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Phylogeny, migration and geographic range size evolution of *Anax* dragonflies (Anisoptera: Aeshnidae)

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The genus *Anax* is a group of cosmopolitan dragonflies noted for its conspicuous migratory behaviours and large size. Here we present the first dated, species-level, multigene, molecular phylogeny for the group to test generic and species-limits, as well as the evolution of migration and range size. Using five mitochondrial and nuclear gene regions (*COI*, *COI*/*COII*, *CYTB*/*ND1*, ITS1 and *PRMT*) from 20 species, we reconstructed a phylogeny of *Anax* using both a Bayesian and maximum likelihood approach. We found that *Anax* (including its hypothesized sister group *Hemianax*) forms a monophyletic group, and that 12 out of 20 species tested positive for monophyly were also monophyletic. The monophyly of several species of *Anax* is less clear. Migratory behaviour, which is known to occur in at least nine species, is recovered as the ancestral behaviour, which was lost and subsequently gained at least three times. Geographic range size seems to be tightly associated with migratory behaviour.

ADDITIONAL KEYWORDS: Anactini – ancestral trait reconstruction – invertebrates – Odonata.

INTRODUCTION

Anax Leach, 1815 is a cosmopolitan genus of dragonfly with species that are used as model organisms for a variety of ecological, behavioural and physiological studies due to their large size and conspicuous behaviour (Folsom & Collins, 1982; Corbet, 1999; Stav *et al.*, 2000; Freeland *et al.*, 2003; May & Matthews, 2008; Crumrine, 2010; Martens *et al.*, 2012; Bybee *et al.*, 2016; Sharkey *et al.*, 2015; May *et al.*, 2017). However, despite the use of *Anax* in both evolutionary and ecological studies, relationships at the species level, including those species previously placed in the sister group *Hemianax* Selys, 1883, remain poorly understood. Little phylogenetic research, outside of understanding the placement of the genus within the Aeshnidae, has been done for *Anax* (Von Ellenrieder, 2002; Dijkstra & Kalkman, 2012). Further, a phylogeny for the genus *Anax* is essential for testing phylogenetic hypotheses about trait evolution and historical biogeography.

One behavioural trait that several species of *Anax* (including *Hemianax* after Paulson & Schorr, 2020) are known to exhibit is migratory behaviour. Many studies have used phylogenies to examine the evolution of migration in vertebrate groups (Chesser & Levey, 1998; Nagy & Tökölyi, 2014); however, fewer studies have considered the evolution of migration in invertebrates (Dingle, 2006; Roff & Fairbairn, 2007; Chapman *et al.*, 2015). Within insects, this migratory syndrome has

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evolved multiple times across at least ten insect orders including in butterflies, hoverflies and dragonflies (Satterfield *et al.*, 2020). Insect migrants face unique challenges compared to vertebrate migrators; shorter lifespans mean that an entire migratory journey may not be completed in one generation, as showcased by the popular example of the monarch butterfly, which can take four generations to complete a migratory cycle (Batalden *et al.*, 2014). Small body size exposes migrating invertebrates to predation or leaves them vulnerable to being carried away by the wind or current (Roff & Fairbairn, 2007). Despite these constraints, migration in insects rivals or outweighs vertebrate migration in the number of species that migrate (thousands), and the number of migrating individuals (1×10^{15} globally, 4–6 billion in a swarm in the darner *Aeshna bonariensis* Rambur, 1842) (Holland *et al.*, 2006; Satterfield *et al.*, 2020). In fact, the farthest-flying insect species, the globe skimmer dragonfly (*Pantala flavescens* Fabricius, 1798) completes a migratory flight of 14 000–18 000 km (Troast *et al.*, 2016), which is comparable to the flight of the farthest bird migrator, the arctic tern *Sterna paradisaea* Pontoppidan, 1763, with a one-way distance of 20 000 km (Alerstam *et al.*, 2003). If the migration distance for *S. paradisaea* and *P. flavescens* is scaled by body size or wingspan, roughly a tenfold difference exists, and compared, *P. flavescens* is the furthest-travelling animal migrant known on earth by a large margin.

Migration within the Odonata is defined as an actively initiated one-way flight, which continues until arrival, with or without navigational cues and results in a non-random redistribution between generations of a species (Corbet, 1999). May (2013) expands this definition using the behavioural criterion of reduced responsiveness to external stimuli (Kennedy, 1985). Using these criteria, nine of the 32 species in *Anax* are considered "migratory" (Table 1) (Corbet, 1999), the most well-known being the North American common green darner *Anax junius* (Drury, 1773) (Fig. 1A-C). Each spring, groups of *A. junius* migrate from the southern USA and Mexico ~650 km north to as far as Canada before laying their eggs (Hallworth *et al.*, 2018). In autumn, masses of the next generation of *A. junius* are reported flying south where their offspring overwinter before laying the eggs of the next generation that will return north in the spring (May, 2013; Hallworth *et al.*, 2018). Other species of *Anax* follow a similar pattern across the globe. In the Eastern Hemisphere, the vagrant emperor, *Anax ephippiger* (Burmeister, 1839) (Fig. 1W) is a predominant migrator that breeds in sub-Saharan Africa, the Middle East and south-west Asia, but commonly migrates in swarms across Morocco to parts of mainland Europe and Britain, and has appeared as far north as Iceland (Silsby, 1993; Mediani *et al.*, 2012). *Anax imperator* Leach in Brewster, 1815 (Fig. 1F-G) has a similar distribution, with sub-Saharan populations that may migrate, but more work is needed to confirm this observation (Corbet, 1999). *A. ephippiger*, *Anax tristis* Hagen, 1867 and *Anax guttatus* (Burmeister, 1839) (Fig. 1W, K, L, respectively) have been observed making transoceanic journeys across the Indian Ocean (Anderson, 2009; Hedlund *et al.*, 2020). Evidence suggests that one Central/South American species, *Anax amazili* (Burmeister, 1839) (Fig. 1S), with a range that extends from Texas, USA, to Uruguay (Dalzochio *et al.*, 2012) may make a similar migratory journey across the Southern Atlantic (Alves *et al.*, 2019). Finally, *Anax parthenope* (Selys, 1839) (Fig. 1H) makes migratory journeys across the Tian Shan Mountains in China (Borisov, 2009), and *A. gibbosulus* Rambur, 1842 (Fig. 1M) and *Anax papuensis* (Burmeister, 1839) (Fig. 1V) exhibit migratory syndromes in the Australian and South Pacific regions (Watson & Theischinger, 1984; Grand *et al.*, 2019).

