Molecular Phylogeny of Holarctic Aeshnidae with a Focus on the West Palaearctic and Some Remarks on Its Genera Worldwide (Aeshnidae, Odonata) [†]

Thomas Schneider [1,*,‡,§], Andy Vierstraete [2,§], Oleg E. Kosterin [3,§], Dietmar Ikemeyer [4], Fang-Shuo Hu [5,6], Nataly Snegovaya [7] and Henri J. Dumont [2]

1. Independent Researcher, Arnold-Knoblauch-Ring 76, 14109 Wannsee, Germany
2. Department of Biology, University of Gent, 9000 Gent, Belgium; andy.vierstraete@ugent.be (A.V.); henri.dumont@ugent.be (H.J.D.)
3. Institute of Cytology & Genetics SB RAS, Academician Lavrentyev Avenue 10, Novosibirsk 630090, Russia; kosterin@bionet.nsc.ru
4. Independent Researcher, Billerbecker Str. 6, 48329 Havixbeck, Germany; dkjikemeyer@t-online.de
5. Department of Biological Sciences, National Sun Yat-Sen University, 70 Lienhai Rd., Kaohsiung 80424, Taiwan; fangshuo_hu@smail.nchu.edu.tw
6. Dragonfly Association of Taiwan, 2F., No. 5, Long'an Rd., Xinzhuang Dist., New Taipei City 242046, Taiwan
7. Zoological Institute NAS of Azerbaijan, Proezd 1128, Kvartal 504, Baku 1073, Azerbaijan; snegovaya@yahoo.com
* Correspondence: thomas.rs@gmx.de
† urn:lsid:zoobank.org:pub:CC2B3DBC-0DF4-49A6-A55D-934F37252D53.
‡ Volunteer at the Museum für Naturkunde Berlin, 10115 Berlin, Germany.
§ These authors contributed equally to this work.

Abstract: Aeshnidae Rambur, 1842 are impressive large insects distributed worldwide. Currently, over 500 species are recognized. Nevertheless, the phylogeny of this family is not completely understood. We applied molecular phylogenetic analysis using two popular phylogenetic markers, the mitochondrial COI gene fragment (barcoding sequence) and the nucleic ITS region, containing the ITS1, 5.8S rRNA, and ITS2 sequences. We used available and credible published sequences and 96 newly sequenced specimens. Our analysis involved all West Palaearctic species, all but one genera of the Holarctic Aeshnidae, and most genera worldwide, and is by far the largest molecular study of this family. The topology of all trees created with different algorithms and genes is in favour of the current taxonomic concept, with some remarkable outcomes. *Aeshna* Fabricius, 1775, was found to be diverged into several branches, especially with respect to the COI gene. Although it appeared not monophyletic in phylogenetic reconstructions based on the ITS region, the analysis of COI and joint analysis suggest its monophyly in the current taxonomical sense, with one notable exception. *Aeshna isoceles* (Müller, 1767) has fallen out of *Aeshna* in all analyses, so a new monophyletic genus, *Isoaeschna* gen. nov. is introduced for it. The genus *Brachytron* Evans, 1845 tightly clustered with *Aeschnophlebia* Selys, 1883, *Epiaeschna* Hagen in Selys, 1883, and *Nasiaeschna* Selys in Förster, 1900. Thus, we suggest subsuming these four genera under the priority name *Brachytron*. *Tetracanthagyna* Selys, 1883 clusters as expected with *Brachytron* in the ITS tree, but is an independent ancient clade of its own in all COI trees. The genus *Polycanthagyna* Fraser, 1933 syn. nov. is synonymised to *Indaeschna* Fraser, 1926. On the species level, we suggest that the American *Aeshna septentrionalis* Burmeister, 1839 be treated as a subspecies of *A. caerulea* (Ström, 1783), *Aeshna caerulea septentrionalis*. We synonymize *Gynacantha hyalina* Selys, 1882 with *Gynacantha subinterrupta* Rambur, 1842. Our analysis provides new insights on the tight relationships of the circumboreal species *Aeshna juncea* and *A. subarctica* and the intraspecies phylogeny of *Aeshna juncea*.

Keywords: revision of Aeshnidae; new genus; *Isoaeschna*; synonymization

1. Introduction

Aeshnidae Rambur, 1842 is a diverse family of Ansioptera (Figure 1) embracing large and robust dragonflies, which are strong fliers; some are even able to cross oceans from one continent to another. The family is distributed worldwide, and the number of species included is steadily increasing and is currently well above 500 [1]. The representatives of Aeshnidae are currently sorted in 54 accepted genera [1]. Less than half of the genera and about one-fifth of species is found in the Holarctic. Several classifications for Aeshnidae were proposed mainly on arbitrarily chosen morphological characteristics and wing venation [2–4]. More selected characters from external morphology have been used in a cladistic analysis of all genera of Aeshnidae known up to 2001 by Natalia von Ellenrieder [5]. Studies using molecular methods mostly addressed other families or the entire order of Odonata, the latter including only a small number of Aeshnidae [6–12]. A recent study on the genus *Anax* Leach in Brewster, 1815 brought some light into the relationship of migration and phylogeny [13]. These studies left the phylogenetic relationships between the Holarctic Aeshnidae unresolved.

A molecular genetic study of Aeshnidae that includes a considerable number of specimens is still missing. Therefore, we made such an attempt, focused on two popular markers, the mitochondrial COI gene fragment and the nuclear ITS regioninvolving both published sequences and 96 newly sequenced specimens. We used several algorithms to obtain the most appropriate results. Our analysis comprises nearly all West Palaearctic species and nearly all genera of the Holarctic Aeshnidae and is by far the largest molecular study of this family so far.
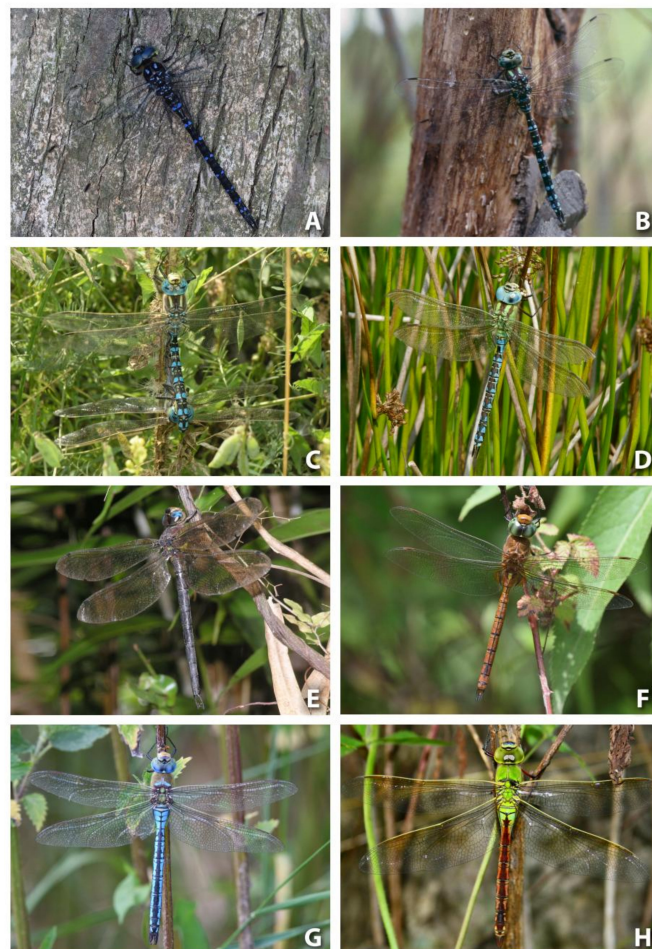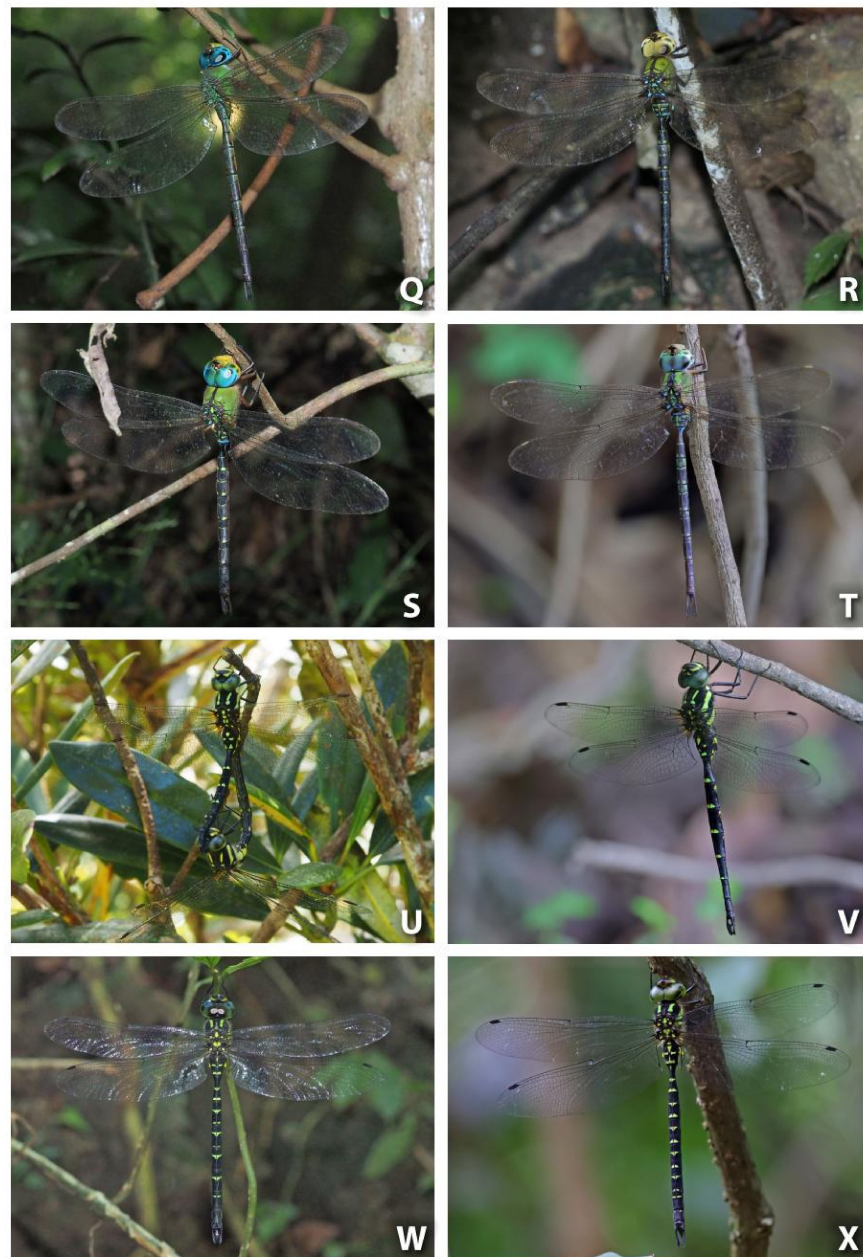

Figure 1. *Cont.*

Figure 1. *Cont.*

Figure 1. Some Aeshnidae in their natural environment: (**A**): *Aeshna vercanica* ♂, Māzandarān Province, Iran. (**B**): *Aeshna crenata* ♂, SE Transbaikalia, Russia. (**C**): *Aeshna serrata*, copula, West Siberia, Russia. (**D**): *Aeshna viridis* ♂, Germany. (**E**): *Tetracanthagyna waterhousei* ♂, Mondulkiri Province, Cambodia. (**F**): *Aeshna isoceles* ♂, Gilān Province, Iran. (**G**): *Anax imperator* ♂, Germany. (**H**): *Anax julius* ♂, Primorye, Russia. (**I**): *Anax guttatus* ♀, Kampot Province, Cambodia. (**J**): *Aeschnophlebia longistigma*, copula, Primorye, Russia. (**K**): *Brachytron pratense* ♂, Germany. (**L**): *Caliaeschna microstigma* ♂, Muğla Province, Turkey. (**M**): *Boyeria irene* ♂, France. (**N**): *Polycanthagyna erythromelas* ♀, Pursat Province, Cambodia. (**O**): *Planaeschna milei* ♂, Shikoku Chiho, Japan. (**P**): *Gynacantha subinterrupta* ♂, Ratanakiri Province, Cambodia. (**Q**): *Gynacantha bayadera* ♂, Pingtung County, Taiwan. (**R**): *Gynacantha ryukyuensis* ♂, Yilan County, Taiwan. (**S**): *Gynacantha japonica* ♂, Nantou County, Taiwan. (**T**): *Gynacantha hyalina* ♂, New Taipei City, Taiwan. (**U**): *Sarasaeschna lieni* copula, Pingtung County, Taiwan. (**V**): *Sarasaeschna tsaopiensis* ♂, Yilan County, Taiwan. (**W**): *Planaeschna risi* ♂, Yilan County, Taiwan. (**X**): *Planaeschna taiwana* ♂, Taipei City, Taiwan. Photos: (**A,D,F,G,K–M**): Dietmar Ikemeyer, (**B,C,E,I,J,N–P**): Oleg Kosterin, (**H**): Vladimir Onishko, (**Q–X**): Fang-Shuo Hu.

2. Materials and Methods

2.1. Materials

Our molecular analysis included a total of 291 Aeshnidae specimens (Tables 1 and S1). Of them, 96 specimens were sequenced in the course of this study, 27 specimens were sequenced and published by us previously [14], and the sequences of 168 specimens were taken from GenBank [15]. The latter were earlier published in [10,16,17], and some other papers (Table 1; for more information, including the PCR numbers mentioned in the figures with trees, consult Table S1). Our analysis involved 28 of 54 currently recognised genera of Aeshnidae, and 17 of 18 genera occurring in the Holarctic region (with *Gomphaeschna* Selys, 1871 being the only genus not involved). Eleven genera from beyond the Holarctic region were also included, as they were supposed to have relatives in the Holarctic region.

Table 1. Information on Aeshnidae specimens used; the boldfaced GenBank reference numbers refer to sequences obtained in the course of this study.

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Aeschnophlebia longistigma Selys, 1883			Korea		[18]	KF257055	no data
Aeschnophlebia longistigma			Japan		[19]	no data	AB706669
Aeschnophlebia longistigma	42.4700	130.6400	Russia	Primorye, Lake Lotos	O. Kosterin leg.	**OR130000**	**OR133899**
Aeschnophlebia longistigma	44.5100	132.7000	Russia	Primorye, Khasan District, Prokhory Village	O. Kosterin leg.	**OR130001**	**OR133898**
Aeschnophlebia anisoptera Selys, 1883			Japan		[19]	no data	AB706668
Aeshna affinis Vander Linden, 1820			Italy		[20]	MT298234	no data
Aeshna affinis			Azerbaijan	Yardimli, Shefekli Village	N. Snegovaya leg.	**OR130002**	**OR133867**
Aeshna affinis			Azerbaijan	Shabran District	N. Snegovaya leg.	**OR130003**	**OR133869**
Aeshna affinis			Azerbaijan	Balaken, Gabagchol	N. Snegovaya leg.	**OR130004**	**OR133868**
Aeshna affinis			Germany		[15]	HM422047	no data
Aeshna caerulea (Ström, 1783)			Italy		[20]	MT298235	no data
Aeshna caerulea			Austria		[16]	MW490272	no data
Aeshna caerulea			Sweden	Lapland	T. Schneider leg.	**OR130006**	**OR133866**
Aeshna caerulea			France		[15]	no data	MN656996
Aeshna caerulea	52.6400	96.8000	Russia	Tuva Republic, Todzha District, Lake Ottug-Khol'	O. Kosterin leg.	**OR130005**	no data
Aeshna canadensis Walker, 1908			Canada		[15]	HM413507	no data
Aeshna canadensis			Canada		[15]	JF839358	no data
Aeshna constricta Say, 1840			Canada		[15]	KM528410	no data
Aeshna constricta			Canada		[15]	KM528706	no data
Aeshna crenata Hagen, 1856			Finland		A. Schröter leg., Senckenberg Museum Frankfurt	**OR130008**	**OR133857**
Aeshna crenata			Finland		A. Schröter leg., Senckenberg Museum Frankfurt	no data	**OR133853**
Aeshna crenata	54.8200	69.7800	Russia	S Ural, Bashkortostan, Uchaly District, at Muldashevo Village	O. Kosterin leg.	**OR130011**	**OR133856**

