are different mechanisms for germ cell production in divergent coral species. However, this would be a significant developmental modification in closely related organisms (within the same order, Scleractinia).

(b) Evolutionary origins of the germ stem cell lineage

One of the prevailing hypotheses on the evolutionary advantage of germ–soma segregation is that it protects the multicellular organism from selection among competing
somatic cell lineages, which proliferate at the expense of the individual [1]. Segregation of germ and somatic cell functions becomes even more critical as an organism increases in size, since an increased number of cell divisions will lead to greater mutation accumulation [12,39]. However, in the basal metazoan phylum Porifera, germ cells as well as other somatic cell types are differentiated from archeocytes or choanocytes [40,41], and thus there is less distinction between cells committed to reproductive and somatic functions (i.e. no GSC lineage). Our results, presented here for the anthozoan cnidarian O. faveolata, as well as previous work in hydrozoans, suggest that a GSC system may be the ancestral mode for partitioning somatic and germ cell functions in Eumetazoa [21–23] (figure 4). Still, a possibility remains that GSC partitioning has been acquired within some cnidarian lineages (including O. faveolata studied here, as well as Hydra [21,22]) independently. To fully establish basal origin of GSCs in Eumetazoa, more studies of potential GSC lineages in non-bilaterian Eumetazoa are required, including more cnidarian taxa, Placozoa and Ctenophora. Nevertheless, regardless of how many times a GSC lineage has evolved, our study shows that it has happened earlier in metazoan evolutionary history than previously thought.

Our results also suggest that that an independent GSC lineage may not have been a necessary precursor to the evolution of multicellularity in Metazoa. However, the emergence of a GSC lineage in Eumetazoa may have been a concomitant developmental change associated with the evolution of greater complexity in tissue organization compared with Porifera. In order to segregate GSCs from MPSCs, there must be mechanisms to not only restrict cell function but also to restrict the spatial location of these cells through regulation of a local cellular microenvironment around the stem cells [42]. Acquisition of this enhanced regulatory capacity in the common ancestor of cnidarians and bilaterians may underlie the ability to segregate a GSC lineage, as well as the ability to organize tissue layers composed of differentiated cell types. Studies on the stem cell system of Ctenophora, which are non-bilaterian metazoans, also support the view that ancestral animal stem cells may have possessed greater functional specificity and spatial restriction than previously recognized [19]. In contrast, cell fate in porifers is much more labile and less stable, since cells can migrate between cell layers and transdifferentiate into other cell types [43].

Our findings also challenge the notion put forth by Buss [2] that asexuality and germline segregation are incompatible developmental states [2]. Asexual development requires stem cells to retain differentiation capacity indefinitely, as somatic and germ cells must constantly be replenished throughout the lifetime of an individual. This constant cellular renewal may explain why interstitial stem cells are indistinguishable from GSCs based on morphological and gene expression signatures in Hydra [20,44,45]. In scleractinian corals, which bud new polyps continuously throughout their lifetime, GSCs need to divide to be supplied to the newly added polyps. In fact, maintenance of GSCs in a mitotically active state could be a derived mode of GSC function in colonial metazoans, since bilterian GSCs are normally mitotically quiescent. However, there is no reason to expect asexuality to preclude the existence of a GSC lineage, as it is well established that multiple independent stem cell lineages are maintained within the asexually budding Hydra [21].

**Ethics.** Coral samples were collected under the Flower Garden Banks National Marine Sanctuary permit no. FGBNMS-2012-02. **Data accessibility.** DNA sequences are available from GenBank under accession nos. SAMN04027586-SAMN04027642. **Authors’ contributions.** M.V.M. designed the study, conducted sampling and contributed to bioinformatic analyses. S.B. performed molecular work, contributed to bioinformatic analyses and wrote the first draft of the manuscript. G.V.A. performed molecular work. M.V.M. and S.B. contributed to revisions. All authors gave final approval for publication. **Competing interests.** The authors declare no competing interests. **Funding.** This study was supported by funding from the National Science Foundation DEB-1054766 to M.V.M. **Acknowledgements.** We thank Eli Meyer for his help in collecting samples from the Flower Garden Banks National Marine Sanctuary.