Along with differences in migratory behaviour, species of *Anax* exhibit variation in size of geographic range. Some species of *Anax* have a multi-continent distribution [e.g. *A. junius*, with a range that extends across North America, Central America, the West Indies, Hawaii, Tahiti and China (Corbet, 1999)], whereas the ranges of other species are limited to a small area (e.g. *Anax strenuus* Hagen, 1867, a Hawaiian endemic, Fig. 1D). Migration may play a role in geographic range size, with migratory species likely having larger geographic ranges due to individuals travelling, and occasionally residing, in the more extreme boundaries of their habitable ranges (Freeland *et al.*, 2003). However, many geographic and environmental barriers also affect the size of dragonfly species ranges (Brown *et al.*, 1996) including mountains, bodies of inhabitable water (Hof *et al.*, 2006; Kalkman *et al.*, 2007), storm prone regions (Kalkman & Orr, 2012) and climate (i.e. temperature and precipitation) (Hickling *et al.*, 2006; Kalkman *et al.*, 2007). Additionally, changes in geographic range size are evident as climate change continues to impact animal distributions around the world (Webb & Gaston, 2000; Raffard *et al.*, 2020). It is also possible that geographic range size may be phylogenetically heritable (traits that influence geographic range size are heritable, and the history of those traits can be understood in a phylogenetic context) and therefore constrained by the evolutionary history of each species (Patiño *et al.*, 2017; Krasnov *et al.*, 2018; Suhonen *et al.*, 2019).

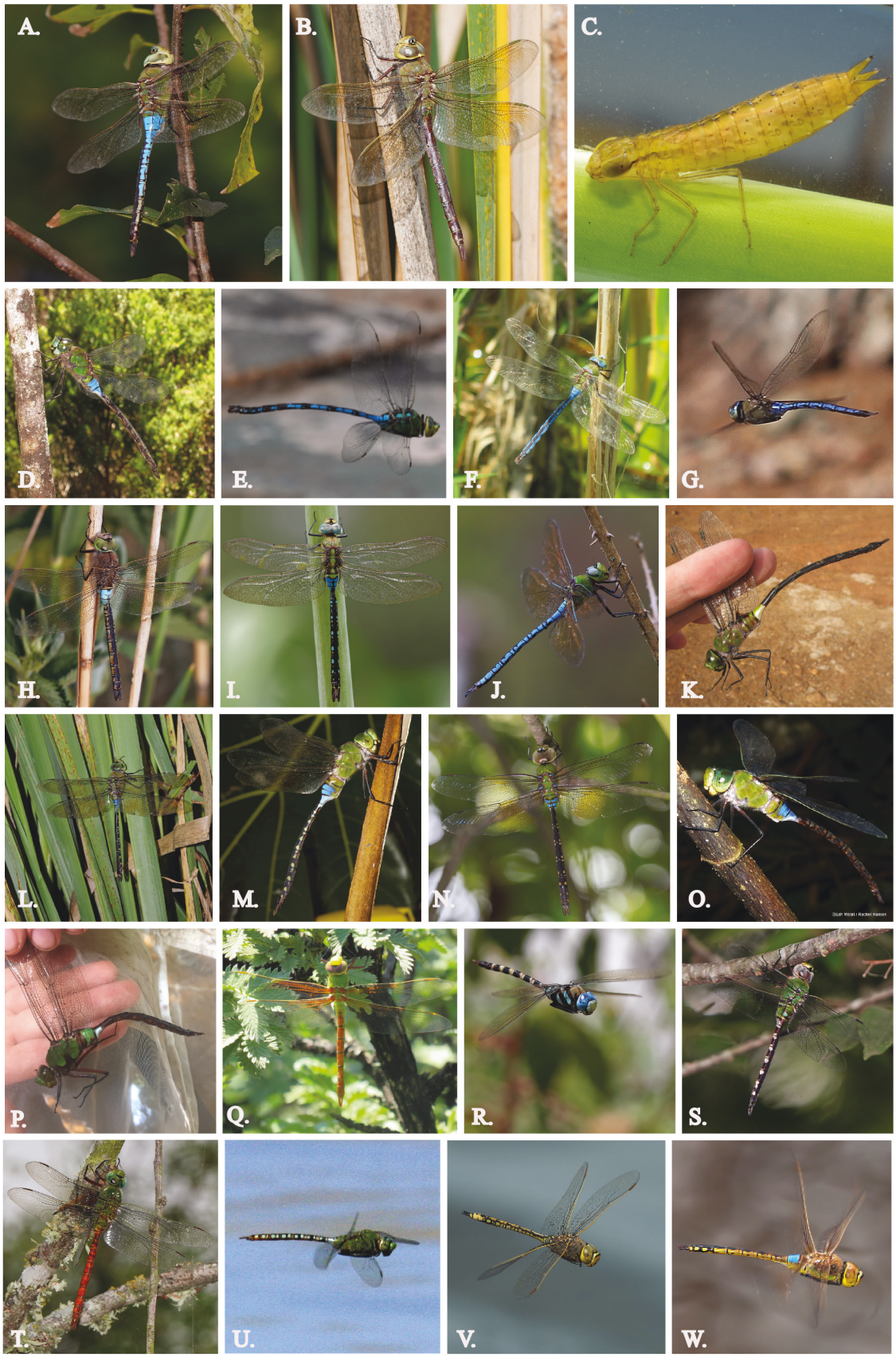
Evidence for phylogenetic heritability of traits influencing range size is seen in closely related groups that often share similar range sizes (Jablonski, 1987). However, the claim that geographic range size is heritable at the species level is debated, perhaps in part due to its link to group selection, with many maintaining that most geographic range sizes are more easily explained by looking at individual species (Webb & Gaston, 2003,

Table 1. Distribution, record of migratory behaviour and range size of currently recognized species of *Anax* (Paulson & Schorr, 2020)