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Aeshna crenata	52.6000	96.8000	Russia	Tuva, Todzha District, Lake Saylyg-Khol'	O. Kosterin leg.	**OR130010**	**OR133859**
Aeshna crenata	58.9900	126.2449	Russia	Yakutia, Tommot Town env.	O. Kosterin leg.	**OR130012**	**OR133854**
Aeshna crenata	45.0600	131.9900	Russia	Primorye, Khanka District, Platono-Aleksandrovskaya Village env.	O. Kosterin leg.	**OR130009**	**OR133858**
Aeshna crenata	53.3040	157.4730	Russia	Kamchatka, Malki Village env.	O. Kosterin leg.	no data	**OR133855**
Aeshna crenata			Korea		[18]	KF257093	no data
Aeshna crenata			Japan		[19]	no data	AB706671
Aeshna crenata			Russia		[19]	no data	AB706672
Aeshna cyanea (Müller, 1764)			France		[16]	MW490109	no data
Aeshna cyanea			Italy		[20]	MT298236	no data
Aeshna cyanea			Azerbaijan	Siyazan, near Galaalty	N. Snegovaya leg.	**OR130013**	**OR133832**
Aeshna cyanea			Azerbaijan	Isailli, Garanokhur Lake	N. Snegovaya leg.	**OR130014**	**OR133833**
Aeshna cyanea			Switzerland		[15]	MN454844	no data
Aeshna cyanea			Italy		[14]	KU180305	KU180377
Aeshna cyanea			Germany		[14]	KU180307	KU180376
Aeshna cyanea			Poland		[14]	KU180308	KU180379
Aeshna cyanea			Algerie		[14]	KU180311	KU180374
Aeshna cyanea			Tunisia		[14]	KU180310	KU180385
Aeshna cyanea			Tunisia		[14]	KU180320	KU180386
Aeshna cyanea			Poland		[14]	KU180306	KU180378
Aeshna cyanea			Spain		[14]	KU180309	KU180384
Aeshna cyanea			Armenia		[14]	KU180316	KU180370
Aeshna cyanea			Russia	Krasnodar Krai, Mostovskiy District, Psebay	[14]	KU180312	KU180380
Aeshna cyanea			Armenia		[14]	KU180319	KU180371
Aeshna cyanea			Russia	Krasnodar Krai, Mostovskiy District, Psebay	[14]	KU180318	KU180383
Aeshna cyanea			Russia	Krasnodar Krai, Mostovskiy District, Psebay	[14]	KU180317	KU180382
Aeshna cyanea			Russia	Krasnodar Krai, Mostovskiy District, Psebay	[14]	KU180313	KU180381
Aeshna cyanea			Georgia		[14]	KU180314	KU180375
Aeshna cyanea			Armenia		[14]	KU180315	KU180369
Aeshna cyanea			Armenia		[14]	KU180321	KU180372
Aeshna cyanea			Belgium		[14]	KU180304	KU180373
Aeshna eremita Scudder, 1866			Canada		[15]	HM381222	no data
Aeshna eremita			USA	Alaska	[21]	KU873985	no data
Aeshna grandis (Linnaeus, 1758)			Germany		[14]	KU180299	KU180363
Aeshna grandis			Italy		[20]	MT298237	no data
Aeshna interrupta Walker, 1908			USA	Alaska	[21]	KU873988	no data
Aeshna interrupta			USA	Alaska	[21]	KU873987	no data
Aeshna interrupta			Canada		[15]	HM381232	no data
Aeshna isoceles (Müller, 1767)			Italy		[20]	MT298239	no data
Aeshna isoceles			Italy		[20]	MT298240	no data
Aeshna isoceles			Lebanon	Bared Brook	T. Schneider leg.	**OR130019**	**OR133827**
Aeshna isoceles			Germany	Brandenburg	T. Schneider leg.	**OR130018**	**OR133825**
Aeshna isoceles			Morocco	Quiouane Middle Atlas	T. Schneider leg.	**OR130020**	**OR133830**

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Aeshna isoceles			Azerbaijan	Zakatala, Geratap	N. Snegovaya leg.	**OR130017**	**OR133826**
Aeshna isoceles			Azerbaijan	Lenkoran, Azfilial Settlement	N. Snegovaya leg.	**OR130016**	**OR133829**
Aeshna isoceles			Azerbaijan	Agstafa, Poylu Village, Kura River	N. Snegovaya leg.	**OR130015**	**OR133828**
Aeshna isoceles			France		[15]	no data	FN356032
Aeshna juncea (Linnaeus, 1758)	58.5000	125.5067	Russia	Yakutia, Aldan District, Lebedinyy Town	O. Kosterin leg.	**OR130030**	**OR133838**
Aeshna juncea	56.4500	160.9500	Russia	Kamchatka, at Klyuchi Village	O. Kosterin leg.	**OR130025**	**OR133843**
Aeshna juncea	42.4600	130.6400	Russia	Primorye, Lake Lotos	O. Kosterin leg.	**OR130027**	**OR133839**
Aeshna juncea	43.3400	41.6740	Russia	N Caucasus, Karachay-Cherkes Republic, Dombay env.	O. Kosterin leg.	**OR130026**	**OR133844**
Aeshna juncea	53.3400	57.7900	Russia	S Ural, Bashkortostan, Sargaya Village env.	O. Kosterin leg.	**OR130028**	**OR133840**
Aeshna juncea	41.8000	47.4100	Russia	Dagestan, Agul District, Lake Debrishara	O. Kosterin leg.	**OR130024**	**OR133845**
Aeshna juncea	54.1500	83.6101	Russia	W Siberia, Ordynskoe District, Spirino Village env.	O. Kosterin leg.	**OR130029**	**OR133841**
Aeshna juncea			Italy		[20]	MT298245	no data
Aeshna juncea			Italy		[20]	MT298244	no data
Aeshna juncea			Italy		[20]	MT298243	no data
Aeshna juncea			Armenia		V. Ananian leg.	**OR130021**	**OR133846**
Aeshna juncea			Pakistan	Hindu Kush	T. Schneider leg.	**OR130023**	**OR133842**
Aeshna juncea			Georgia	Lesser Caucasus	T. Schneider leg.	**OR130022**	**OR133847**
Aeshna juncea			Germany		[14]	KU180297	KU180364
Aeshna juncea			Canada		[15]	JF839255	no data
Aeshna juncea			Canada		[22]	KR143341	no data
Aeshna juncea			Japan		[19]	no data	AB706686
Aeshna juncea			Russia		[19]	no data	AB706688
Aeshna juncea			Russia	Buryatia	[19]	no data	AB711414
Aeshna juncea			South Korea		[19]	no data	AB711415
Aeshna mixta Latreille, 1805			Austria		[17]	MW208418	no data
Aeshna mixta			Montenegro		[17]	MW208417	no data
Aeshna mixta			Austria		[17]	MW208419	no data
Aeshna mixta			Bosnia and Herzegovina		[17]	MW208416	no data
Aeshna mixta			Montenegro		[20]	MT298246	no data
Aeshna mixta			Azerbaijan	Kedabek, Novoivanovka Village	N. Snegovaya leg.	**OR130031**	**OR133863**
Aeshna mixta			Azerbaijan	Yardimli, Avash Village	N. Snegovaya leg.	**OR130032**	**OR133864**
Aeshna mixta			Greece		D.A.L. Davies leg.	**OR130033**	**OR133865**
Aeshna mixta	55.6960	37.5200	Russia	Moskow	[23]	OM089772	no data
Aeshna palmata Hagen, 1856			Canada		[15]	JF839302	no data
Aeshna palmata			Canada		[15]	JN294467	no data
Aeshna palmata			Canada		[15]	JF839296	no data
Aeshna petalura Martin, 1908			Taiwan	Yilan Co. SanXing Pond	Fang-Shuo Hu leg.	**OR130034**	**OR133834**
Aeshna petalura			Taiwan	Yilan Co. SanXing Pond	Fang-Shuo Hu leg.	**OR130035**	**OR133835**

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Aeshna septentrionalis Burmeister, 1839			Canada		[15]	GU714053	no data
Aeshna septentrionalis			Canada		[15]	JF839362	no data
Aeshna serrata Hagen, 1856			Finland		A. Schröter leg., Senckenberg Museum Frankfurt	**OR130036**	**OR133851**
Aeshna serrata	54.8600	83.0740	Russia	W Siberia, Novosibirsk	O. Kosterin leg.	**OR130039**	**OR133850**
Aeshna serrata	54.1400	61.2900	Russia	S Ural, Chelyabinsk Province, Troitsk District	O. Kosterin leg.	**OR130038**	**OR133848**
Aeshna serrata	56.4500	160.9500	Russia	Kamchatka, at Klyuchi Village	O. Kosterin leg.	**OR130037**	**OR133849**
Aeshna serrata			Russia		[19]	no data	AB706698
Aeshna serrata			Russia		[19]	no data	AB706699
Aeshna sitchensis Hagen, 1861			Canada		[15]	HM413523	no data
Aeshna sitchensis			Canada		[22]	KR143430	no data
Aeshna soneharai (Asahina, 1988)	55.5400	60.5800	Russia	S Ural, Lake Bol'shaya Akulya	O. Kosterin leg.	**OR130041**	**OR133860**
Aeshna soneharai	55.7000	37.5200	Russia	Moscow	V. Onishko leg.	**OR130040**	**OR133861**
Aeshna soneharai	54.1500	83.6101	Russia	W Siberia, Ordynskoe District, Spirino Village env.	O. Kosterin leg.	**OR130042**	**OR133862**
Aeshna soneharai			South Korea		[18]	KF257096	no data
Aeshna soneharai			Japan		[19]	no data	AB706697
Aeshna subarctica Walker, 1908			Germany		[14]	KU180298	KU180362
Aeshna subarctica	52.6400	96.8900	Russia	Tuva, Lake Saylyg-Khol'	O. Kosterin leg.	**OR130043**	**OR133837**
Aeshna subarctica	54.8800	83.0484	Russia	W Siberia, Novosibirsk	O. Kosterin leg.	**OR130044**	**OR133836**
Aeshna subarctica			Italy		[20]	MT298250	no data
Aeshna subarctica			Italy		[20]	MT298249	no data
Aeshna subarctica			Japan		[19]	no data	AB711410
Aeshna subarctica			Finland		[19]	no data	AB711413
Aeshna subarctica			Japan		[19]	no data	AB711411
Aeshna tuberculifera Walker, 1908			Canada		[15]	HM413600	no data
Aeshna tuberculifera			Canada		[15]	JF839364	no data
Aeshna umbrosa Walker, 1908			Canada		[15]	GU712955	no data
Aeshna umbrosa			Canada		[15]	GU713003	no data
Aeshna vercanica Schneider et al. 2015			Azerbaijan		[14]	KU180322	KU180361
Aeshna vercanica			Iran		[14]	KU180302	KU180365
Aeshna vercanica			Iran		[14]	KU180303	KU180368
Aeshna vercanica			Azerbaijan	Lenkoran, Azfilial Settlement	N. Snegovaya leg.	no data	**OR133831**
Aeshna vercanica			Azerbaijan		[24]	no data	FN356031
Aeshna verticalis Hagen, 1861			Canada		[15]	HM413554	no data
Aeshna verticalis			Canada		[15]	HM413555	no data
Aeshna viridis Eversmann, 1836			Germany		[14]	KU180301	KU180366
Aeshna viridis			Germany		[14]	KU180300	KU180366
Aeshna viridis			Germany		[14]	no data	KU180387
Anaciaeschna jaspidea (Burmeister, 1839)			India		[15]	KT879909	no data
Anaciaeschna jaspidea			Japan		[19]	no data	AB706701
Anaciaeschna martini (Selys, 1897)			Japan		[25]	LC466165	LC466157
Anaciaeschna martini			Nepal		[25]	LC466163	LC466154
Anaciaeschna martini			Japan		[19]	no data	AB706702

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Anaciaeschna triangulifera McLachlan, 1896			Tanzania		[26]	KC912207	no data
Anax congoliath Fraser, 1953			Angola		[27]	KU565906	no data
Anax congoliath			Gabon		[27]	KU565907	no data
Anax ephippiger (Burmeister, 1839)			Italy		[20]	MT298252	no data
Anax ephippiger			Italy		[20]	MT298251	no data
Anax ephippiger			Israel	Jordan Valley, near Had Nes	A. Leirich leg. coll. T. Schneider	**OR130045**	**OR133824**
Anax ephippiger			Tanzania		[19]	no data	AB706703
Anax ephippiger			Japan		[28]	no data	LC511177
Anax ephippiger			Cameroon		[24]	no data	FN356093
Anax gibbosulus Rambur, 1842			Samoa		[13]	MW810906	no data
Anax gladiator Dijkstra & Kipping, 2015			Zambia		[27]	KU565911	no data
Anax gladiator			Congo		[27]	KU565913	no data
Anax guttatus (Burmeister, 1839)			Japan		[19]	no data	AB706704
Anax guttatus			Borneo		[13]	MW810901	no data
Anax immaculifrons Rambur, 1842			India		[15]	MG544869	no data
Anax immaculifrons			Lebanon		T. Schneider leg.	**OR346685**	**OR350846**
Anax imperator Leach in Brewster, 1815			Liberia		[27]	KU565916	no data
Anax imperator			Germany	Brandenburg	T. Schneider leg.	**OR130046**	**OR133816**
Anax imperator			Germany	Brandenburg	T. Schneider leg.	**OR130047**	**OR133817**
Anax junius (Drury, 1773)			USA		[29]	AY555548	no data
Anax junius			USA		[15]	HQ986154	no data
Anax junius			USA		[30]	no data	LC366224
Anax junius			USA		[30]	no data	LC366223
Anax julius Brauer, 1865	45.2200	133.5130	Russia	Primorye, Kirovskiy District, Gornye Klyuchi Village env.	O. Kosterin leg.	**OR130048**	**OR133818**
Anax julius	45.0600	131.9900	Russia	Primorye, Khanka District, Platono-Aleksandrovka Village env.	O. Kosterin leg.	**OR130049**	**OR133820**
Anax julius			Vietnam	Yen Bai	Dang leg. coll. T. Schneider	**OR130051**	**OR133821**
Anax julius			Vietnam	Yen Bai	Dang leg. coll. T. Schneider	**OR130050**	**OR133819**
Anax julius			Japan		[19]	no data	AB706711
Anax julius			Japan		[19]	no data	AB706713
Anax nigrofasciatus Oguma, 1915			Korea		[15]	MN609590	no data
Anax nigrofasciatus			Korea		[15]	MN609591	no data
Anax nigrofasciatus			Vietnam	Sa Pa	Dang leg. coll. T. Schneider	**OR130052**	**OR133823**
Anax panybeus Hagen, 1867			Japan		[19]	no data	AB706710
Anax panybeus			Japan		[19]	no data	AB706709
Anax parthenope (Selys, 1839)			Poland		[31]	MN701501	no data
Anax parthenope			Poland		[31]	MN701506	no data
Anax parthenope			Germany	Brandenburg	T. Schneider leg.	**OR130053**	**OR133822**
Anax piraticus Kennedy, 1934			Guam		[30]	no data	LC366251

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Anax rutherfordi McLachlan, 1883			Liberia		[27]	KU565918	no data
Anax rutherfordi			Liberia		[27]	KU565917	no data
Anax speratus Hagen, 1867			Congo		[27]	KU565921	no data
Anax speratus			South Africa		[27]	KU565923	no data
Anax tristis Hagen, 1867			Congo		[27]	KU565931	no data
Anax walsinghami McLachlan, 1883			USA	Utha	[13]	MW810898	no data
Anax walsinghami			USA	California	[13]	MW810942	no data
Andaeschna occidentalis Bota-Sierra, 2019			Colombia		C.A.Bota-Sierra leg.	**OR346684**	**OR350845**
Austrogynacantha heterogena Tillyard, 1908	−19.2828	146.8010	Australia	Queensland	[15]	HQ986475	no data
Austrogynacantha heterogena			Australia	Queensland	Australian Museum (D. Smith)	**OR130054**	**OR133872**
Basiaeschna janata (Say, 1840)			Canada		[15]	GU712962	no data
Basiaeschna janata	40.9100	−73.7300	USA	New York	[15]	MT947635	no data
Basiaeschna janata			Canada		[15]	JN419315	no data
Boyeria cretensis Peters, 1991			Greece	Crete, Zakros	A. Martens leg.	**OR130055**	**OR133883**
Boyeria grafiana Williamson, 1907			Canada		[15]	GU713103	no data
Boyeria grafiana			Canada		[15]	JN419353	no data
Boyeria irene (Fonscolombe, 1838)			Italy		[20]	MT298265	no data
Boyeria irene			Italy		[20]	MT298267	no data
Boyeria irene			France		[24]	no data	FN356042
Boyeria irene			France		[16]	MW490313	no data
Boyeria irene			Switzerland	Ennetburgen	S. Kohl leg.	**OR130057**	**OR133881**
Boyeria irene			Switzerland	Ennetburgen	S. Kohl leg.	**OR130056**	no data
Boyeria irene			Germany	Lower Saxon	F. Suhling leg. coll. T. Schneider	no data	**OR133880**
Boyeria irene			Italy	Calabria	T. Schneider leg.	no data	**OR133882**
Boyeria karubei Yokoi, 2002			Vietnam		[32]	no data	LC612603
Boyeria maclachlani (Selys, 1883)			Japan		[19]	no data	AB706723
Boyeria vinosa (Say, 1840)			Canada		[15]	HM395246	no data
Brachytron pratense (Müller, 1764)			Italy		[20]	MT298272	no data
Brachytron pratense			Italy		[20]	MT298273	no data
Brachytron pratense	44.7300	37.4600	Russia	W Caucasus, Abrau Peninsula, Malyy Utrish Village env.	O. Kosterin leg.	**OR130061**	**OR133896**
Brachytron pratense	44.7300	37.4600	Russia	W Caucasus, Abrau Peninsula, Malyy Utrish Village env.	O. Kosterin leg.	**OR130060**	**OR133895**
Brachytron pratense			Germany	Potsdam	T. Schneider leg.	**OR130059**	**OR133894**
Brachytron pratense			Germany	Potsdam	T. Schneider leg.	**OR130058**	**OR133893**
Brachytron pratense			Azerbaijan	Lenkoran, Azfilial Settlement	N. Snegovaya leg.	no data	**OR133897**
Caliaeschna microstigma (Schneider, 1845)			Montenegro		[20]	MT298276	no data
Caliaeschna microstigma			Lebanon	Bared Brook	T. Schneider leg.	**OR130064**	**OR133889**