Species	Distribution	Evidence of migratory behaviour	Range size (km^2)
Anax amazili (Burmeister, 1839)	North and South America	Dunkle, 1989; Corbet, 1999	165 000
Anax bangweuluensis Kimmins, 1955*	Africa	–†	5000
Anax chloromelas Ris, 1911*	Africa	–	5000
Anax concolor Brauer, 1865	North and South America	–	40 000
Anax congoliath Fraser, 1953	Africa	–	17 500
Anax dubius Lacroix, 1921*	Korea	–	n/a
Anax ephippiger (Burmeister, 1839)	Europe and Africa	Corbet, 1984; Parr, 1997; Corbet, 1999; Anderson, 2009; Borisov, 2009	700 000
Anax fumosus Hagen, 1867*	Sulawesi, Solomon Islands	–	5000
Anax georgius Selys, 1872	Timor, Western Australia	–	7500
Anax gibbosulus Rambur, 1842	Asia, south-west Pacific	–	90 000
Anax gladiator Dijkstra & Kipping, 2015*	Africa	–	7500
Anax guttatus (Burmeister, 1839)	Oceania, Asia	Corbet, 1999; Anderson, 2009	230 000
Anax immaculifrons Rambur, 1842*	Asia	–	92 500
Anax imperator Leach in Brewster, 1815	Europe, Africa	Parr, 1997; Corbet, 1999	782 500
Anax indicus Lieftinck, 1942*	Asia	–	50 000
Anax junius Drury, 1773	North America	Corbet, 1999; Freeland *et al.*, 2003; May & Matthews, 2008; May, 2013	855 000
Anax longipes Hagen, 1861	North and South America	–	392 500
Anax maclachlani Förster, 1898*	Indonesia	–	25 000
Anax mandrakae Gauthier, 1988*	Madagascar	–	n/a
Anax nigrofasciatus Oguma, 1915	Asia	–	120 000
Anax panybeus Hagen, 1867	Southeast Asia	–	37 500
Anax papuensis (Burmeister, 1839)	Australia	Rowe, 1987; Corbet, 1999	675 000
Anax parthenope (Selys, 1839)	Asia	Parr, 1997; Corbet, 1999; Borisov, 2009	687 500
Anax piraticus Kennedy, 1934	Guam	–	2500
Anax pugnax Lieftinck, 1942*	Asia	–	2500
Anax rutherfordi McLachlan, 1883*	Africa	–	5000
Anax selysii Förster, 1900*	Indonesia	–	5000
Anax speratus Hagen, 1867	Africa	–	155 000
Anax strenuus Hagen, 1867	Hawaii	–	35 000
Anax tristis Hagen, 1867	Africa	Gambles, 1960; Corbet, 1999	85 000
Anax tumorifer McLachlan, 1885	Madagascar	–	20 000
Anax walsinghami McLachlan, 1883	North America	–	142 500

*Not included in the current study.†Where no evidence of migratory behaviour is available, we indicate this with a dash.

2005; Hunt *et al.*, 2005; Waldron, 2007; Borregaard *et al.*, 2012). Geographic range size is affected by a variety of factors including historical geographic barriers and climate. Observing the range size of *Anax* dragonflies in a phylogenetic context, due to the many similar traits closely related species share, provides insight into the history of this group, as long as limitations are recognized.



In addition to enabling study of migration and range size evolution, a robust phylogeny for this group is necessary to address taxonomic issues in odonate systematics, especially within the Anactini tribe. One of the foremost challenges in European dragonfly phylogenetics is the disputed generic identity of *Anax ephippiger* and *Anax papuensis* (Fig. 1V-W, formerly part of the genus *Hemianax*) (Dijkstra & Kalkman, 2012). Some researchers argue that *Hemianax* should be sunk into *Anax* (Gentilini & Peters, 1993) whereas others insist that distinct characters distinguish it as a separate genus (Von Ellenrieder, 2002). The most recent phylogenies of the Anisoptera identify the Anactini tribe as a distinct clade within the Aeshnidae, but do not have the taxon sampling required to fully resolve the *Anax*/*Hemianax* debate (Carle *et al.*, 2015; Letsch *et al.*, 2016).

Several members of the genus also have disputed species limits. For example, Seehausen (2017) suggested that the species *Anax panybeus* Hagen, 1867 and *A. gibbosulus* could be conspecific, noting that one problem lies in the original descriptions, which lack specific diagnostic features needed to make confident identifications between either species. Another potential taxonomic problem is with *Anax concolor* Brauer, 1865 and *Anax longipes* Hagen, 1867, which are currently both valid species. In the past, Hagen (1890) listed *A. concolor* as a synonym for *A. longipes*. The two species are similar in colour (with a green thorax and dark red-brown to bright red abdomen) and have a characteristic, unmarked frons (Geijskes, 1968). However, *A. concolor* differs from *A. longipes* by dark stripes along the dorsal and lateral carinae as well as the presence of lighter spots that range from yellowish to bright blue (Fig. 1U). On the other hand, mature adult *A. longipes* are solid red after the 3rd segment of the abdomen (Fig. 1T). They also differ by range, with *A. concolor* in South America and *A. longipes* largely in North America. Part of the confusion between these two species stems from the fact that *A. longipes* was originally described based on a female specimen, making taxonomic comparisons between the two species more difficult.

There are also recognized subspecies in this group (e.g. *Anax parthenope julius* Brauer, 1865 and *Anax nigrofasciatus nigrolineatus* Fraser, 1935), which some researchers have suggested may be distinct from the nominal species (Sahito *et al.*, 2017). The species boundaries here are ambiguous as Tennessen (1982) recorded hybrid individuals between *Anax nigrofasciatus* Oguma, 1915, *A. parthenope* and *A. imperator*. Furthermore, recent barcode analyses were unable to differentiate between individuals of *A. imperator* and *A. parthenope* (Galimberti *et al.*, 2020; Rewicz *et al.*, 2021). A robust phylogeny is necessary to begin to resolve taxonomic issues in this group for the study of their traits in an evolutionary context.

Here we present the first dated, multigene, molecular analysis of *Anax* to test the monophyly of the genus (including the former *Hemianax*) and species limits. We use this analysis to look at the evolution of migration in this group, including how many times migration evolved, when migration originated and if there is a correlation with migration and geographic range size. The results of this study provide a reference for future work on this genus from both an evolutionary and taxonomic standpoint.

MATERIAL AND METHODS

Taxon sampling

We acquired 127 specimens from 33 countries from the Florida State Collection of Arthropods, the Brigham Young University (BYU) cryo-collection and personal collections (Supporting Information, Table S1). These specimens included 20 species of *Anax* and ten outgroup taxa. All specimens were authoritatively identified and verified in the Bybee laboratory at BYU before extractions. We extracted DNA from the specimens using a Qiagen DNeasy extraction kit following a

Figure 1. Species of *Anax*. A, *A. junius* (common green darner) male, Brownsville, Texas, USA. Photo by Benjamin Schwarz. B, *A. junius* (common green darner) female, San Marcos, Texas, USA. Photo by Benjamin Schwarz. C, *A. junius* (common green darner) nymph, Utah, USA. Photo by C. Riley Nelson. D, *A. strenuus* (giant Hawaiian darner, pinao), Hawaii, USA. Photo by Karl Magnacca. E, *A. walsinghami* (giant darner), Presidio Co., Texas, USA. Photo by Benjamin Schwarz. F, *A. imperator* (blue emperor), South Africa. Photo by Andrew Kruger. G, *A. imperator,* variant with brown thorax, Socotra, Yemen. Photo by Robert Ketelaar. H, *A. parthenope* (lesser emperor), the Netherlands. Photo by Antoine van der Heijden. I, *A. nigrofasciatus* (blue-spotted emperor), Nepal. Photo by Antoin van der Heijden. J, *A. tumorifer* (Madagascar emperor), Madagascar. Photo by Erland Refling Nielsen. K, *A. tristis* (black emperor), Grand Gedeh Co. Liberia. Photo by KD Dijkstra. L, *A. guttatus* (pale-spotted emperor), New Caledonia. Photo by Daniel Grand. M, *A. gibbosulus* (green emperor), Maupiti. Photo by C. Riley Nelson. N, *A. panybeus* (arrow emperor), Singapore. Photo by Marcus FC Ng. O, *A. piraticus*, Guam. Photo by Elijah Wostl. P, *A. congoliath* (dark emperor), Cameroon. Photo by Rebecca Clement. Q, *A. speratus* (orange emperor), South Africa. Photo by Andrew Kruger. R, *A. immaculifrons* (magnificent emperor), Nepal. Photo by Antoine van der Heijden. S, *A. amazili* (Amazon darner), Lockhart, Texas. Photo by Benjamin Schwarz. T, *A. longipes* (comet darner), Hays Co., Texas. Photo by Benjamin Schwarz. U, *A. concolor* (blue-spotted comet darner), Santa Ana, Texas. Photo by Martin Reid. V, *A. papuensis* (Australian emperor), New Zealand. Photo by Michael Ashbee. W, *A. ephippiger* (vagrant emperor), Spain. Photo by Jorge Pérez.

standard protocol for insect extractions, using only the femur on most specimens, as specimens are large. For museum specimens, we soaked the legs overnight in Qiagen buffer solution before performing the extraction. We amplified portions of the mitochondrial genes cytochrome oxidase subunits 1 and 2 (*COI*, *COI/COII*), cytochrome B and NADH dehydrogenase 1 (*CYTB/ND1*) and nuclear regions for internal transcribed spacer 1 (ITS1) and protein arginine methyltransferase (*PRMT*) using polymerase chain reaction (PCR). The primers and amplification conditions are listed in the Supporting Information (Table S2). Samples were sequenced at the BYU Sequencing Center with an ABI3730xl machine and are available on GenBank with the accessions MW810869–MW810955, MW814732–MW814844, MW850754–MW850895, MW756723–MW756841 and MW844047–MW844124 (Supporting Information, Table S1). Resulting sequences were aligned in MAFFT (Katoh & Standley, 2013) using default settings and trimmed and concatenated in Geneious (Biomatters, http://www.geneious.com/).

DATA AVAILABILITY STATEMENT

The data underlying this article are available on GenBank with the accessions MW810869–MW810955, MW814732–MW814844, MW850754–MW850895, MW756723–MW756841 and MW844047–MW844124 (Supporting Information, Table S1). Alignments and trees can be accessed on the Dryad Digital Repository doi:10.5061/dryad.9ghx3ffgx

PHYLOGENETIC ANALYSES

We reconstructed trees using maximum likelihood (ML) and Bayesian methods. First, we ran ML analyses on the IQ-TREE web server (Trifinopoulos *et al.*, 2016), using both ModelFinder and 1000 ultrafast bootstraps (Kalyaanamoorthy *et al.*, 2017; Hoang *et al.*, 2018). After initial analyses showed partitioning by nucleotide position made little difference in tree or topology, we chose not to partition our data set (Susko & Roger, 2021). We used BEAST v.2.5 (Bouckaert *et al.*, 2019) to simultaneously estimate a Bayesian topology and divergence times. First, BEAUti was used to set analysis parameters including the model of evolution obtained in ModelFinder (GTR+F+I+G4). We applied a relaxed log normal molecular clock and birth-death tree model after running nested sampling analyses to determine the best fit models for our data (Russel *et al.*, 2019) (Supporting Information, Table S3 shows results of nested sampling analysis in BEAST). Relative ages of the clades of *A. imperator* and *H. ephippiger* were dated using an exponential fossil calibration with a hard minimum age (5.33 Mya). Although *A. parthenope* also has a fossil representative we chose not to include it because the species was recovered as paraphyletic in all our ML reconstructions, making fossil placement difficult. The root of our tree was constrained with a hard maximum uniform prior of 139.8 Mya corresponding to the oldest known crown fossil for the Anactini tribe (*Merlax bohemicus* Prokop & Nel, 2000). The resulting file was run in BEAST v.2.6.0 (Bouckaert *et al.*, 2019) with a random starting tree for 50 000 000 generations. Tracer v.1.7.1 (Rambaut *et al.*, 2018) was used to ensure sufficient effective sample size (ESS) values as well as convergence of our trees. Finally, a consensus tree was generated using TreeAnnotator v.1.10.4 (Bouckaert *et al.*, 2014) with a burn-in of 5 000 000 states consistent with the burn-in observed in Tracer.

TRAIT EVOLUTION ANALYSES

We used RASP 4 (Yu *et al.*, 2020) to reconstruct ancestral states on our dated tree. The results of our BEAST consensus tree were trimmed so each well-defined species clade could be represented by a single terminal in order to visualize the results more easily (Fig. 2). Migratory behaviour was coded as a discrete character (i.e. present/absent) based on the *Anax* species listed by Corbet (1999), who also defines this behaviour within the order Odonata. We acknowledge that there are multiple definitions of migration and traits associated with migratory behaviour. Here we follow Corbet's designation, allowing us to clearly identify migratory vs. non-migratory species. A different definition may influence the extent to which a species is migratory (e.g. not all individuals from a species may migrate or migrate far, etc.). Next, we performed a Bayesian Markov chain Monte Carlo (MCMC) analysis, with a limit of a single state per ancestor for a total of 500 000 generations.

To estimate the geographic range size of each species, we downloaded occurrence data for each *Anax* species from the Global Biodiversity Information Facility (www.gbif.org). *Anax piraticus* Kennedy, 1934 was excluded from the analysis because it had only two georeferenced records. These data points were imported into the Geospatial Conservation Assessment Tool (GeoCAT), which generates a Quickhull algorithm based on occurrence points in order to estimate the Area of Occupancy (AOO) and Extent of Occurrence (EOO) (Bachman *et al.*, 2011). We used a modified version of the AOO such that each data point encompassed a 50 km diameter. Three data points were removed because they were clear outliers (potential misidentification or vagrants) that vastly expanded the range of species. These included data points that were found on distant continents outside the normal known range of a species, as well as points found in the middle of oceans not near known ranges. Although this conservative approach may underestimate the geographic range size of some