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/Reference	GenBank COI	GenBank ITS
Caliaeschna microstigma			Azerbaijan	Balaken District, Djidjikhana	N. Snegovaya leg. coll. T. Schneider	**OR130063**	**OR133888**
Caliaeschna microstigma			Azerbaijan	Ordubad District, Agdere	N. Snegovaya leg. coll. T. Schneider	**OR130062**	**OR133887**
Cephalaeschna risi Asahina, 1981			Taiwan	Qilan, Yilan Co.	Fang-Shuo Hu leg.	**OR130065**	no data
Cephalaeschna risi			Taiwan		Fang-Shuo Hu leg.	**OR130066**	**OR133931**
Coryphaeschna adnexa (Hagen, 1861)			Bolivia	Nuflo de Chavez, San Julian	[15]	MN345399	no data
Epiaeschna heros (Fabricius, 1798)			USA	Virginia	[15]	MN345091	no data
Gynacantha africana (Palisot de Beauvois, 1807)			Liberia	Grand Bassa County	[27]	KU566102	no data
Gynacantha bayadera Selys, 1892	13.6000	105.9300	Cambodia	Stung Treng Province, Srae Ruessei Village env.	O. Kosterin leg.	**OR130067**	**OR133874**
Gynacantha bispina Rambur, 1842			Mauritius		[27]	KU566103	no data
Gynacantha bullata Karsch, 1891			Democratic Republic Congo	Kisangani	[27]	KU566105	no data
Gynacantha bullata			Cameroon		[24]	no data	FN356092
Gynacantha congolica Dijkstra, 2015			Democratic Republic Congo	Orientale	[27]	KU566118	no data
Gynacantha demeter Ris, 1911	10.5240	103.7130	Cambodia	Preah Sihanouk Province, Ream Peninsula	O. Kosterin leg.	**OR130068**	**OR133873**
Gynacantha dravida Lieftinck, 1960			India		[15]	MK990607	no data
Gynacantha hyalina Selys, 1882	25.2643	121.5840	Taiwan	New Taipei, Aliban Ecological Farm	C. H. Ma & I. L. Lee leg.	**OR130069**	**OR133877**
Gynacantha japonica Bartenev, 1910			Korea		[18]	KF257090	no data
Gynacantha japonica			Japan		[19]		AB706724
Gynacantha manderica Grünberg, 1902			South Africa		[27]	KU566119	no data
Gynacantha nigeriensis (Gambles, 1956)			Uganda		[27]	KU566123	no data
Gynacantha pupillata Dijkstra, 2015			Democratic Republic Congo	Orientale	[27]	KU566133	no data
Gynacantha ryukyuensis Asahina, 1962			Japan		[19]	no data	AB706729
Gynacantha saltatrix Martin, 1909	13.6000	105.9300	Cambodia	Stung Treng Province, Srae Ruessei Village env.	O. Kosterin leg.	**OR130070**	no data
Gynacantha subinterrupta Rambur, 1842	10.5010	193.7220	Cambodia	Preah Sihanouk Province, Ream Peninsula	O. Kosterin leg.	**OR130071**	**OR133875**
Gynacantha subinterrupta			Vietnam	Bao Loc, Lam Dong	Dang leg. coll. T. Schneider	**OR130072**	no data
Gynacantha usambarica Sjöstedt, 1909			Liberia		[27]	KU566134	no data
Gynacantha vesiculata Karsch, 1891			Liberia		[27]	KU566139	no data
Heliaeschna crassa Krüger, 1899	11.5810	103.1280	Cambodia	Koh Kong Province, Tatai Commune	G. Chartier leg.	no data	**OR133878**
Indaeschna grubaueri (Förster, 1904)			Indonesia	Kalimantan	coll. T. Schneider	**OR130073**	no data

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Nasiaeschna pentacantha (Rambur, 1842)	37.8667	−76.8000	USA	Virginia	[15]	MN345309	no data
Oplonaeschna armata (Hagen, 1861)			Mexico		[15]	MN345104	no data
Oplonaeschna armata			Mexico	Oaxaca	[15]	MN346016	no data
Periaeschna magdalena Martin, 1909			Taiwan	New Taipei City	C. H. Ma leg.	**OR130074**	**OR133890**
Periaeschna magdalena			Vietnam		Dang leg. coll. T. Schneider	**OR130075**	**OR133891**
Pinheyschna subpupillata (McLachlan, 1896)			South Africa		[15]	AF429287	no data
Pinheyschna yemenensis (Waterston, 1985)			Saudi Arabia	Soudah Waterfall	M. Waldhauser leg.	**OR130076**	**OR133871**
Planaeschna asahinai Karube, 2011			Vietnam		[32]	LC612707	no data
Planaeschna celia Wilson & Reels, 2001			Vietnam		[32]	LC612709	no data
Planaeschna cucphuongensis Karube, 1999			Vietnam		[32]	no data	LC612627
Planaeschna cucphuongensis			Vietnam		Dang leg. coll. T. Schneider	**OR130077**	**OR133885**
Planaeschna milnei (Selys, 1883)			Japan		[19]	AB708630	AB706740
Planaeschna risi Asahina, 1964			Japan		[19]	AB708637	AB706742
Planaeschna risi	24.8804	121.6645	Taiwan	New Taipei, Jingualio	Fang-Shuo Hu leg.	**OR130079**	**OR133884**
Planaeschna taiwana Asahina, 1951			Taiwan		[19]	no data	AB706746
Planaeschna tamdaoensis Asahina, 1996			Vietnam		[32]	LC612720	LC612639
Planaeschna tomokunii Asahina, 1996			Vietnam		[32]	no data	LC612643
Planaeschna ishigakiana Asahina, 1951			Taiwan		[30]	no data	LC366218
Planaeschna ishigakiana			Vietnam		[32]	no data	LC612631
Planaeschna ishigakiana	24.7499	121.5585	Taiwan	New Taipei, Fushan	Fang-Shuo Hu leg.	**OR130078**	**OR133886**
Planaeschna viridis Karube, 2004			Vietnam		[32]	no data	LC612644
Polycanthagyna erythromelas (McLachlan, 1896)	12.3860	103.0550	Cambodia	Pursat Province, Phnom Tumpor Mt	O. Kosterin leg.	**OR130080**	**OR133870**
Polycanthagyna melanictera (Selys, 1883)			Korea		[18]	KF257100	no data
Polycanthagyna melanictera			Japan		[30]	no data	LC366042
Polycanthagyna melanictera			Japan		[19]	no data	AB706747
Polycanthagyna melanictera			Japan		[19]	no data	AB706748
Remartinia luteipennis (Burmeister, 1839)			Panama		[15]	MN344834	no data
Rhionaeschna californica (Calvert, 1895)			Canada		[15]	JF839371	no data
Rhioaeschna diffinis (Rambur, 1842)			Chile	Parc National Le Campenie, Cajan Grande	Ch. Pineda leg. coll. T. Schneider	**OR130081**	**OR133879**
Rhionaeschna multicolor (Hagen, 1861)			Canada		[15]	JF839373	no data
Sarasaeschna kunigamiensis (Ishida, 1972)			Japan		[19]	no data	AB706753
Sarasaeschna kunigamiensis			Japan		[19]	AB708646	no data
Sarasaeschna lieni (Yeh & Chen, 2000)			Taiwan		[19]	AB708649	no data
Sarasaeschna lieni			Taiwan	Yilan Co., Yunshan Township	Fang-Shuo Hu leg.	**OR130082**	no data

Table 1. *Cont.*

Species	Latitude	Longitude	Country	Region	Collector/ Reference	GenBank COI	GenBank ITS
Sarasaeschna lieni			Taiwan		[19]	no data	AB706754
Sarasaeschna pryeri (Martin, 1909)			Japan		[19]	no data	AB706755
Sarasaeschna pryeri			Japan		[19]	AB708650	no data
Sarasaeschna tsaopiensis (Yeh & Chen, 2000)			Taiwan	Yilan Co., Yunshan Township	Fang-Shuo Hu leg.	no data	**OR133900**
Staurophlebia reticulata (Burmeister, 1839)	−12.8833	−71.2333	Peru	Madre de Dios	[15]	MN343860	no data
Tetracanthagyna plagiata (Waterhouse, 1877)			Malaysia		[15]	no data	AB706758
Tetracanthagyna waterhousei McLachlan, 1898	12.5700	107.4150	Cambodia	Mondulkiri Province, at Buu Sraa Waterfalls	O. Kosterin leg.	**OR130083**	no data
Tetracanthagyna waterhousei			Vietnam	Bao Loc, Lam Dong	Dang leg. coll. T. Schneider	**OR130085**	no data
Tetracanthagyna waterhousei			Vietnam	Bao Loc, Lam Dong	Dang leg. coll. T. Schneider	**OR130084**	**OR133892**
Triacanthagyna septima (Selys in Sagra, 1857)			Dominican Republic	Santiago	[15]	MN345267	no data

For the ITS region, which contains the ITS1, 5.8S rRNA, and ITS2 sequences, the sequences are between 648 and 1006 bp long, depending on the species (Figure 2). For the barcoding fragment of the COI gene, we used two alignments: one with sequences with a length of 632 bp (Figure 3) and the other with shorter sequences, also available in GenBank [15], so that all sequences were trimmed to the same length of the shortest, 341 bp (see Section 3.2.2). We used *Orthetrum melania* (Selys, 1883) and *Neopetalia punctata* (Hagen in Selys, 1854) as outgroups for the ITS and COI analyses, respectively.

2.2. DNA Extraction and Sequencing

Per specimen, a 1 mm section of a leg was transferred to a tube with 20 μL 0.05 N NaOH and 2 μL 5% Tween 20. This was heated for 15 min at 95 °C and cooled on ice. A volume of 100 μL sterile water was added to the tube and mixed. The amount of 1 to 5 μL of this solution was used in a PCR reaction. For details of PCR and sequencing see [33,34]; for the COI barcoding fragment we used the primers CO1490F (50-GGT CAA ATC ATA AAG ATA TTG G-30) and CO2198R (50-TAA ACT TCA GGG TGA CCA AAA AAT CA-30) and for the ITS region we used the primers Vrain2F (50-CTT TGT ACA CAC CGC CCG TCG CT-30) and 28R1 (50-TGA TAT GCT TAA NTT CAG CGG GT-30).

2.3. Reducing Artefacts

All sequences used were checked for plausibility of determination. Each sequence was blasted to check for sequencing contamination and in case of doubts, it was checked to determine that it was not a pseudogene by comparing it with other sequences and translating it to protein. This was also done for some GenBank sequences that appeared in unexpected branches in the tree. Some sequences were revealed to be pseudogenes and were discarded. The alignments with a COI sequence of *Austrogynacantha heterogena* Tillyard, 1908 from GenBank revealed a strange, close relationship with *Aeshna isosceles*; therefore, we isolated DNA from an additional specimen of this species we received from the Australian Museum and approved the correctness of the GenBank sequence.

We also eliminated positions in the alignment that were saturated with multiple substitutions with the program Gblocks (Figure S2) [35] and constructed trees with the so-reduced alignment (see below).

2.4. Phylogenetic Analysis

Alignments were made with the online version of MAFFT [36] with default settings. The model of DNA evolution that best fit the data was determined with JMODELTEST version 2.1.10 [37]. Based on the Bayesian information criteria (BIC), the best model was chosen (nst = 6, rates = gamma for both COI and ITS analysis). With this model of evolution, trees were constructed using MRBAYES 3.2.7a [38]. The settings were as follows: 10 million generations, a sample frequency of 1000, and a burnin value of 5000 trees. For more detail, see [33,34].

Since the COI-based trees were less resolved than the ITS-based tree, we also tried to eliminate positions in the alignment that were saturated with multiple substitutions with the program Gblocks (Figure S1) [35] and constructed trees with the so-reduced alignment that was 315 bp long.

In addition, both sequences altogether were analysed with StarBeast3 v1.1.7 [39], which is a multi-individual multi-locus species tree estimation program, using Bayesian coalescent analysis, as implemented in the BEAST v2.7.3 package [40]. This approach takes into account that sequences do not evolve alone but are always present in some species which may originate from each other by divergence. Xml input files were created in BEAUTI v2.7.4, using the HKY + Γ + I model for both markers. The following settings were used for all analysis: base frequencies: 'empirical'; clock model: 'Strict clock Clock.rate: 1'; TreePrior: 'Yule Model'; popMean: Log Normal with M: −5 ans S: 1.2; clockRates: 'Exponential'. The analyses were run on BEAST software. Analyses were run for 10 million generations, sampling every 5000th generation. Tracer v. 1.7.1 [41] was used for examining the effective sample size (ESS) for parameters and determining the burnin. Trees and posterior probabilities were summarized using TreeAnnotator v. 2.7.3 and shown on the maximum clade credibility tree with median heights, with a posterior probability limit = 0.5 and burnin percentage = 10. The trees were drawn in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 1 June 2023).

2.5. Haplotype Network Analysis

Haplotype networks were built based on the COI alignment using POPART 1.7 [42] software with the TCS network inference method. A haplotype network is the sum of the shortest evolutionary pathways between the current haplotypes via subsequent mutations that connect the current DNA molecules via putative intermediate molecules.

3. Results and Discussion

3.1. Analysis Based on the ITS Region

We begin our analysis with the more conserved gene fragment of the nuclear ribosomal RNA region including the intervening ITS region: ITS1, the 5.8S rRNA coding sequence, and ITS2. A phylogenetic tree reconstructed on the base of the ITS regions by a Bayesian approach is provided in Figure 2. The left part of this figure presents the tree as a phylogram, in which lengths of branches are proportional to the number of accumulated nucleotide substitutions, which depends on both the time of divergence and the rate of molecular evolution. The right part of Figure 2 is the same mirrored tree as a cladogram, which shows only the tree topology. The cladogram is added to visualise divergence and the clustering of sequences differing in few substitutions only, which is not seen in the phylogram (on the left) because of too-short branches.

In general, the tree based on the ITS region corresponds well to the current taxonomy of Aeshnidae and shows most of the current genera as monophyletic, with one notable exception of the largest and most familiar genus, *Aeshna* Fabricius, 1775. In this tree, 16 clades can be recognized.

Clade 1 (*juncea*-clade) comprises two species, *Aeshna juncea* (Linnaeus, 1758) and *Aeshna subarctica* Walker, 1908. Surprisingly, *A. juncea* was represented by two clusters (not well seen in the phylogram on the left side of Figure 2 but visualised in the cladogram of its right side), one with specimens from the Caucasus and Transcaucasia region and the other

including the rest of specimens from elsewhere. Unfortunately, no American representative of *A. juncea* was available for the ITS region analysis. *A. juncea* and *A. subarctica* appeared as sister species, as expected.