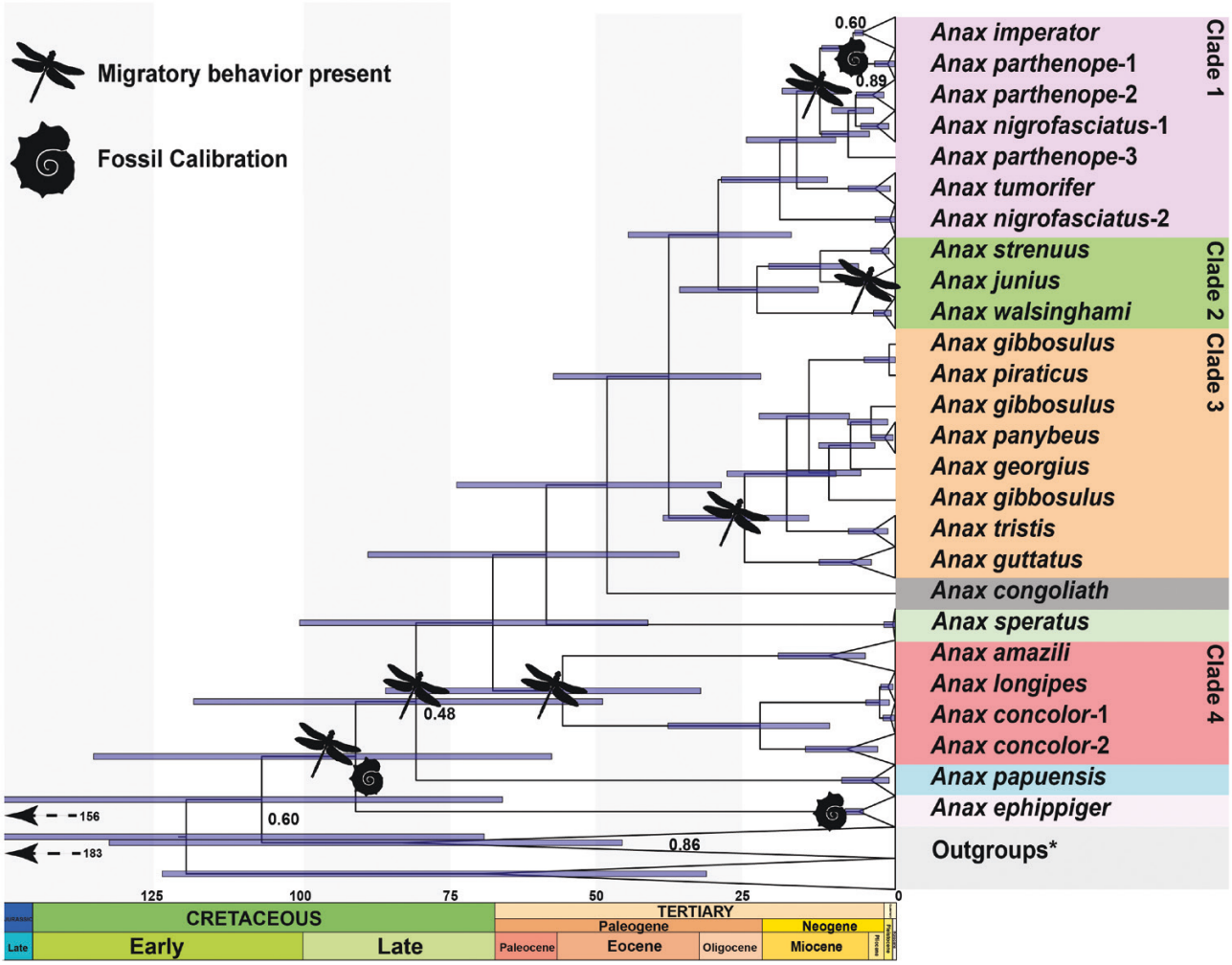


Figure 2. Bayesian time-calibrated tree of five gene regions with placement of fossil taxa. Clades with dragonflies have at least one migratory taxon. Monophyletic species are condensed to show relationships. Posterior Probabilities > 0.90 not shown. *Outgroups include species from *Aeshna*, *Oplonaeschna*, *Anaciaeschna* and *Gynacantha.* See the Supporting Information (Table S1) for more details.

species, it excludes uninhabitable areas that the EOO readily incorporates into its estimate. Furthermore, the prevalence of records for dragonflies in databases such as GBIF suggests that a lack of geographic data is not an issue for most of these species and therefore the underestimating of range in this case should be limited.

We first tested for phylogenetic signal of geographic range size in RASP 4 using Pagel's λ to ensure that the character was phylogenetically informative for this group (Yu *et al.*, 2020). Pagel's λ estimates the phylogenetic signal of a trait on a scale of 0–1, with 0 indicating no phylogenetic signal was detected. We also tested whether there was directional change in this trait by treating it as a continuous character in BayesTraits v.3 using a stepping-stone analysis (Meade & Pagel, 2017). We then uploaded our trimmed tree generated in RASP to RStudio (v.4.0.2) in conjunction with the geographical range size for each species. Next, the geographic range size for each species was transformed using the log function in order to better visualize the large variance in size. We reconstructed the estimated geographic range size as a continuous character using the *phytools* package (Revell, 2012), which uses methods laid out in Felsenstein (1985) to estimate internal nodal states using a ML approach.

RESULTS

The results of our ML and Bayesian analyses were congruent with regard to the species composition in all clades. *Anax+Hemianax* formed a clade with a 0.91 posterior probability (Supporting Information, Figs S1-S2). The former Hemianax species (*A. papuensis* and

A. ephippiger) did not cluster but formed a gradation leading up to all *Anax* species. We recovered 12 of the 20 species as monophyletic, with a posterior probability of > 0.9. *A. parthenope* was recovered as polyphyletic in three clades, largely based on geographical region while *A. nigrofaciatus* also formed a polyphyletic group of two clades (Fig. 2, Clade 1). *A. gibbosulus*, *A. piraticus*, *A. panybeus* and *A. georgius* Selys, 1872 formed a monophyletic group (Fig. 2, Clade 3), but *A. gibbosulus* was rendered polyphyletic by the other three species. Finally, *A. longipes* and *A. concolor* formed a monophyletic group with *A. longipes* embedded in *A. concolor*. The genus *Anax* was estimated to have originated in the Late Cretaceous period (~90 Mya); however, the majority of extant species did not originate until the Late Oligocene or Early Miocene.

Geographical ranges within *Anax* varied widely and were conservatively estimated to range from 7500 km² to 855 000 km². The smallest range of a species included in our phylogeny was that of *A. georgius* which was estimated at 7500 km². *A. junius* had the largest range of 855 000 km². Despite this difference, range size as a continuous character was found to be phylogenetically informative with a Pagel's λ value of 0.97 (P = .316). Using Jeffrey's scale (Meade & Pagel, 2017) there was "substantial evidence" for directional selection of range size, with a Bayes factor of 5.52. The ancestral state reconstruction of geographic range size found a moderately large sized geographic range (~400 000 km²) for the most recent common ancestor of *Anax*. Moderately sized geographic range was also