Clade 2 (*grandis*-clade) is composed of *Aeshna grandis* (Linnaeus, 1758), which is the type species of the genus *Aeshna* Cowely (1934), *Aeshna viridis* Eversmann, 1836, *Aeshna serrata* Hagen, 1856, and *Aeshna crenata* Hagen, 1856. Strikingly, this clade of these four species diverges in only two monophyletic clusters, that for *A. crenata* and that for, altogether, *A. serrata*, *A. grandis* and *A. viridis* (Figure 2, see the cladogram on the right). Curiously, the three latter species, so different in appearance and ecology, look in the tree as if they were a single species, while *A. crenata*, representing the other cluster, is a species strongly resembling *A. serrata* in appearance and even sometimes confused with it.
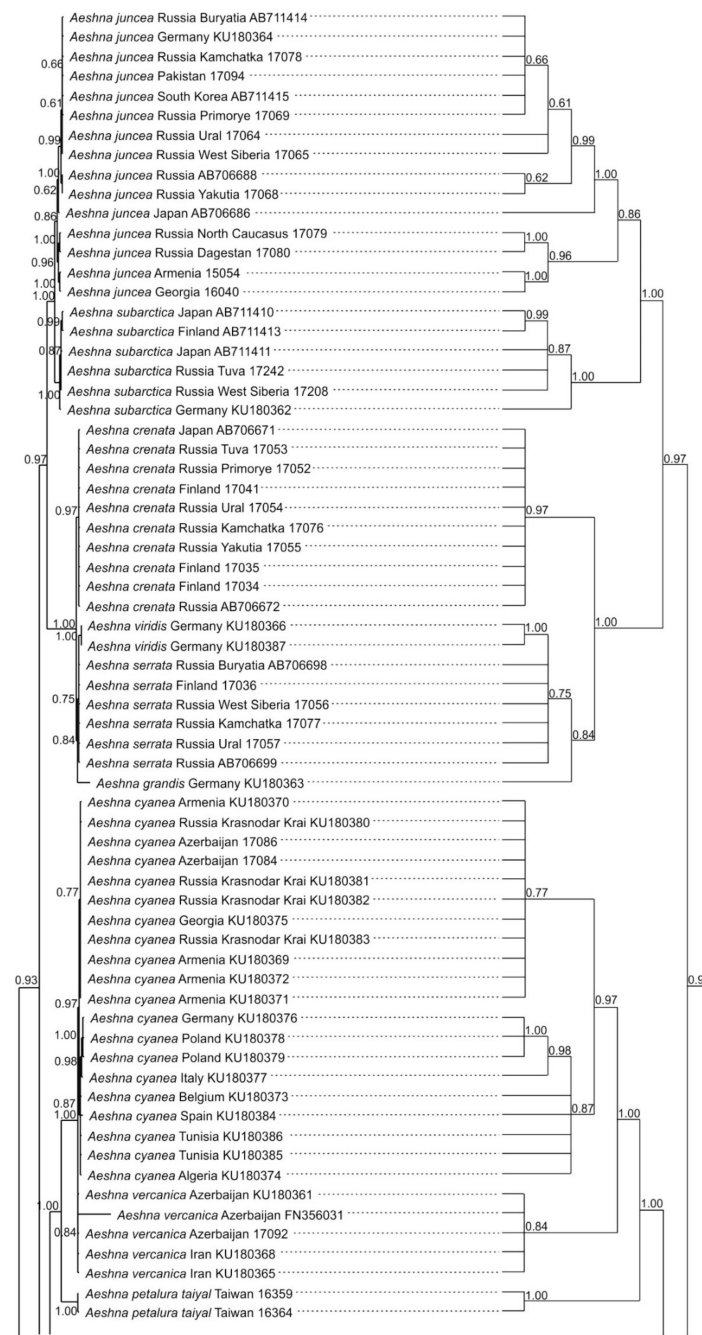
Figure 2. *Cont.*
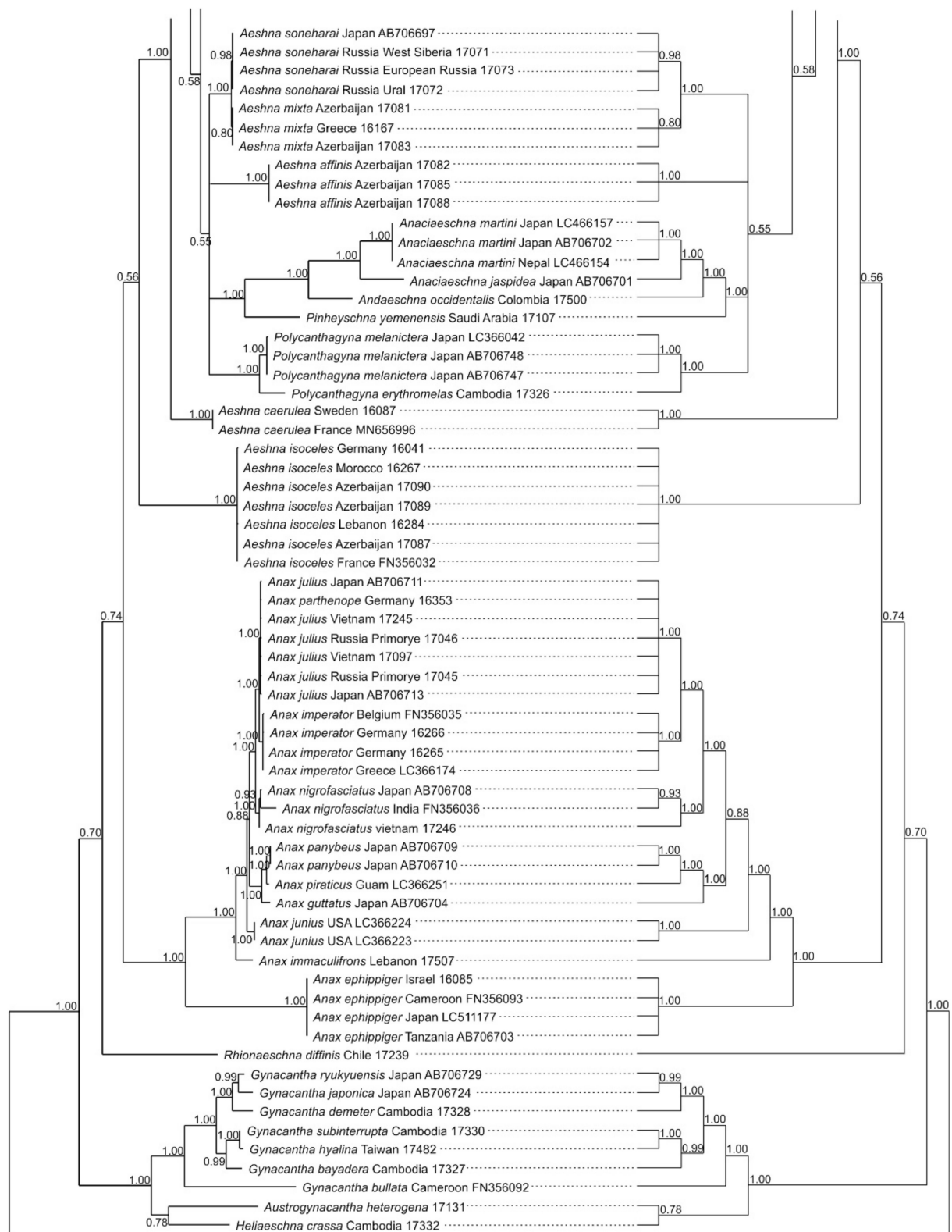
Figure 2. *Cont.*
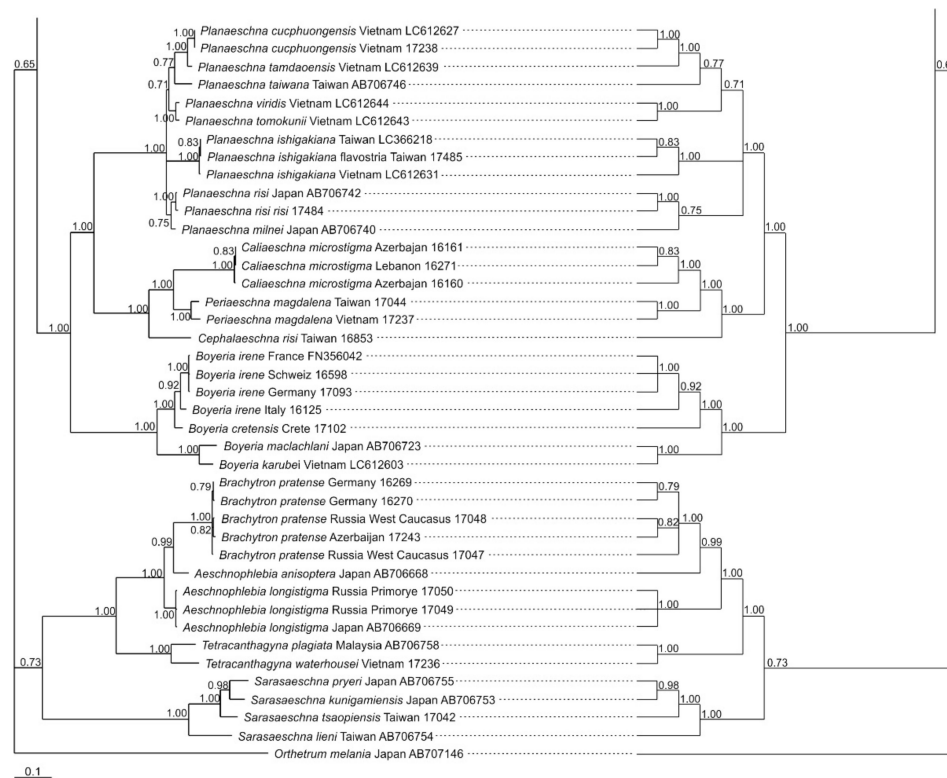
Figure 2. Bayesian phylogenetic tree reconstructed from the ITS region of representatives of Aeshnidae using MRBAYES 3.2.7a, shown as a phylogram on the left side and the mirrored cladogram on the right side. Bayesian posterior probability values are depicted at the nodes. Included are our own sequences (PCR number next to the name) and those retrieved from GenBank (accession number next to the name).

Clade 3 (*cyanea*-clade) includes *Aeshna cyanea* (Müller, 1764), *Aeshna vercanica* Schneider et al., 2015, and the East Asian *Aeshna petalura* Martin, 1908. Males of all these three species have a particular morphology of the upper appendages, which are broad and have a downward terminal hook, which resembles a raptor's beak. The possible relationship of *A. petalura* with *A. cyanea* was already suggested by Erich Schmidt [43]. Two clusters of *A. cyanea* can be recognized, one including specimens from the Caucasus and Transcaucasia and the other with specimens from Europe and North Africa (Figure 2, see the cladogram on the right side). This dichotomy has been recognized before [14,44], and is paralleled by the above-discussed divergence of *A. juncea* from the Caucasus and Transcaucasia versus from elsewhere. The American members of the '*umbrosa* group' ('paddle-tails') were not available for the ITS analysis.

Clade 4 includes *Aeshna mixta* Latreille, 1805 and *Aeshna soneharai* (Asahina, 1988) as very closely related sister taxa. Actually, their ITS region differs in just two substitutions in the ITS region. It is noteworthy that Onishko et al. [23], who raised the taxon *A. soneharai* to species level based on external characters, behaviour, sympatric occurrence, and differences in the mitochondrial COII sequences, also sequenced the ITS2 spacer and found it to be identical to *A. mixta*. We now have sequenced the broader ITS region and found that *Aeshna soneharai* differs in three nucleotide substitutions from *A. mixta*.

Clade 5 includes *Aeshna affinis* Vander Linden, 1820 only.

Clade 6 is represented by the two species of the genus *Anaciaeschna* Selys, 1878, *Andaeschna occidentalis* Bota-Sierra, 2019, and *Pinheyschna yemenensis* (Waterston, 1985). This clade represents members of three continents. Such a relation was already discussed by von Ellenrieder [45].

Clade 7 contains both species of the genus *Polycanthagyna* Fraser, 1933 available.

Clade 8 is represented solely by the species *Aeshna caerulea* (Ström, 1783).

Clade 9 is distinctly separated from all the above-mentioned clades and includes only one species: *Aeshna isoceles* (Müller, 1767), with all its sequences identical, although our analysis contains specimens comprising the whole geographical distribution of this species, including Europe, the Near East, West Asia, and North Africa.

It is noteworthy that the cluster uniting clade 9 with clades 1–8 has the negligible support of a 0.56 posterior probability value, which provides a strong argument against inclusion of *A. isoceles* into the genus *Aeshna*. The cluster uniting clades 1–8 has the highest support, 1.0, and could be considered as one genus named *Aeshna*, but this, at the same time, would imply synonymization of the genera *Anaciaeschna, Polycanthagyna, Andaeschna,* and *Pinheyschna* Peters et Theischinger, 2011 with *Aeshna*. This view is, however, not supported by the COI analysis (see below).

Clade 10 represents the genus *Anax*. The clade includes *Anax ephippiger* (Burmeister, 1839) and makes an additional genus as *Hemianax* Selys, 1883 unnecessary, which is in line with previous studies, for example the most recent one by [13]. *Anax imperator* Leach in Brewster, 1815 and *Anax parthenope* (Selys, 1839) are well separated, while *A. parthenope* (unfortunately represented in the ITS tree by a single specimen only) and *Anax julius* Brauer, 1865 are not separated.

Clade 11 is represented in our analysis only by one specimen and species, *Rhionaeschna diffinis* (Rambur, 1842) from Chile. Few members of this genus reach the Holarctic in southern North America. A better relation of this genus to the others can be seen in the StarBeast analysis of the combined genes (see below).

Clade 12 is composed of three genera: *Gynacantha* Rambur, 1842, *Austrogynacantha* Tillyard, 1908 and *Heliaeschna* Selys, 1882. Members of the last two genera are not present in the Holarctic.

Clades 13, 14 and **15** are an interesting complex of genera. All these genera have very strong supports on the tree and could be made clades of their own, so our subdivision of this complex into clades is rather arbitrary. Clade 13 includes *Planaeschna* McLachlan, 1896, *Caliaeschna* Selys, 1883, *Periaeschna* Martin, 1908, and *Cephalaeschna* Selys, 1883. Clade 14 is represented by the genus *Boyeria* McLachlan, 1896. Clade 15 is composed of *Brachytron* Evans, 1845, *Aeschnophlebia* Selys, 1883 and *Tetracanthagyna* Selys, 1883. These three clades strongly resemble clade 2 of the morphological cladistic tree by Ellenrieder [5], as containing the first two of its three groups. These three clades may be assumed as the subfamily Brachytroninae, as suggested previously [4]. Besides the morphological similarity, the members of this subfamily share also similarities in behaviour and habitat selection, with most of them preferring shady stream sections or marshes.

Clade 16 is represented by *Sarasaeschna* McLachlan, 1896 only (the node uniting it with clade 15 is scarcely supported, the posterior probability being 0.73).

3.2. Analysis Based on the COI Gene

For the analysis based on the COI gene fragment, we attempted several options. First, we reconstructed phylogenies based on long (632 bp) and short (339 bp) fragments of the COI gene. For the purpose of this study, we sequenced the long fragment. The short fragment was naturally less informative but had an advantage of having much better representation in GenBank [15], so that we were able to include many more species. We also attempted a Gblocks analysis, which removes positions in the alignment that are saturated by substitutions or poorly aligned. These three analyses yielded similar results, and although the long fragment tree included fewer species, it was highly representative for the Holarctic because of our efforts to de novo sequence relevant specimens. Therefore, we will describe below the phylogenetic tree reconstructed on the base of the long fragment (Figure 3).

3.2.1. Analysis Based on Long COI Fragment

Compared to the ITS tree, the COI tree (Figure 3) contained much more basic clades, including smaller number of sequences. Furthermore, the COI sequence did not resolve the

phylogenetic relationships of quite a number of those basic clades, thus revealing polytomy. Nevertheless, grouping of genera in this tree appeared very interesting.
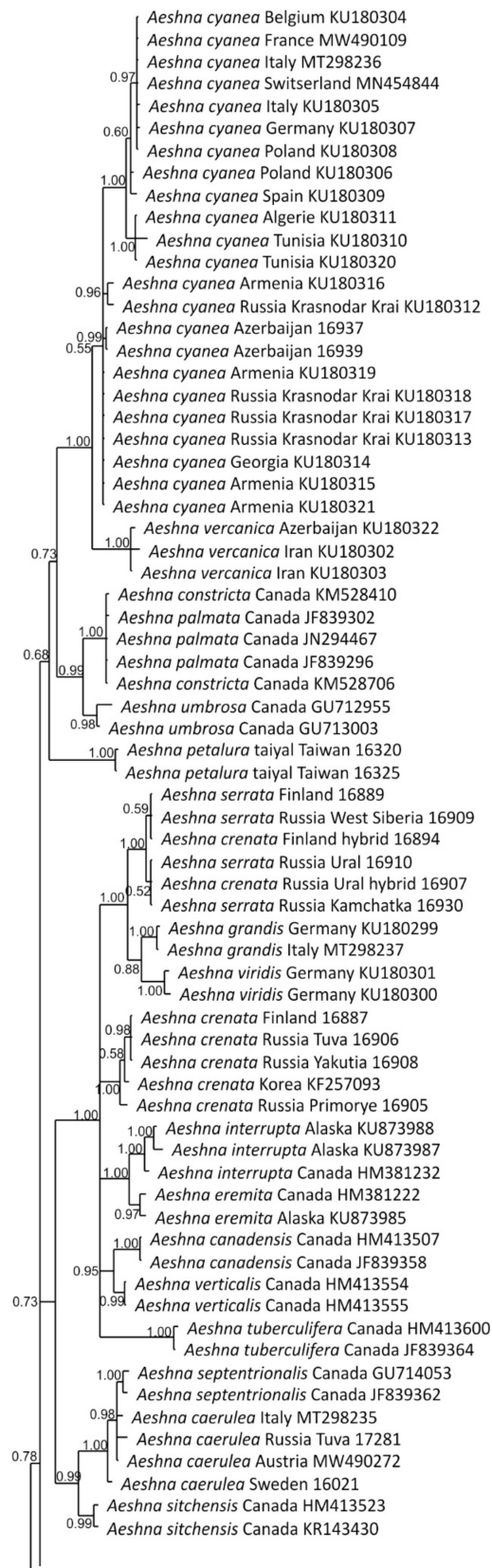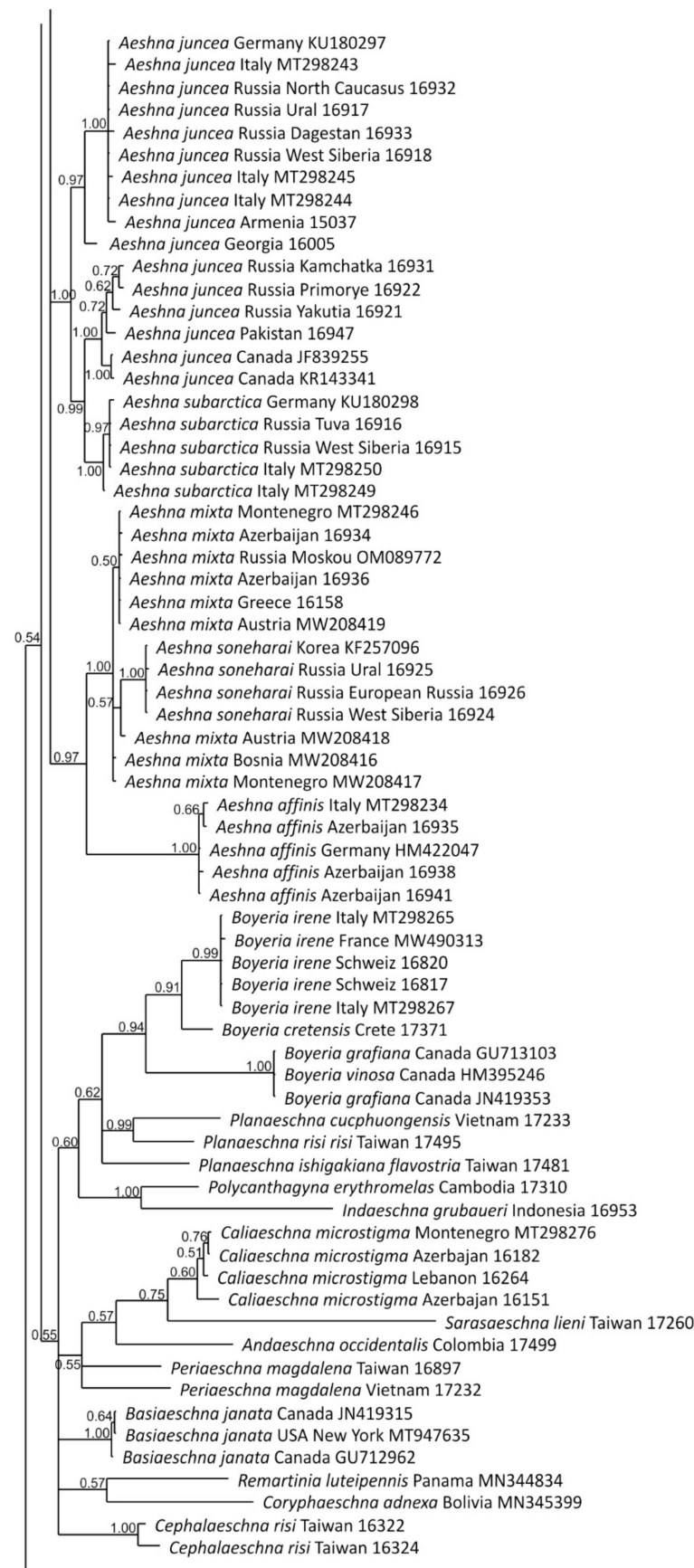