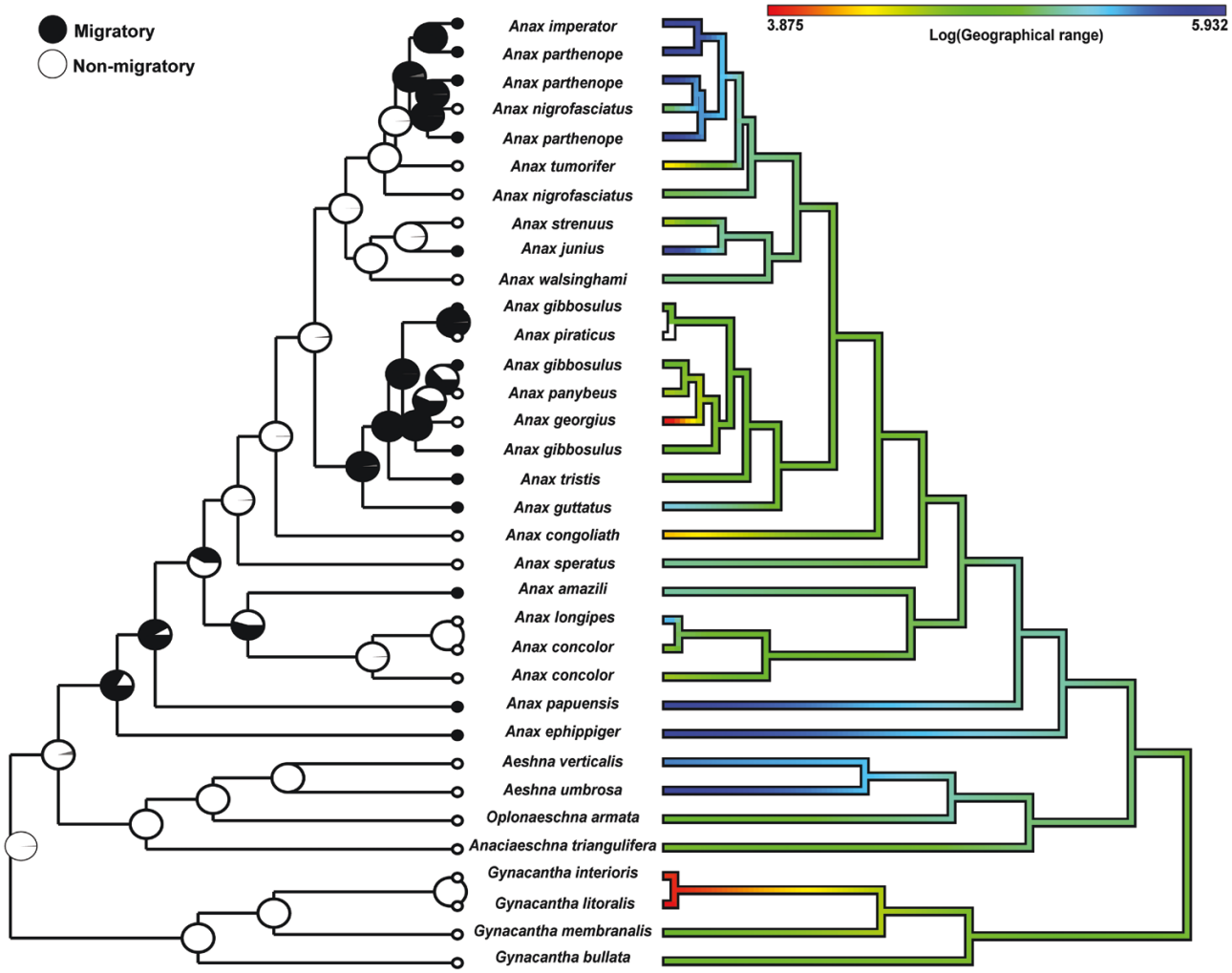


Figure 3. Maximum likelihood ancestral state reconstruction of migratory behaviour and geographical range of *Anax* on a Bayesian tree from five gene regions. The fraction of the circle that is shaded indicates the likelihood that the ancestor was migratory.

recovered along the backbone of the topology, with more extreme ranges at the tips of the topology.

Migratory behaviour was reconstructed as the ancestral condition for *Anax* with five total losses. There were four additional gains of migratory behaviour: in Clade 1, *A. junius* in Clade 2, in Clade 3 and in Clade 4 (Figs 2 and 3). The evolution of migratory behaviour also appeared to be closely associated with a larger geographic range size (Fig. 3). There were several exceptions, including *A. gibbosulus* and *A. tristis*, which have relatively small distributions, and *A. guttatus* and *A. amazili* that have moderately small-sized geographic ranges (Fig. 3).

DISCUSSION

TAXONOMY

There is high support for the tribe Anactini including *Anax* and *Hemianax,* but the paraphyly of *Hemianax* provides additional support for formally synonymizing *Hemianax with Anax.* However, *A. papuensis* was only placed sister to the other *Anax* species with a bootstrap value of 0.48. Improving both genetic and taxonomic sampling would likely provide more confidence for this relationship. The results of our phylogeny confirm several outstanding taxonomic questions, while also highlighting areas that need further investigation.

Furthermore, while the non-monophyly of several species in this genus is apparent, 12 of 20 species within *Anax* were monophyletic in our study. Within *Anax*, we find four well-supported monophyletic species groups. First, Clade 1, a largely Eurasian clade extending into Africa with one Madagascan species, consists of *A. imperator*, *Anax tumorifer* McLachlan, 1885, *A. parthenope* and *A. nigrofasciatus* (Fig. 2). The first two species in this clade, *A. imperator* and *A. tumorifer*, are grouped monophyletically. The remaining two species are not: *A. parthenope* is polyphyletic, with clades largely sorted following geographic region, with south-eastern European and western Asian specimens forming *A. parthenope-1* and specimens collected from Japan forming *A. parthenope-2* and *A. parthenope-3* (Fig. 2). A subspecies of *A. parthenope* (*A. parthenope julius*) is recognized in eastern Asia, including Japan, and has been proposed to be a distinct species in the past (Peters, 1986). Our phylogeny suggests that there is a distinct genetic difference between this subspecies and *A. parthenope*. These results are similar to Rewicz *et al.* (2021) who found difficulty in sorting out barcoding sequences from *A. imperator* and *A. parthenope*, and only found distinct genetic differences in Southeast Asian specimens of *A. parthenope*. However, the East Asian *A. parthenope* are paraphyletic (see *A. parthenope*-2 and *A. parthenope*-3) with respect to *A. nigrofasciatus-1*, thus the story of *Anax* in eastern Asia is likely even more complicated and more work is needed to unravel the species limitations here.

A. nigrofasciatus-2 is sister to the rest of Clade 1. This species also has a known subspecies, *A. nigrofasciatus nigrolineatus* (Kumar, 1973), in addition to the nominate subspecies. We recovered two clades of *A. nigrofasciatus*, rendering this species non-monophyletic. It is unclear whether the current groups correspond to the two subspecies, because all *A. nigrofasciatus* specimens were collected in Japan and none were identified to the subspecies level. Much of the non-monophyly in Clade 1 may be explained by the observation that *A. parthenope* has been known to produce hybrids with both *A. nigrofasciatus* and *A. imperator* (Tennessen, 1982). Sympatric species that interbreed make it difficult to define and identify species, especially when using mitochondrial barcoding genes (Galimberti *et al.*, 2020; Rewicz *et al.*, 2021). More work is needed to understand species-level relationships and potential hybridization here.