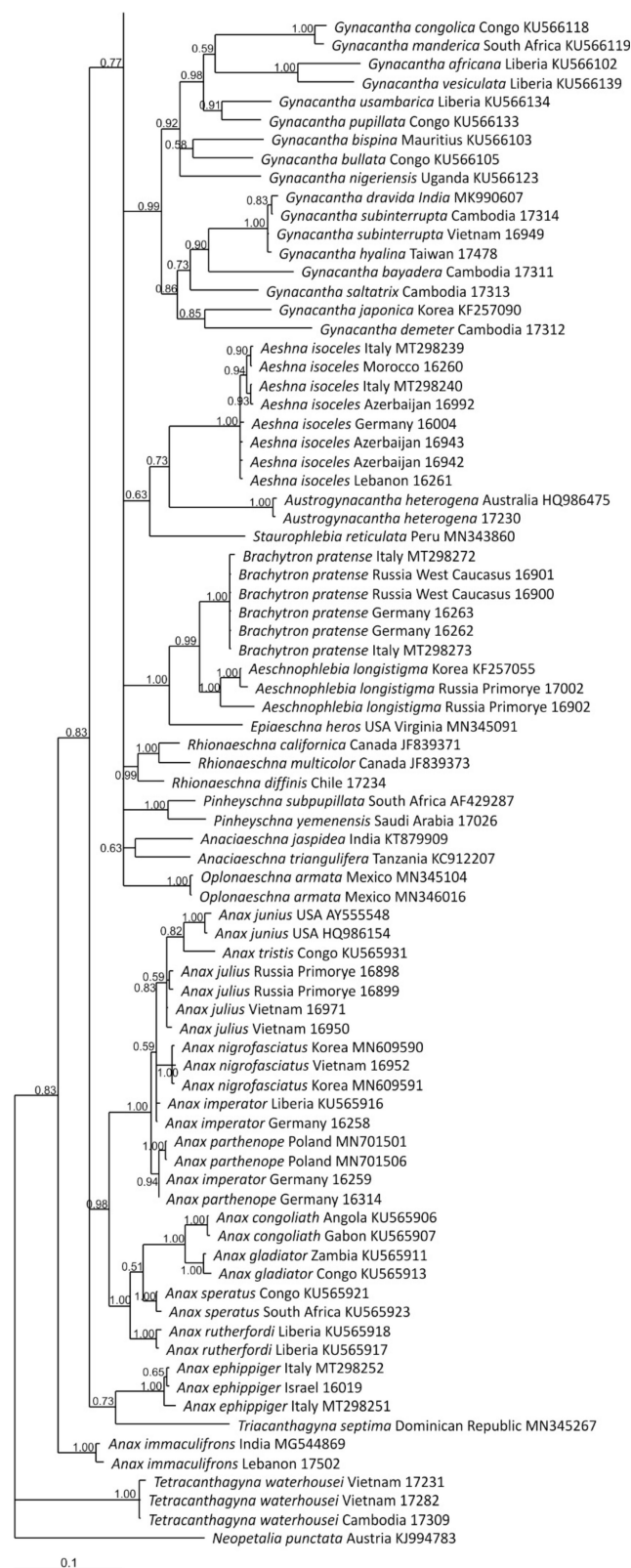**Figure 3.** *Cont.*

Figure 3. *Cont.*

Figure 3. Bayesian tree reconstructed from the long (632 bp) fragment of the COI gene of representatives of Aeshnidae using MRBAYESs 3.2.7a, shown as a phylogram on the left side and the mirrored cladogram on the right side. Bayesian posterior probability values are depicted at the nodes. Included are our own sequences (PCR number next to the name) and those retrieved from GenBank (accession number next to the name).

The uppermost main node of the tree (Figure 3), with a weak support of 0.68, corresponds to the ITS clade 3 and can be called the *cyanea*-clade. It contains three well supported branches. The first of them is composed of *A. cyanea* and *A. vercanica*; the second includes the North American members of the '*umbrosa* group' ('paddle-tails'), *Aeshna umbrosa* Walker, 1908, *Aeshna constricta* Say, 1840, and *Aeshna palmata* Hagen, 1856, the sequences of which were available in GenBank (2023). The third branch includes the Asian *A. petalura*.

Specimens of *A. constricta* Say, 1840 and *A. palmata* Hagen, 1856 are interspersed in the tree (Figure 3), as if they were the same species. The haplotype network (Figure 4) shows that they share the most common allele of the studied COI fragment.
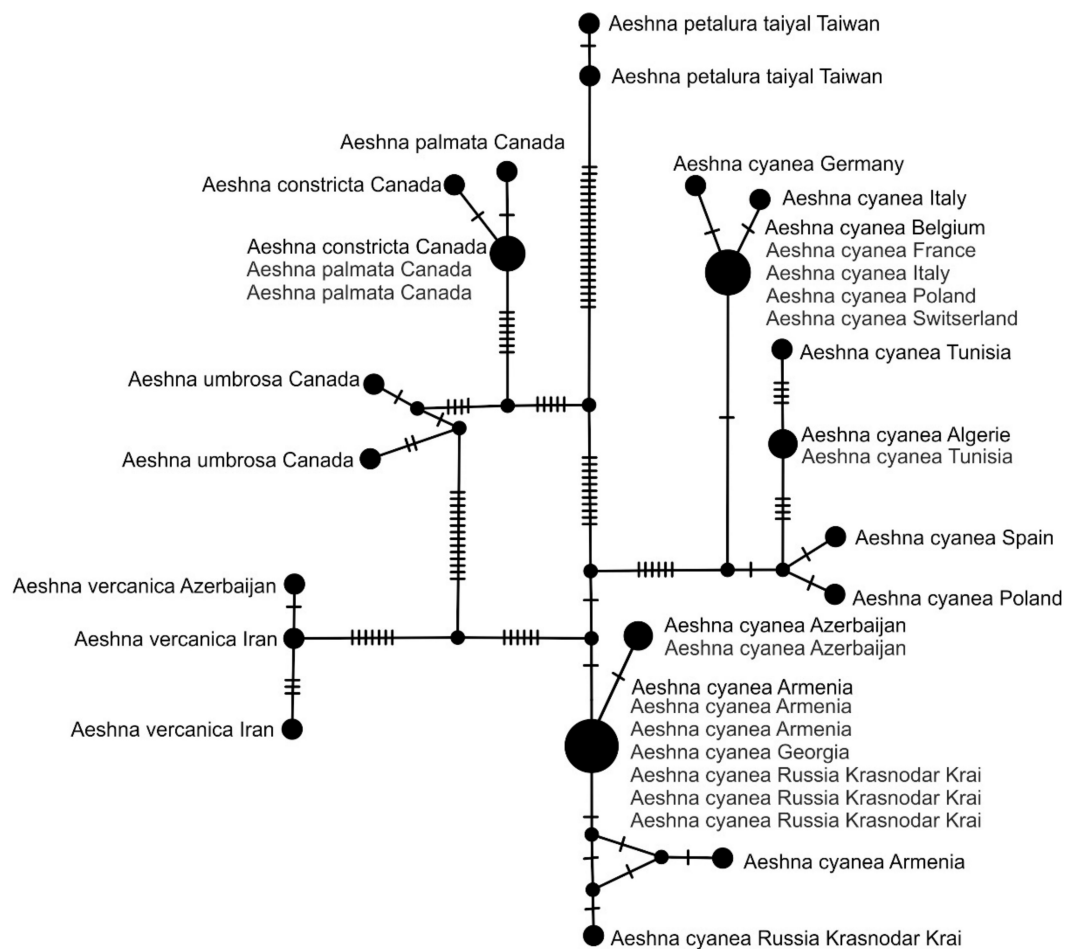
Figure 4. Haplotype network of the long COI fragment for *Aeshna cyanea*, *A. vecranica*, *C. umbrosa*, *A. constricta*, *A. palmata*, and *A. petal.* Like in the ITS analysis, specimens of *A. cyanea* from North Africa and Europe cluster together. However, specimens of *A. cyanea* from the Caucasus and Transcaucasia region on one hand and from the rest of the range on the other had no longer form sister clades as in the ITS tree (Figure 2). Instead, the Caucasian plus Transcaucasian specimens radiate from the base of the *A. cyanea* cluster, while the European plus African cluster is now internal, as a sprouting among them. This can be interpreted as the species *A. cyanea* having originated in the Caucasian area and then one of its lineages having spread to the west and occupied vast European and North African territories. *A. vercanica* and *A. cyanea* are sister branches in the COI tree (Figure 3), but the node of *A. cyanea* has a weak support of 0.55.

The next clade, with the highest possible support of 1.0 (the *grandis*-clade) corresponds to the ITS clade 2. Besides the West Palaearctic *A. grandis*, *A. viridis*, *A. serrata*, and *A. crenata*, it also includes the North American *Aeshna interrupta* Walker, 1908, *Aeshna eremita* Scudder, 1866, *Aeshna canadensis* Walker, 1908, *Aeshna verticalis* Hagen, 1861, and *Aeshna tuberculifera*

Walker, 1908 in the COI tree (Figure 3). The species *A. grandis* and *A. viridis* form clusters of their own, having rather recently diverged but having the maximum support of 1.0, and are not united with *A. serrata* as they were in the ITS tree (Figure 2).

Two putative cases of introgression between *A. serrata* and *A. crenata* were detected. Two specimens, from Finland and the Ural Mountains (Russia), were identified as *A. crenata* but had a COI sequence identical to *A. serrata*. Since their identification by morphological means was unequivocal (the specimen from Ural was collected and examined by one of us), we may suggest that this was an old mitochondrial introgression from *A. serrata* into *A. crenata* rather than recent hybridization.

The next well supported clade includes the Eurasian species *A. caerulea* and the North American species *Aeshna septentrionalis* Burmeister, 1839 and *A. sitchensis* Hagen, 1861. The node uniting this clade to the previous one is not supported (0.73), so should not be taken into account. The branch of *A. septentrionalis* is not a sister one to any other species, but appeared as an inner branch inside *A. caerulea*. This is also well illustrated by the haplotype network constructed for this clade (Figure 5), where alleles revealed in the two Canadian species appeared to independently originate from that found in a specimen from Austria. So, our data rule out the species level of *A. septentrionalis.* Therefore, we synonymize it with *A. caerulea* at the species level as suggested before [46–48], downgrading it to the subspecies of the latter.
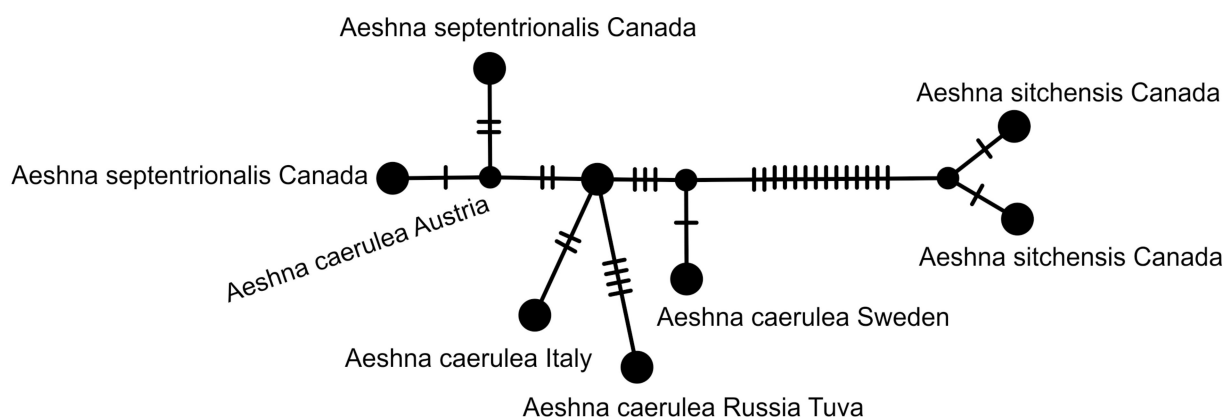
Figure 5. Haplotype network of the long COI fragment for *Aeshna caerulea*, *A. septentrionalis*, and *A. sitchensis*.

The next large clade (*juncea*-clade) in the COI analysis corresponds to the ITS clade 1 and comprises the same two species, *A. juncea* and *A. subarctica*. Like in the ITS analysis, there are two clusters, but their content is striking. One of them comprises specimens of *A. juncea* originating from the West Palaearctic, up to West Siberia to the east. The specimens from the Caucasus region (Armenia, Georgia, North Caucasus) are no longer separated but are interspersed with European specimens. The second cluster contains specimens of *A. juncea* originating from the eastern half of Eurasia (Pakistan and the Russian Far East), and from Canada (North America). This result is in agreement with that reported by Kohli et al. [11], who found common COI haplotypes of North American and East Asian (Japan and China) specimens of *A. juncea*, which were different from those of European specimens. Unexpectedly, the second cluster contains, also, all specimens of *A. subarctica*. Thus, the structure of the *juncea*-clade in the COI tree contradicts not only that in the ITS tree but also the long-established morphological systematics. Obviously, we faced a case wherein mitochondria exhibit a phylogeny of their own, discordant to that of nuclear sequences and that resulting from morphological data. Such cases frequently appear in Odonata [20,49,50]. This also underlines the close relationship between *A. juncea* and *A. subarctica*. The COI haplotype network of the two species shows, from a different aspect, the same pattern, in which the Far Eastern and North American specimens of *A. juncea* are more separated from the West Palaearctic *A. juncea* than from *A. subarctica* (Figure 6).
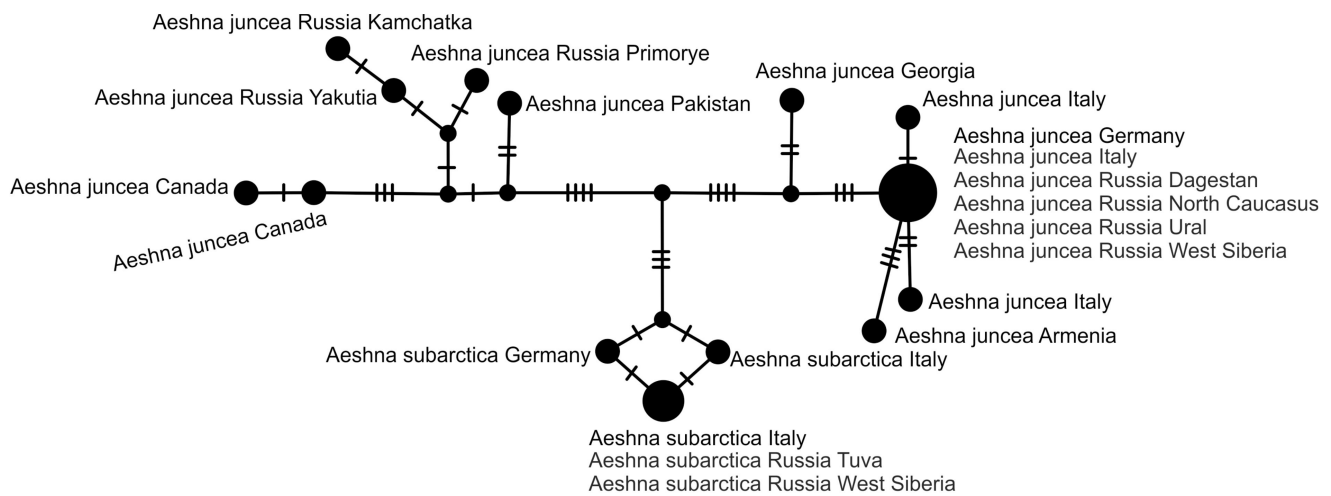
Figure 6. Haplotype network of the long COI fragment for *Aeshna juncea* and *A. subarctica*.