Clade 2 includes the North American species *A. junius* and *Anax walsinghami* McLachlan, 1883 as well as the Hawaiian endemic species *A. strenuus.* Although the range of *A. junius* extends into the range of the other two, these three species form distinct subclades. There are populations of *A. junius* in Hawaii that have spatial overlap with *A. strenuus*; however, there is no evidence of hybridization. Local researchers have observed that *A. junius* appears to be largely a lowland species and *A. strenuus* a highland species, although *A. strenuus* appears to have more flexibility in its range as we have caught both species together near sea level (S.M.B. & D.R.B.). The origin of *A. junius* as a species (~5 Mya) is recovered as older than the origin of *A. strenuus* (~3.5 Mya). The present-day Hawaiian Islands are relatively young, with volcanic islands ranging from ~2–5 Mya, although Kure Atoll formed much earlier ~25 Mya, followed by the rest of the currently uninhabited Northwest Hawaiian Islands. The origin of *A. strenuus* corresponds to just after the emergence of the oldest of the main Hawaiian Islands, Kaua‘i that is estimated to be 5.1 Mya (Neall & Trewick, 2008). Within *A. strenuus*, the molecular markers used showed no clear genetic structure between individuals from different islands. The lack of structure likely indicates that there is one large population of *A. strenuus,* and that the stretches of ocean between individual islands is not a sufficient barrier for such strong fliers.

Clade 3 has distributions throughout Africa, East Asia and Oceania, and includes *A. guttatus*, *A. tristis*, *A. gibbosulus*, *A. piraticus*, *A. panybeus* and *A. georgius* (Fig. 2). Although *A. guttatus* and *A. tristis* are monophyletic, the remaining species in Clade 3 (*A. gibbosulus*+*A. piraticus*+*A. panybeus*+*A. georgius*) do not conform to clades. Part of this lack of monophyly could be due to challenges in identifying these species.

For example, *A. gibbosulus* and *A. panybeus* are easily mistaken and misleading morphological characters used in past descriptive keys have led to subsequent misidentifications and confusion in current identification efforts (Hämäläinen & Müller, 1997; Seehausen, 2017). A revisiting of type specimens is likely needed in these cases to guide future taxonomic studies. *A. piraticus* was described from a single male specimen from Guam, but after comparing the species to *A. panybeus* from Sulawesi, the author of *A. piraticus* thought they may be the same species (Kennedy, 1934; Swezey & Williams, 1942). *A. georgius* was also redescribed and re-diagnosed with an additional specimen collected in Australia because of a labelling error (Watson & Theischinger, 1987; Asahina, 1990). Our study suggests that *A. gibbosulus*, *A. piraticus* and *A. panybeus* may be conspecifics. Nevertheless, we are hesitant to include *A. georgius* here at this time as it is represented by fewer genes in our phylogeny.

Finally, a fourth monophyletic grouping within *Anax* is a Western Hemisphere clade that includes *A. concolor*, *A. longipes* and *A. amazili* (Clade 4, Fig. 2). *A. amazili* forms a monophyletic group, but *A. longipes* was recovered within *A. concolor*. The relationship between *A. concolor* and *A. longipes* has been examined several times in the literature with *A. concolor* even being treated as a subspecies of *A. longipes* (Paulson, 1966; Abbott, 2005). Paulson (1966) noted that these species might represent two allopatric forms of the same species. These taxa are morphologically similar and are most easily differentiated based on geography, with *A. longipes* occurring north of Mexico and *A. concolor* found from Mexico to South America (Paulson, 1966). However, the presence of *A. concolor* in Santa Ana National Wildlife Refuge, Texas, further complicates this distinction (Paulson, 2005). Traditionally, delineation and identification of the two species has been difficult, with abdominal colour and size being the main morphological characters used (Paulson, 1966). Geijskes (1968) cited support for two species based on thorough morphological comparison. Our analysis, conversely, suggests that the two actually form one group, which supports Hagen (1890) who considered them conspecific.

The African species *Anax congoliath* Fraser, 1953 and *Anax speratus* Hagen, 1867 did not cluster with any other species in this study, a finding congruent with the low support value that Dijkstra *et al.* (2015) found between clades of *A. speratus*+*A. ruthorfordi* and *A. congoliath*+*A. gladiator* in their *COI* gene tree. Although to date this is the most comprehensive molecular phylogeny of *Anax*, we were unable to obtain DNA for 12 of the 32 species, including several from Africa which were not included in this study due to low gene coverage or lack of access to material (Table 1). To completely resolve the species relationships in *Anax*, deeper taxon sampling is needed.

Several of the divergence time estimates for the four clades above correspond with historical geological events which may have influenced speciation. By the time the Anactini tribe evolved ~90 Mya, the break-up of the Gondwanan supercontinent was already well-underway. This break-up may have played a role in the evolution of lentic groups of dragonflies like *Anax* (Letsch *et al.*, 2016). Clade 4 diverged from the other clades around the time of the Cretaceous-Palaeogene extinction event ~66 Mya (Condamine *et al.*, 2016), and the divergence time estimates for Clades 1, 2 and 3 from each other happened near the time of the Eocene-Oligocene transition cooling event ~33 Mya (Condamine *et al.*, 2016), which was a major cause of extinction.

Migration

The ancestor of *Anax* was a migratory dragonfly and arose in the Early to Late Cretaceous period (~90 Mya). That migratory behaviour was the ancestral state at a time in geological history when Pangaea was nearly 100 Myr into its breakup is interesting. Migratory behaviour may have provided groups like *Anax* and their ancestors the ability to cover vast amounts of geography across continents that were much closer in proximity than they are today. However, looking across the backbone of the phylogeny, migration is quickly lost (Fig. 3). Following this loss, the results of our analysis suggest that there are four major origins of migration (including single species gains in *A. junius* and *A. amazili*). The first gain appears in the ancestor of *A. amazili* at the end of the Cretaceous (~55 Mya). The results of our maximum likelihood (ML) reconstruction suggest that the ancestor of *A. amazili* and *A. concolor*+*A. longipes* was migratory, but that it was lost in *A. concolor*+*A. longipes*. There is a well-documented warming period around this time that has been associated with the northward migration of several different types of taxa, including insects (Currano *et al.*, 2008; Erwin, 2009).

The second gain, which appears in Clade 3, occurred around 25 Mya. This period (23–25 Mya) is also associated with a drastic climatic warming event during the Late Oligocene (Wappler, 2010). As discussed before, taxonomic issues prevent us from gaining a complete understanding of the evolution of this group, but three of the six species in this clade are migratory. The polyphyly present in this part of the phylogeny suggests this clade may be a single species with at least some migratory populations; however, deep branches suggest there are multiple species with both migratory and non-migratory behaviour. To fully understand the evolution of migration in this clade and actual species diversity, taxonomic revision is needed. Thus, depending on the taxonomy of this clade there are either four or five losses of migration within *Anax* (Fig. 3).