The next clade in the COI tree corresponds to the ITS clade 4 but includes *A. affinis* as its first divergence, as expected. In the COI tree, *A. mixta* and *A. soneharai* are not two sister branches, as in the ITS tree, but *A. soneharai* appeared as an inner branch inside *A. mixta*. At the same time, in the haplotype network (Figure 7), these species are independent branches. The Kimura 2-parameter distance (for details see [33]) between *A. mixta* and *A. soneharai* is small (0.02), suggesting very recent separation of these taxa (Figure 7). For sequences with so few substitutions, a haplotype network is a more adequate representation than a phylogenetic tree, since correct phylogenetic analysis demands a considerable signal from many substitutions.
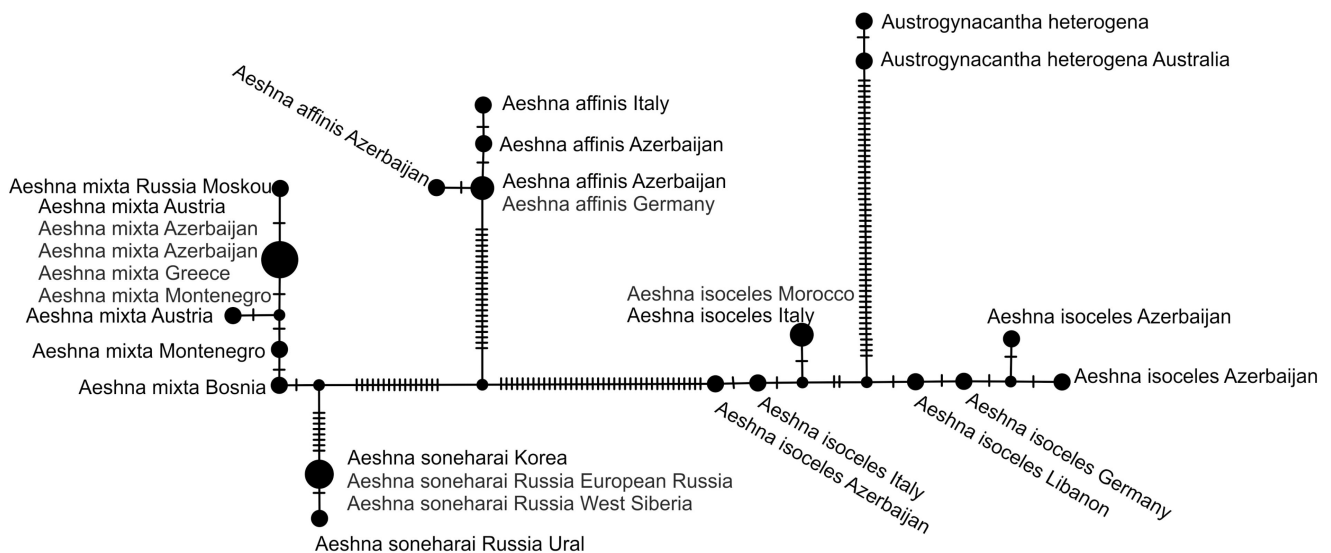
Figure 7. Haplotype network of the long COI fragment for *Aeshna mixta*, *A. soneharai*, *A. affinis*, *A. isosceles*, and *Austrogynacantha heterogena*.

The next large cluster in the COI tree is not supported. Inside this, a well supported branch represents the genus *Boyeria*, with four Holarctic species; the next one contains two species of *Planaeschna*, *P. cucphuongensis* Karube, 1999 and *P. risi* Asahina, 1981. The third species of this genus, *P. ishigakiana* Asahina, 1951, fell aside in a polytomic cluster, with a negligible support of 0.62, with the two previous species and *Boyeria*. The next well supported branch is represented by *Polycanthagyna erythromelas* (McLachlan, 1896) and *Indaeschna grubaueri* (Förster, 1904), tightly clustering together with the highest possible

support, 1.00. The same clustering of these two species from different genera is supported also by the combined anaylsis by StarBeast (see below). The following group of eight sequences represents the genera *Caliaeschna*, *Sarasaeschna*, *Andaeschna*, and *Periaeschna* but it does not form a clade, as neither of its nodes is supported. Even representatives of the same species do not show significant clustering. For instance, all the four specimens of *Caliaeschna microstigma* (Schneider, 1845) cluster to each other with supports not higher than 0.76, with a specimen from Azerbaijan distant from the other three (including the second specimen from Azerbaijan). The two specimens of *Periaeschna magdalena* Martin, 1909 do not cluster with each other at all. The genera *Cephalaeschna*, *Periaeschna,* and *Planaeschna*, which clustered with *Caliaeschna* in the ITS tree, are now sorted apart. The haplotype analysis also showed that *Caliaeschna* is not closely related to *Cephalaeschna*, *Planaeschna*, *Sarasaeschna*, and *Remartinia* Navás, 1911 (Figure 8). Thus, *Caliaeschna* cannot be united with *Cephalaeschna*, as discussed previously [51].
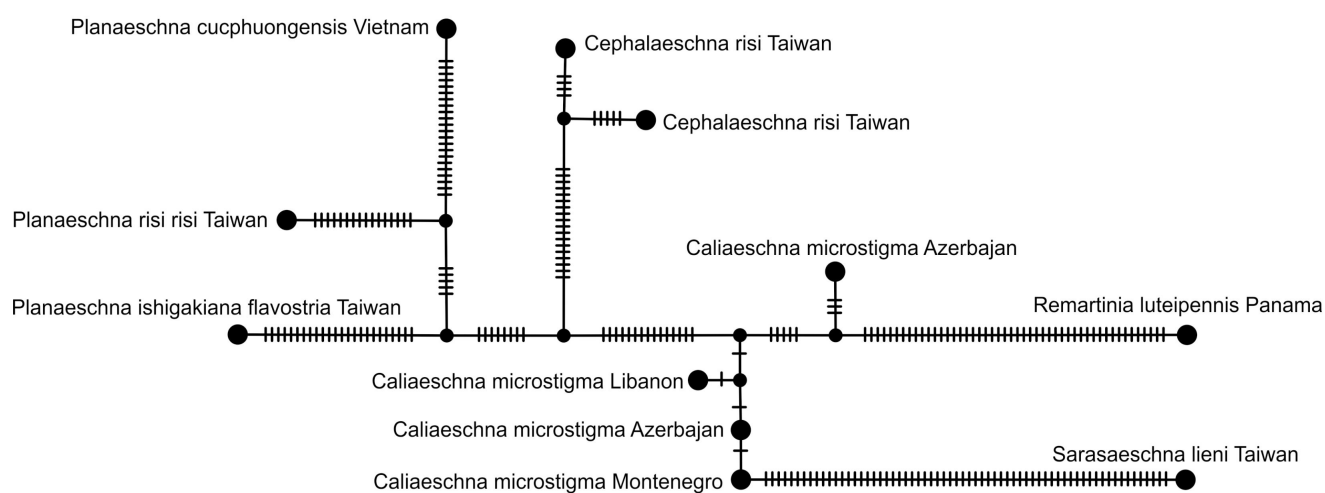
Figure 8. Haplotype network of the long COI fragment for the genera *Planaeschna*, *Cephalaeschna*, *Caliaeschna*, *Remartinia*, and *Sarasaeschna*.

Our specimens of *Caliaschna microstigma* (the only species of its genus) originate from all over its range, from Montenegro to Azerbaijan. Their cluster is not supported, although the sequences do not differ much, as suggested by the haplotypic network (Figure 8). In particular, a specimen from Balakan District of Azerbaijan (sequence 16161) represents a local population characterised by strongly reduced antehumeral stripes, not paralleled by shrinkage of other pale markings [52]. At the same time, the specimen from Ordubad District of Azerbaijan (sequence 16160) has normal, not reduced antehumeral stripes. The reduced stripe was observed in the neighbouring Georgia [53], eastern parts of Turkey (unpublished), while in some populations of Dagestan (Russia) and SE Turkey, individuals with both stripe versions fly together (unpublished). Both our analyses, of ITS (Figure 2) and COI (Figure 3), did not reveal any divergence of the Balakan specimen from those from elsewhere; its COI sequence is most close to the specimen from Montenegro. This fact suggests that the reduction of the antehumeral stripe does not manifest a special Caucasian taxon, even of a subspecific rank.

The next three isolated branches are composed of (i) three specimens of *Basiaeschna janata* (Say, 1840), representing the monotypical American genus *Basiaeschna* Selys, 1883, (ii) *Remartinia liteipennis* (Burmeister, 1839) and *Coryphaeschna adnexa* (Hagen, 1861), but the support of this branch of 0.57 is negligible; (iii) two specimens of *Cephalaeschna risi* Asahina, 1851, the only representative of its large genus in our analysis.

The next large and well supported clade includes only the genus *Gynacantha*, represented in our analysis by quite a number of species. Two major, well supported branches can be recognized in this clade, one representing the African members, the other the Asian members of the genus (as seen from country annotations at the species). A corresponding topology is seen in the haplotype network, with the species *Gynacantha bispina* Rambur, 1842 from Mauritius placed in between (Figure 9). The taxonomy of this genus in Africa is problematic [54]. Looking deeper on the species level, a high similarity of *Gynacantha congolica* and *G. manderica* was seen, and the difference in the haplotype tree was beyond the species level (Figure 9); however, we did not have the corresponding ITS sequences to definitively synonymize them. At the same time we propose to synonymize the Asian *Gynacantha hyalina* Selys, 1882 with *Gynacantha subinterrupta* Rambur, 1842; this is supported by the ITS (Figure 2) and COI (Figure 3) trees and the haplotype network (Figure 9).
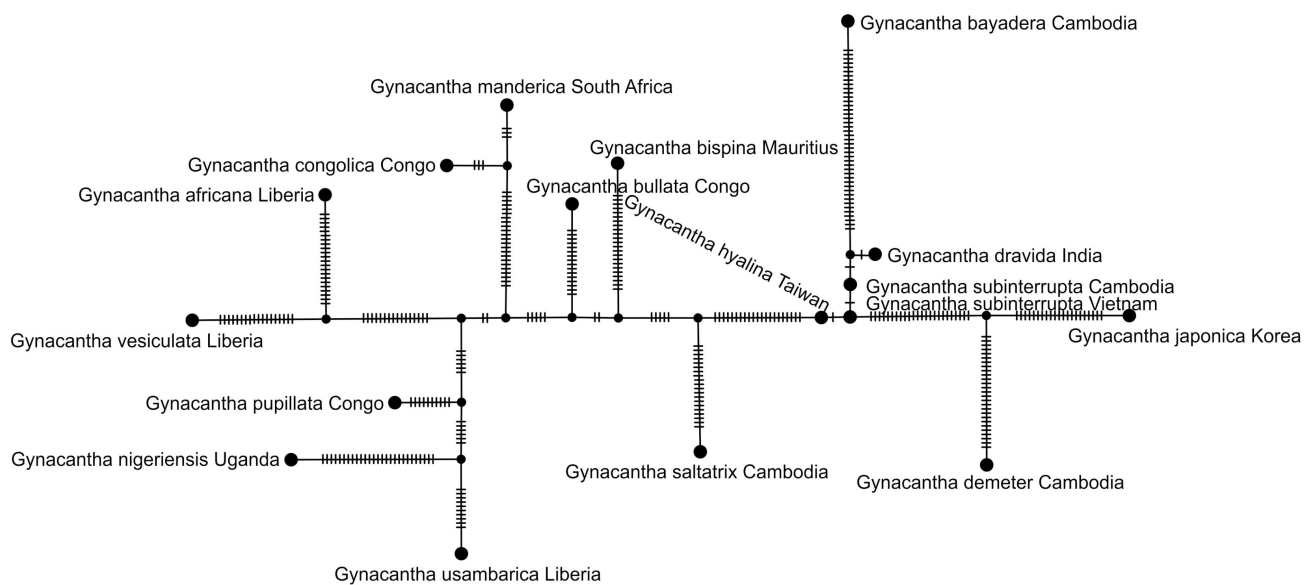
Figure 9. Haplotype network of the long COI fragment for representatives of the genus *Gynacantha*.

The next clade includes *A. isoceles* only, so corresponds to the ITS clade 9. We have analysed specimens from distant geographical regions comprising the whole range of the species and found it highly homogeneous. There is no place for subspecies like *A. isoceles antehumeralis* Schmidt, 1950 (alternatively *Anaciaeschna isoceles antehumerlis*) as suggested by Schmidt [43]. Both markers analysed did not reveal any relationship of this species to *Anaciaeschna*, as supposed previously [43,55]. The relationship to *Austrogynacantha* (Figure 3), as also previously illustrated by Carle [9]'s supplement (COI tree), seems to be an artefact, since the node is weakly supported (the posterior probability being 0.73). It is noteworthy that this node is no longer present in the Gblock tree (Figure S3). Also, the haplotype network shows a far separation of both genera (Figure 10). Furthermore, this relation was not indicated by the ITS analysis. The clustering of the branch formed by a single specimen of *Staurophlebia reticulata* (Burmeister, 1839) with *A. isoceles* and *Austrogynacantha* is insignificant (0.73).
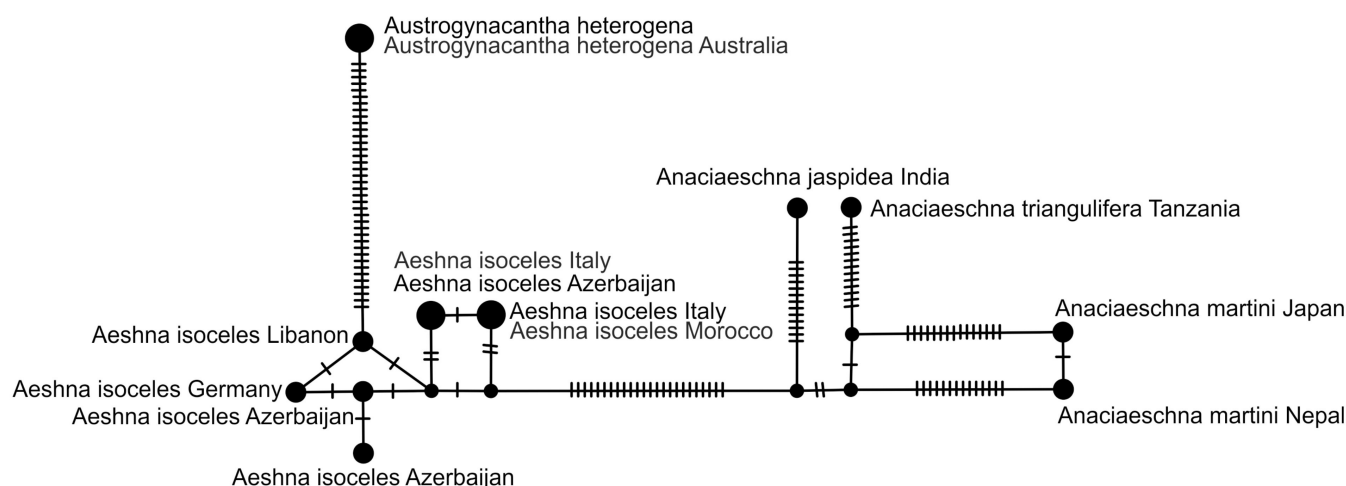
Figure 10. Haplotype network of the long COI fragment for *A. isoceles, Austrogynacantha heterogena,* and *Anaciaeschna* spp.

While the ITS tree showed well the subfamily Brachytroninae, represented by three clades 13–15 (see above), in the COI tree it is no longer traced, but split in as many as six branches (Figure 3).

A robust clade with the maximum support is composed of the genera *Brachytron, Aeschnophlebia,* and *Epiaeschna* Hagen in Selys, 1883. The West Palaearctic genus *Brachytron* consists of a single species, *Brachytron pratense* (Müller, 1764), which is represented in our analysis by specimens from throughout its geographical range. The haplotype network (Figure 11) also suggests a very close proximity of representatives of the above genera. The genus *Tetracanthagyna,* which clustered in the ITS analysis with the genus *Brachytron,* is now far outside this cluster, forming the most basic clade of the Aeshnidae tree (Figure 3). Such different sorting of the genus *Tetracanthagyna* in these two analyses is not seen with any other genus of the family, and the reason for this discrepancy remains unclear. However, the ITS's sorting of it together with *Brachytron* fits much better with the morphological and biological criteria [5].

The next four clades each consist of 2–3 specimens of one genus, respectively. These are *Rhionaeschna* Förster, 1909, represented by three species, *Pinheyschna* (two species), *Anaciaeschna* (two species), and *Oplonaeschna* Selys, 1883 (two specimens of *O. armata* (Hagen, 1861)). Three of these nodes are well supported, but that of *Anaciaeschna* is not, and neither did it showed affinity for *A. isoceles,* sometimes attributed to this genus (see above).

The following robust clade consists of the members of the genus *Anax* (corresponding to clade 10 in the ITS analysis); however, without *A. immaculifrons* (see below) and *A. ephippiger,* both not associated with other *Anax* and the latter loosely clustering with the genus *Triacanthagyna* Selys, 1883. The latter strange sorting seems to be an artefact, as it is no longer present if we remove positions in the alignment that are saturated by substitutions or are poorly aligned (Figure S2), and neither is observed in the tree based on the short COI fragment (see below).

Two clusters can be recognized in the main *Anax* clade: one consists of *Anax tristis* Hagen, 1867, *A. junius* (Drury, 1773), *A. julius, A. nigrofasciatus* Oguma, 1915, *A. imperator* and *A. parthenope;* the second consists of *A. congoliath* Fraser, 1953, *A. gladiator* Dijkstra et Kipping, 2015, *A. speratus* Hagen, 1867 and *A. rutherfordi* McLachlan, 1883. The same relationships between the *Anax* species are seen in the haplotype network (Figure 11). In the combined gene analysis by StarBeast (see below), *Anax walsinghami* McLachlan, 1883 is added to the first group, and *A. immaculifrons* is sorted between all *Anax* species and *A. ephippiger.*
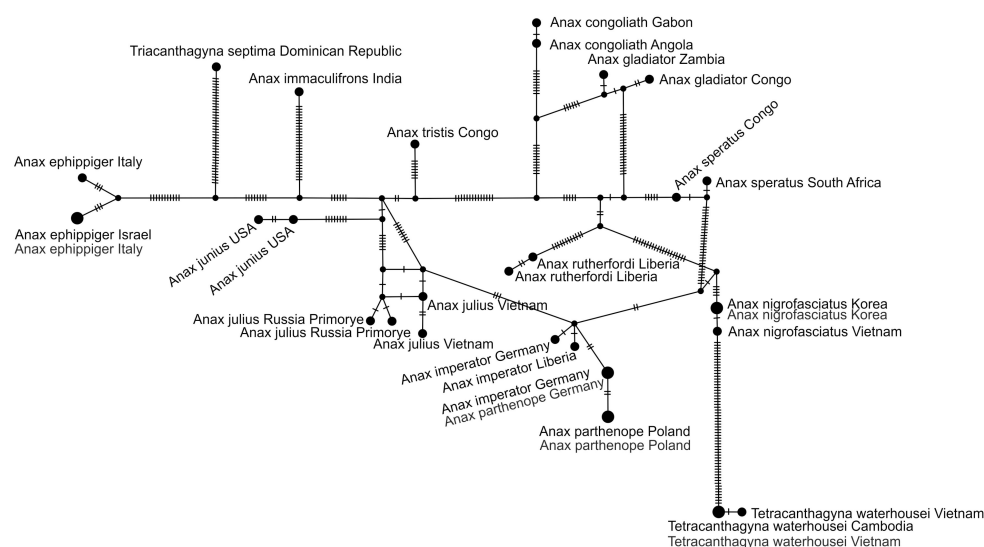
Figure 11. Haplotype network of representatives of the genera *Anax, Triacanthagyna*, and *Tetracanthagyna* for the COI long fragment.

It was reported before that *Anax imperator* and the European *Anax parthenope* s. str. are a rare case of a pair of very different species of Aeshnidae that are not separated based on the COI sequence because of haplotype sharing [10]. This partly concerns our COI tree as well (Figure 3), where the sequence '*Anax imperator* Germany 16259' does not cluster with the two other sequences of this species (including the specimen from the same place) but appears identical to that of *A. parthenope* s. str., and so gets to the cluster of the latter. This sequence was obtained by us from a doubtless male specimen of *A. imperator*. Moreover, the sequence '*Anax imperator* Germany 16258' from another male specimen of the same series appeared close to that of *A. imperator* from Liberia (Figure 3). We have to consider this as a case of introgression of mitochondria from *A. parthenope* s. str. to *A. imperator*, similarly to the above-discussed case of the introgression from *A. serrata* to *A. crenata*. It is noteworthy that this introgression case was recognised in Germany, whereas Geiger et al. [16] did not register a COI haplotype sharing of *A. imperator* and *A. parthenope* in Central Europe. *A. imperator* and *A. parthenope* s. str. are clearly separated by the ITS analysis (Figure 2).

Anax parthenope, in the hitherto prevailing broad sense. is not monophyletic in the COI tree, as was also shown before [13], but is split into the West Eurasian and the East Asian branches. The former represents *A. parthenope* s. str., while the East Asian (including the Far Eastern Russian) specimens represent the taxon *A. julius* Brauer, 1865. Therefore, we assume the latter as a separate species *A. julius*, as originally described in detail by Brauer [56] and later again supported by different authors [57–60].

Strikingly, in the COI tree, *Anax immaculifrons* forms a lineage which branches from the Aeshnidae stem very early, just after the branching of the *Tetracanthagyna* clade (Figures 3 and 4). This result appeared to be robust and is reproduced in all our phylogenetic attempts based on the COI gene. This is very strange, not only because it contradicts the well established taxonomy, but also up to the haplotype network (Figure 11), where *A. immaculifrons* is set apart of other *Anax* but obviously related to them, its root being at the point where *Tetracanthagyna* branches off. The BLAST search in GenBank [15] for the homology to the COI sequence of *A. immaculifrons* unequivocally reveals sequences of other species of *Anax* as most closely related to it. This result is difficult to interpret and the most likely explanation is again an artefact of similarity by chance, which could be facilitated by, e.g., some abnormal substitution rate in the evolutionary lineage leading to *A. immaculifrons*, or by some structural rearrangement(s). The problem is resolved in the StarBeast analysis (see below).

The last clade is represented by two species and three specimens of *Tetracanthagyna waterhousei.* In contrast to our ITS phylogenetic reconstruction, it does not cluster with

Brachytron, but appears to be the most ancient branch of Aeshnidae. This topology is also robust and is retained in the Gblocks tree reconstructed after the removal of positions by substitutions or due to poor alignment (Figure S1).

3.2.2. Analysis Based on a Short COI Fragment

More sequences are available in GenBank [15] of a shorter (339 bp) fragment of the COI gene, so that more species and genera could be included.

The main topology of the tree reconstructed on its base (Figure 12) did not change as compared to that using the longer COI fragment, with some diverging aspects, as follows.
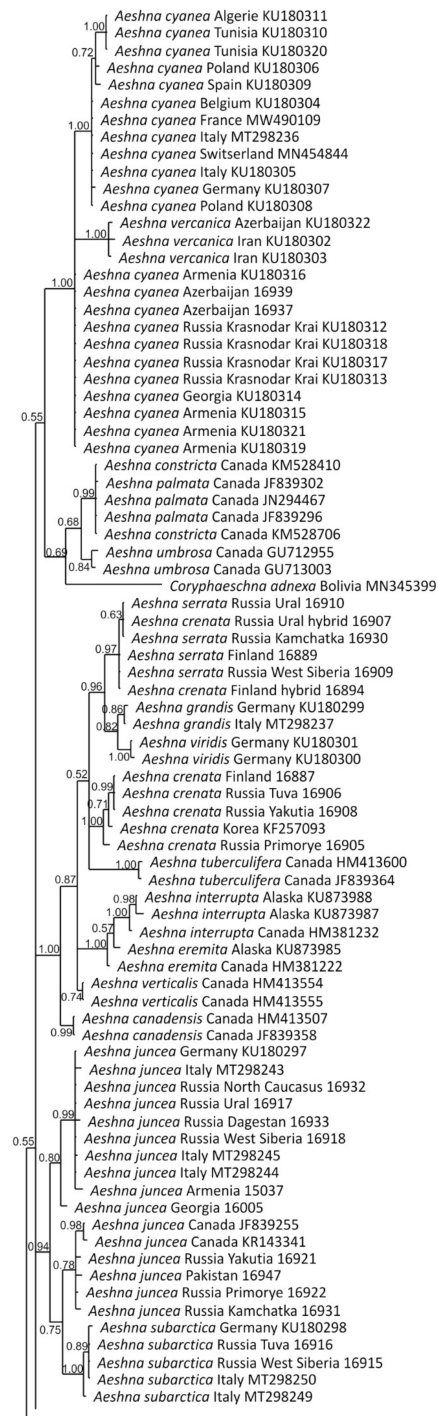
Figure 12. *Cont.*
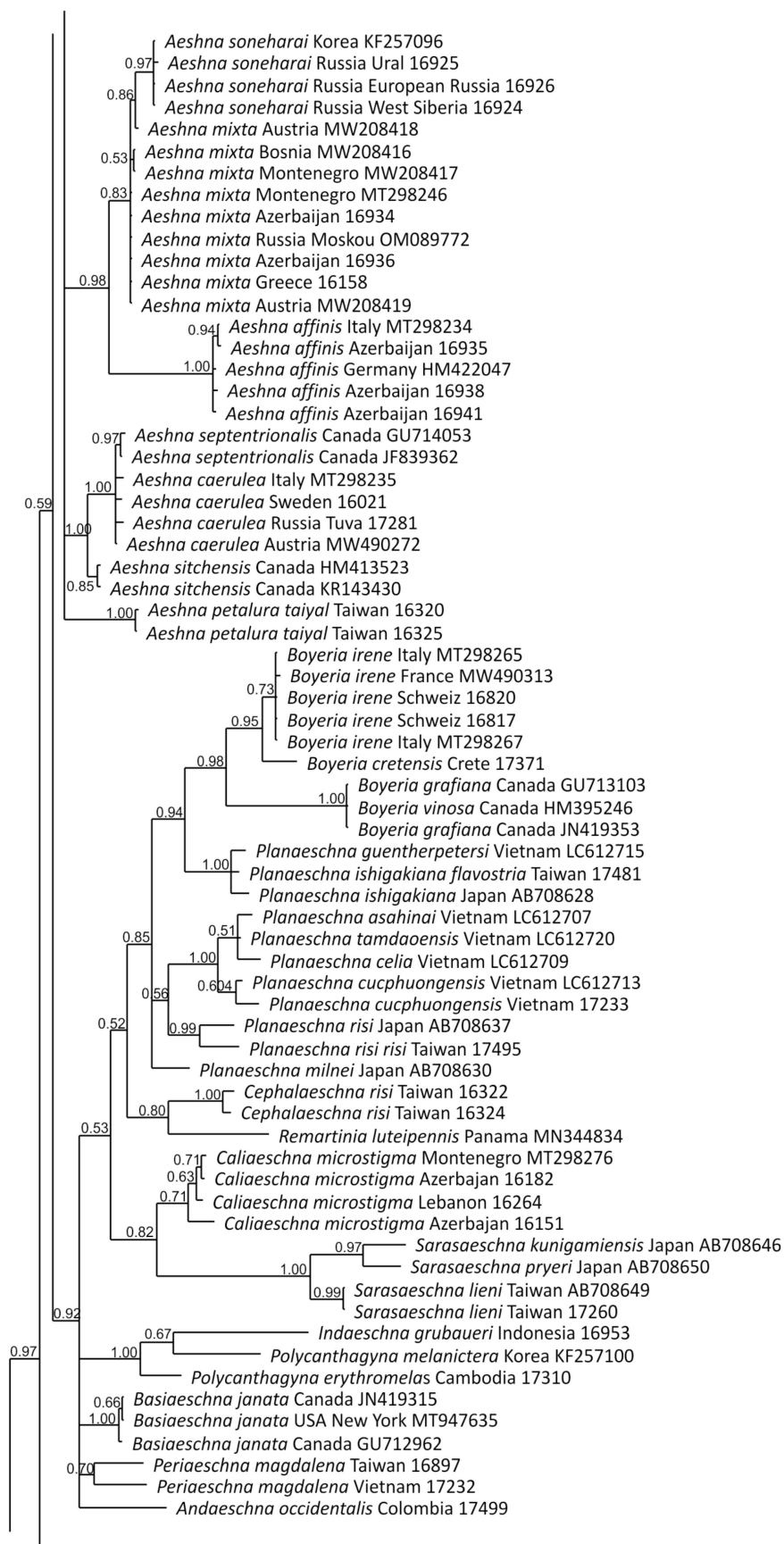
Figure 12. *Cont.*
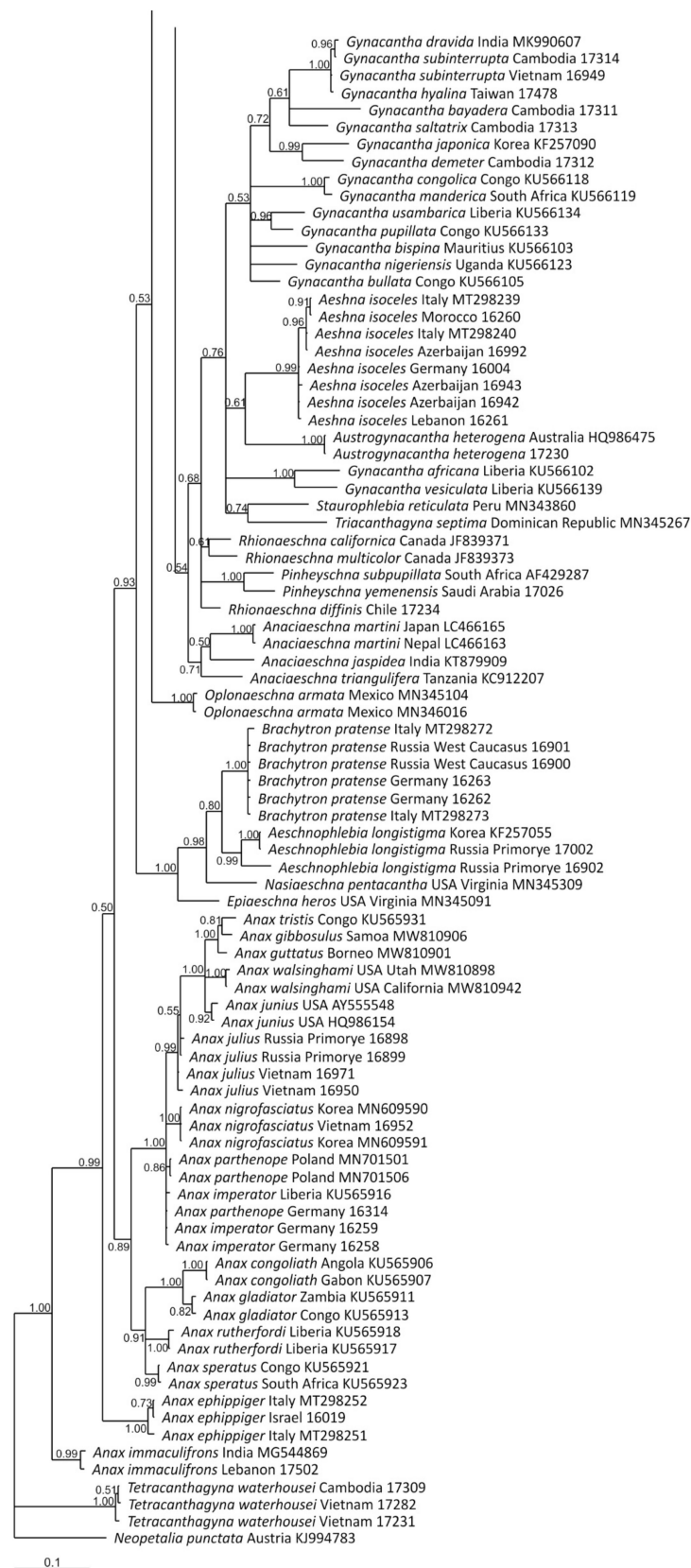
Figure 12. Bayesian tree reconstructed from the short (341 bp) COI gene fragment of representatives of Aeshnidae using MRBAYESs 3.2.7a. Bayesian posterior probability values are depicted at the nodes. Included are our own sequences (PCR number next to the name) and those retrieved from GenBank (accession number next to the name).

A strange sorting of *Coryphaeschna adnexa* (Hagen, 1861) in the clade of *A. cyanea* appeared. But this is no longer present in the Gblock tree (Figure S1), so has to be interpreted as an artefact. The sorting of *A. petalura* outside the *A. cyanea* clade seems to be due to shorter sequences of other members in the clade.

In the tree recognised for the shorter COI, some affinity reappeared (but with rather weak support of 0.53) between *Caliaeschna*, *Sarasaeschna*, *Planaeschna*, *Cephalaeschna*, *Remartinia*, and *Boyeria*, so resembling the results of the ITS analysis (clades 13–15, see Figure 2).

The clade including the West Palaearctic genus *Brachytron*, besides the expected *Aeschnophlebia* and *Epiaeschna*, is now updated with *Nasiaeschna* Selys in Förster, 1900. All these genera share similar biology, being on the wing in spring and preferring strongly vegetated lentic or slowly flowing habitats. For this case, we constructed a haplotype tree with both the short and long fragments of COI, resulting in the same topology. To show also the relationship with the genus *Nasiaeschna*, the short COI version is given (Figure 13), while the long version is provided in Figure S1.
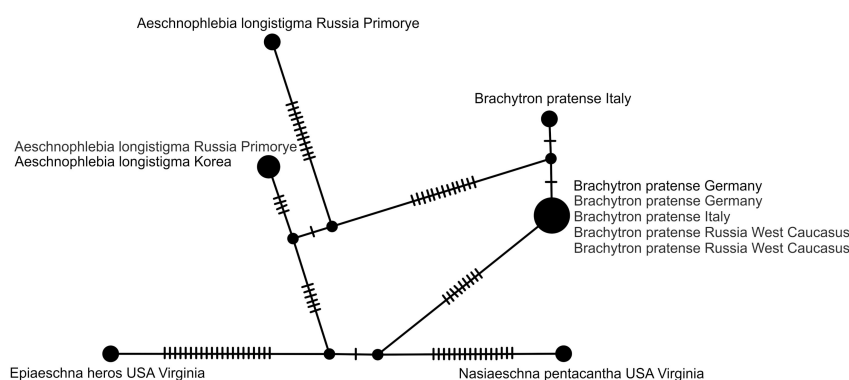
Figure 13. Haplotype network of the short COI gene fragment for the genera *Brachytron*, *Aeschnophlebia*, *Epiaeschna*, and *Nasiaeschna*.

In the short COI fragment tree, *Gynacantha africana* (Palesot de Belauvois, 1807) and *G. vesiculata* Karsch, 1891 are found outside of the other *Gynacantha*, which should be an artifact of insufficient information provided by the short sequence.

In the *Anax* clade, *A. walsinghami*, *Anax guttatus* (Burmeister, 1839), and *Anax gibbosulus* Rambur, 1842 are now grouped in the first cluster (see above), while the clustering of *A. ephippiger* with the main *Anax* cluster has no support (Figure 12).