The third major gain of migration occurred around 13 Mya in Clade 1. This gain occurred just after a significant cooling event (~14–15 Mya) that is often associated with the Antarctic ice sheet expanding (Pearson & Palmer, 2000). This clade is composed of species that are found across the Eurasian continent, are both obligate and non-obligate migrators, and that have the largest geographical ranges (Borisov, 2009; May *et al.*, 2017). For example, *A. imperator* has some populations in northern Africa that migrate to Europe, while *A. parthenope* is an obligatory migrant in Asia with what are likely multiple migratory routes (Corbet, 1999; Nelson *et al.*, 2003; Borisov, 2009). *A. parthenope* and *A. imperator* have some overlap in their respective geographical ranges and, as mentioned previously, have been known to hybridize (Tennessen, 1982).

The fourth major gain only occurred around 5 Mya in *A. junius*, which also corresponds to glaciation events that occurred around 2–4 Mya (Pearson *et al.*, 2000). There is well-documented variability in migratory tendency within *A. junius* populations, which may also be present in other species (May *et al.*, 2017). Its closely related sister species *A. strenuus* does not exhibit migratory behaviour despite being a strong flyer and one of the largest dragonflies in the world (Roderick & Gillespie, 1998; Kalniņš, 2009). *A. strenuus* is an endemic of the Hawaiian Islands with a range of only 35 000 km^2 (compared to 855 000 km^2 of *A. junius*), with populations that extend across the archipelago. *A. junius* is also found in Hawaii, but is both morphologically and genetically distinct from *A. strenuus* (Fig. 2).

The results of our ancestral state reconstruction show that migration has been lost and gained multiple times and is similar to what has been recovered for groups of migratory birds (Winker & Pruett, 2006; do Amaral *et al.*, 2009; Zink, 2011). It is hypothesized that migration is more of a flexible "syndrome" (Alerstam *et al.*, 2003). In fact, migratory behaviour has been shown to appear and disappear within a few generations (Eggeman *et al.*, 2016; Ponti *et al.*, 2020). Flexible migration may be a useful trait for organisms like dragonflies that need to escape rapid changes in climate (e.g. drought or cooling), as well as more broad climatic change such as aridification (Tennessen, 2009). Climate-induced migration has been documented in many species (Juhász *et al.*, 2020). In the dragonfly genus *Trithemis* Brauer, 1868, largely known from Africa, their broad range in conjunction with their ability to disperse during climatic change may be a factor in their continued success today (Damm *et al.*, 2010). The results of our analysis provide possible evidence for climate-induced migration in *Anax* dragonflies with four major gains of this behaviour corresponding with global warming and cooling events. We estimated the age of *Anax* to be approximately 90 Myr. The ability to cope with geographic and global climate change via migration throughout their evolutionary history could be an important factor in the survival of this genus. Because the natural history of some species of *Anax* is poorly understood, it is possible that there may have been even more gains and losses than our analysis shows. Recent work shows that outside *Anax* at least one species of Aeshnidae (*Aeshna canadensis* Walker, 1908) is also migratory (Schilling *et al.*, 2021), and development and use of tools like stable isotope analysis may bring to light other migratory Aeshnidae or *Anax* species.

Range size

The ability of a species to migrate and thus disperse is likely correlated with a larger geographic range (Lees & Gilroy, 2014). Migratory species within the Odonata that have wing characteristics typical of long and sustained flight (e.g. expanded wing base), have been shown to have larger ranges (Outomuro & Johansson, 2019). However, other studies have found that migration is not always an indicator of larger geographic range size, but rather that factors such as latitude are better predictors (Pegan & Winger, 2020). However, these studies often only concern seasonal migration, which is not always the case within odonates. We found geographic range size was tightly correlated with migratory behaviour in *Anax* with only a few exceptions.

The largest range size, found in *A. junius*, is not surprising considering the wide dispersal abilities of this species (Freeland *et al.*, 2003). This species has been recorded to cross oceans and has populations ranging from throughout North America to Hawaii, Tahiti, China and Kamchatka (Corbet, 1999). The next two largest range sizes are found in *A. imperator* and *A. parthenope*, both from Eurasia, with *A. imperator* also found throughout Africa. As Eurasia is the largest landmass on earth it provides the opportunity for these two species to have large ranges, especially as these are also the two species from this region that are migratory. *A. nigrofasciatus* and *A. tumorifer* found in Asia and Madagascar respectively, are not migratory and have smaller range sizes.

Anax gibbosulus and *A. tristis* have relatively small distributions despite being migratory. One factor that may be biasing our results is that African species such as *A. tristis* likely have fewer data available due to being collected less frequently. This species is known to be a tropical migrant with ranges throughout sub-Saharan Africa. It is not frequently encountered in the field and is poorly represented in collections (Parr, 1984; Prendergast, 1998). *Anax gibbosulus* is found in northern Australia and throughout the South Pacific Islands. Perhaps, the ocean and smaller land masses with fewer suitable habitats make it more difficult to establish populations in this region. Furthermore, vagrant organisms are not likely to establish populations due to lack of other individuals to mate with (as known in birds) making

establishment more difficult (Lees & Gilroy, 2014). There is some evidence that *A. gibbosulus* can be found near brackish water, which may be an adaptation to inhabit this oceanic region (Theischinger & Hawking, 2006).

CONCLUSION

Our study provides the first molecular phylogeny of the genus *Anax*. We find evidence supporting the synonymy of *Hemianax* with *Anax*. Our tree shows that there are at least four well-defined clades of *Anax*. In these clades, we find possible hybridizations and several non-monophyletic species, indicating a need for further taxonomic work on these groups using morphological and genetic data sets. Hybridization may be a particular problem to the taxonomy of the group. Our results are compelling and provide a deep insight not only into taxonomic issues but also into the evolution of migration within this genus.

We also find a large amount of range size variation among different species of *Anax*, much of which is closely associated with migratory behaviour. Migratory behaviour is the ancestral state with subsequent losses and gains. Further work in this area should include looking at individual aspects of migratory behaviour and identifying specific migratory traits that are gained and lost in each species. However, this may prove difficult because the rarer species are restricted in both distribution and number, and often their behaviour is not well documented in the literature. Despite taxonomic problems in *Anax*, it remains an important animal group for understanding evolution, particularly in invertebrate systems.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Bayesian time calibration reconstruction of all taxa used in this study with placement of fossil taxa. Posterior probabilities shown at the nodes.
Figure S2. Maximum likelihood tree reconstruction. Bootstrap values shown at the nodes.
Table S1. Taxon sampling, localities and accession numbers for gene regions used for this study.
Table S2. Primers used for PCR reactions.
Table S3. Results of nested sampling analysis in BEAST.