3.3. StarBeast Analysis of COI and ITS Gene Fragments Together

Thus, we have seen that the Bayesian phylogenetic analysis based on the COI fragment using MRBAYES software provided a good resolution on the species level but also some implausible relationships on a higher, genus level. However, it is a matter of fact that any phylogenetic tree based on the investigated gene (the so called 'gene tree') does not necessarily correspond with a phylogenetic tree based on other genes from the same species. Because of insufficient phylogenetic information provided by a particular gene, it is rarely the case that a gene tree is 100% correct. To avoid this problem, StarBeast co-estimates a species tree and several gene trees in one and the same analysis. We have used the StarBeast software to co-estimate species trees based simultaneously on both markers we investigated, COI and ITS. Both entered the analysis for species we sequenced by ourselves, whereas other species entered the analysis with only COI sequences taken from GenBank. We also make species trees where we combined the ITS sequences with the short and the long COI fragments.

The combined analysis by StarBeast of the short COI fragment and ITS region, having more species, as well as that using the long COI fragment and ITS region (Figures 14 and S3) revealed rather a credible topology of the family. The former generally resembles the tree reconstructed with the short COI fragment, as many species entered the joint analysis only

with this sequence. In both StarBeast trees, the genus *Aeshna* (without *A. isosceles*) is restored as monophyletic, with a good support of 0.83–0.9. These trees also better resolve the clade formed by *Rhionaeschna*, *Anaciaeschna*, and *Pinheyschna*, as already suggested by Ellenrieder [45]. *Sarasaeschna* forms an extra clade away from *Planaeschna*, *Periaeschna*, *Caliaeschna*, and *Cephalaeschna*, the latter three forming a loose extra clade. *Andaeschna* is clustered, but without a sound support, in the short COI-ITS tree with *Caliaeschna*, and forms an isolated branch in the long COI-ITS tree.
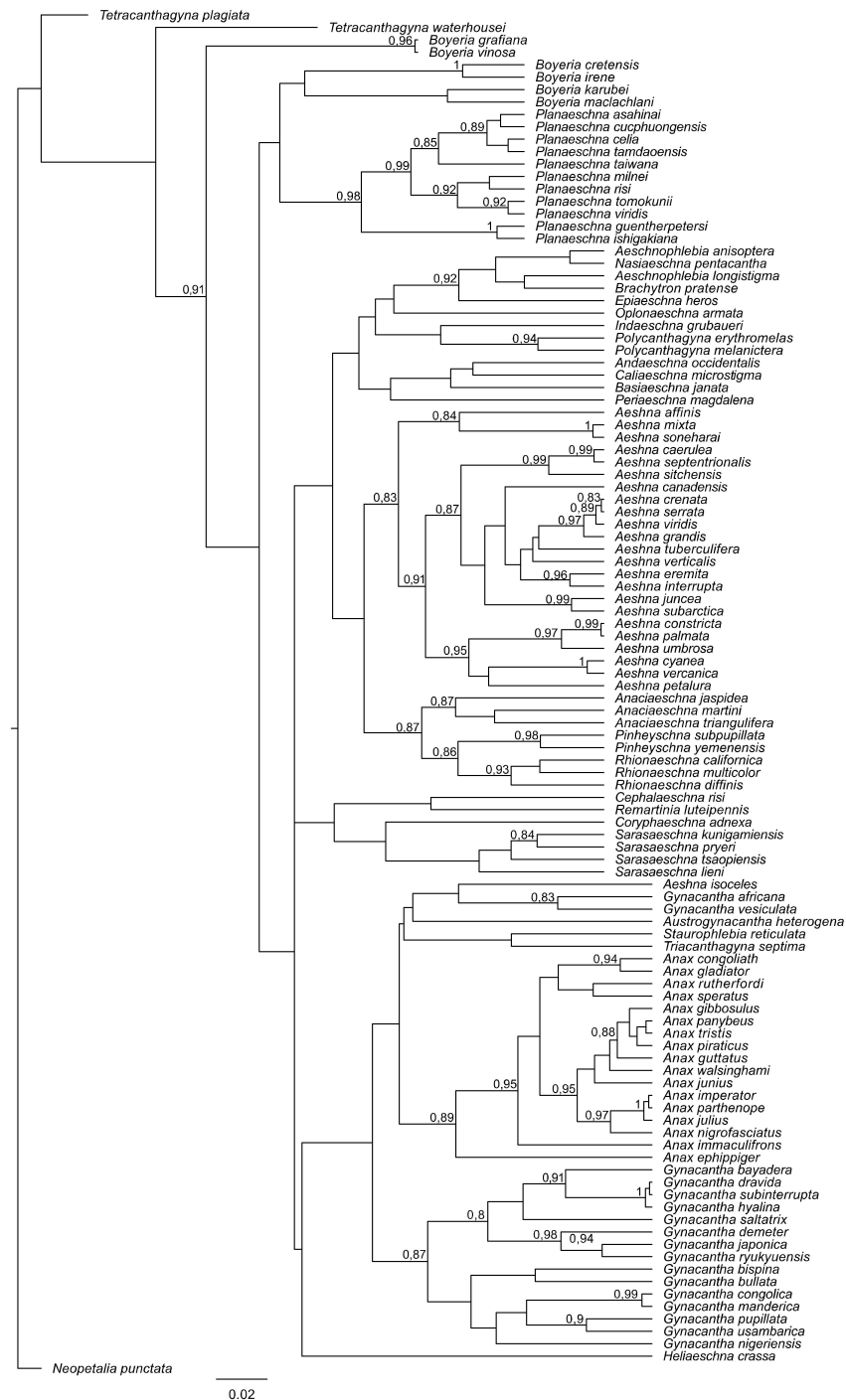
Figure 14. Multi-locus sequence species tree reconstructed with StarBeast3 v 1.1.7 based on the short COI gene fragment and the ITS region of representatives of Aeshnidae. Bayesian posterior probabilities values are depicted at the nodes and as colour in the branches.

4. Overall Discussion

The main difference between the COI tree and the ITS tree is a greater polytomy of the former, with more basic clades with unresolved phylogenetic relationships, which contain few genera. This could be explained if we suppose that in Aeshnidae, the studied COI fragment probably evolved faster than the ITS region, so that variable positions become saturated with substitutions and the phylogenetic signal is lost at evolutionary distances at which the basic divergence of Aeshnidae took place.

Also, different groups of the genus *Aeshna* are diverged much deeper than some other well established genera not related to it, e.g., *Gynacantha*, *Heliaeschna,* and *Austrogynacantha*, as well as the genera of Brachytroninae. There are some cases of discordance between the ITS and COI trees. The most striking is the position of the genus *Tetracanthagyna*, which in the ITS tree is among other Brachytroninae (Figure 2), as also suggested by the morphology-based phylogenetic analysis [5], but appears to be the most ancient divergence of the Aeshnidae stem on the COI tree (Figure 3). This discordance also persisted in the joint StarBeast analysis (Figures 13 and S3). The second discordance concerns *Austrogynacantha*, which was placed in the ITS based tree as expected, with *Gynacantha* (Figure 2), but had no relation to *Gynacantha* in the COI-based trees. The next evident problem in the COI tree was the position of *A. immaculifrons*, which clusters outside *Anax* in the COI tree (Figure 3). The joint StarBeast analysis resolved this problem and reintegrated this species into *Anax* (Figures 5 and S3).

At the low taxonomic level of species, we may point to the discordant phylogenetic pattern in the *A. juncea/A. subarctica* group, wherein the ITS sequences separated these two species (Figure 2), while the COI sequences also formed two clades, but one of them included both *A. subarctica* and *A. juncea* from eastern Eurasia and North America and the other included *A. juncea* from western Eurasia (Figure 3). *A. parthenope* does not diverge from *A. julius* in the ITS tree (Figure 2) or from *A. imperator* in the COI tree (Figure 3), but not vice versa.

Extensive molecular phylogeny of insects at the levels of species and genera started using the mitochondrial gene COI as the most popular marker, which was suggested for insect barcoding. It had such advantages as fast evolution, sometimes allowing researchers to trace divergence even at intra-species level (e.g., *A. cyanea* and *A. juncea*), existing in numerous copies of the cell and strictly (in animals, with few exceptions) maternal inheritance excluding recombination. With time, evidence accumulated that mitochondria evolution may be oddly discordant to that of the nuclear genome and hence phenotype [61]. This concerns Odonata as well, with most examples coming from Coenagrionidae [49,50]. In our analysis we found a possible case of introgression between *A. serrata* and *A. crenata*, evidenced by two specimens, from Finland and the Ural Mountains, which were morphologically *A. crenata* and had the ITS sequences of *A. crenata*, but clustered in the COI analysis within *A. serrata* (Figure 3). Another putative case of introgression of COI was supposed to take place from *A. parthenope* to *A. imperator*.

5. Taxonomic Implications

Our phylogenetic trees based on ITS sequences suggest that the genus *Aeshna* in the current sense is not monophyletic. According to the ITS tree (Figure 2), it can be made monophyletic if we synonymize with it the genera *Pinheyschna* and *Polycanthagyna* (the next node of which unites the current *Aeshna* spp., and *Pinheyschna* only has a weak support of 0.68). Even this broader solution would still place *A. isoceles* outside *Aeshna*. At the same time, in both COI trees (Figures 3 and S2), *Aeshna* is monophyletic (although with a weak support), but again without *A. isoceles*. In none of our analysis did *A. isoceles* show a closer relationship to the genus *Anaciaescha*, as has sometimes been suggested before [43,55]. Therefore, we had to erect a new genus solely for *A. isoceles*.

This is not surprising, as this has been discussed for about 100 years, when Friedrich Ris asked Erich Schmidt "What is *Aeshna isosceles*?" [43]. However, the placement in *Anaciaeschna*, as suggested by him [43], cannot be followed, as none of our gene fragments investigated by

different algorithms correspond to this assumption. Our results (Figures 2 and 3) also did not support a relation of *A. isosceles* to the genus *Andaeschna* De Marmels, 1994, as discussed by von Ellenrieder [45].

We, therefore, suggest for *A. isoceles* the new genus:

***Isoaeschna* gen. nov.**

Type species: *Libellula isoceles* Müller, 1767.

Ethymology: 'ίσος'—a Greek prefix meaning 'equal', *Aeshna*—the name of the genus to which the type species was attributed for a long time.

Differential diagnosis (based on [43,45]): This monotypic genus has some unique combinations of morphological and colourational characters: a transverse ridge on the sternum of S2 (shared with *Anaciaeschna*); narrow, parallel-sided auriculae (shared with *Anaciaeschna*); rounded hindwing anal angle (shared with *Andaeschna* and *Anax*); anterior and posterior veins of anal triangle fused at a point, without prolongation of the fused vein (shared with *Andaeschna* and *Anax*); membranule length comprising 75–100% of the wing anal margin (shared with *Anaciaeschna* and *Andaeschna*) [45]; green eyes without any trace of blue, presence of amber hindwing basal spots [43], the absence of the T-marking on the frons (present in most genera of Aeshnidae but also absent in *Andaeschna* and most *Anax*) [45]; a yellow dorsal triangle on S3. According to von Ellenrieder [45], the new genus appears most close to *Andaeschna*, differing from it in the presence of the transversal ridge on the S2 sternum (a conical tubercle bearing denticles in *Andaeschna*) and the parallel-sided auriculae (triangular or quadrangular with denticles in *Andaeschna*). However, our molecular analysis did not prove this affinity.

Polycanthagyna and *Indaeschna* cluster together very closely in the COI trees and in the combined gene analysis by StarBeast. The ITS sequence of *Indaeschna* is still missing; but we nevertheless suggest to synonymize these two genera:

Indaeschna Fraser, 1926 = *Polycanthagyna* Fraser, 1933, **syn. nov**.

All our results are unequivocally in favour of subsuming the genera *Aeschnophlebia* Selys, 1883, *Nasiaeschna* Selys in Förster, 1900, and *Epiaeschna* Hagen in Selys, 1883 under the genus *Brachytron* Evans, 1845, which has so far been monotypic. This is also not surprising and was already discussed by others [5,51]. All these dragonflies show great similarity in morphology and autecology. Thus, the following synonymies are put forward:

Brachytron Evans, 1845, valid name = *Aeschnophlebia* Selys, 1883 syn. nov. = *Epiaeschna* Hagen in Selys, 1883 syn. nov. = *Nasiaeschna* Selys in Förster, 1900 **syn. nov.**

More complicated and unresolved remains the position of the genus *Tetracanthagyna*. While in the ITS tree, it clusters with *Brachytron*, as discussed earlier [5]; in all COI analyses, it was sorted outside as a primeval clade.

Caliaeschna clusters with *Periaeschna* and *Cephalaeschna* in the ITS tree (Figure 2), but in the long COI tree the clustering of *Caliaeschna* with other genera is too loose and the closest genus is *Sarasaeschna* (Figure 3), for which we have no ITS sequence. Thus, our results are too equivocal for a definitive taxonomic merger of *Caliaeschna* and *Cephalaeschna*, as suggested earlier [5,48].

The genus *Anax* was found to be monophyletic in the ITS tree (Figure 2), whereas in the COI trees (Figures 3 and 12), two species fall outside: *A. ephippiger* is not clustered or loosely clusters with the rest of *Anax*, while *Anax immaculifroms* is found near the base of the tree. However, the joint StarBeast analysis restores its position among *Anax*.

Boyeria unequivocally forms an extra clade in all our analyses.

In some genera, a deeper divergence can be recognized, so, for example, the African and Asian members of the genus *Gynacantha* form two subclades in the COI trees, respectively.

The genera *Anaciaeschna*, *Rhionaeschna*, and *Pinheyschna* are in the same clade in the COI and StarBeast analysis and may be regarded as related, despite their geographical separation, as discussed earlier [58]. Unfortunately, we had no sequences of the genera *Zosteraeschna* Peters at Theischinger, 2011 to check to see if they would belong to the same clade, as expected.

Some taxonomic inferences at the species level can be made. *Anax julius* is well separated from *A. parthenope* in the COI tree (Figure 3) but not in the ITS tree (Figure 2); the former is in favour of there being different species, as proposed earlier [56–60,62]. The lack of divergence of their ITS region could be ascribed to the above-mentioned putative slower evolution of the ITS region in Aeshnidae.

All our analyses unequivocally suggest that the North American *A. septentrionalis* and the Eurasian *A. caerulea* are extremely close to each other. As has been repeatedly stated [40,47,48,62–64], they have no substantial morphological differences, while the reported ones were scarcely distinctive. Therefore, following [47,48], we treat the American populations as the subspecies *Aeshna caerulea septentrionalis*.

The American species *A. palmata* and *A. constricta* share the most common COI allele (Figure 14) and look like the same species. We, however, abstain from their synonymization, as both are known to broadly co-occur and to differ by a number of characters. Maybe our result reflects some mitochondrial introgression between these species.

We synonymize *Gynacantha hyalina* Selys, 1882 **syn. nov.** with *Gynacantha subinterrupta* Rambur, 1842. *G. dravida* Lieftinck, 1960 looks like the same species as *Gynacantha subinterrupta* Rambur, 1842 in the COI and haplotype tree; however, we did not have the ITS sequence to decide this definitively.

The two recently proposed [25], closely related but separate species, *A mixta* and *A. soneharai*, are well separated in the ITS tree (although by three substitutions only) (Figure 2) and the COI haplotype networks (Figure 8), while in the COI tree the latter looks like an in-group inside the former.

The ITS analysis of *A. juncea* revealed a separation of *A. juncea* from the Caucasian/Transcaucasia region versus elsewhere. This may deserve taxonomical fixation at the subspecies level. Two available names were proposed for *A. juncea* from the Caucasus: *A. juncea crenatoides* Bartenev, 1925 and *A. juncea atshischgho* Bartenev, 1929 [65–67]. They were claimed to share such unfortunately quantitative characters as broad thoracic stripes and shallowly incised vulvar lamina, and to differ in the absence (in the former) versus presence (in the latter) of the so-called 'lateral genital plates' in the female ovipositor [67]. It is noteworthy that we managed to involve into our analysis specimens from a population where those 'lateral genital plates' were present, from North Caucasus, and from populations where they are absent, from Dagestan [68] and Georgia (from where *A. juncea crenatoides* was described) [52]. Both their ITS and COI sequences appeared identical. Although subspecies are entities of geographical variation usually differing in some single character and so do not need to be diverged all over their genomes, our result is in favour of treating these subspecies as synonyms; *A. juncea crenatoides* = *A. juncea atshischgho* **syn. nov.**

The divergence of the COI gene of the same *A. juncea* by longitude, from the West Palaearctic east to West Siberia and America plus the East Palaearctic west to Pakistan, with the species *A. subarctica* clustering to the latter, as can be seen in Figure 3, is striking. In the ITS tree, *A. juncea* and *A. subarctica* perform as well diverged monophyletic species (Figure 2). We may suppose the following scenario which could have taken place during the repeated coolings and warmings of the Pleistocene/Holocene. First, both species diverged from their common, most probably Eurasian ancestor in different continents to become *A. juncea* in Eurasia and *A. subarctica* in North America. Then, after some of the repeated restorations of Beringia, both expanded to the other continent. The expansion of *A. juncea* to America was accompanied by mitochondrial introgression from *A. subarctica* to *A. juncea*. Then, those 'contaminated' populations of *A. juncea* expanded back to Eurasia, occupying its eastern regions. This is more or less concordant with the results of an attempt at phylogeographical analysis of the same COI gene by the same two species [11], but the data lost most geographical information due to operating in such a huge 'region' as 'Russia', which occupies more than half of the Holarctic, so they hardly provide an informative geographical resolution.

Our suggestions for taxonomical changes in Aeshnidae, as discussed above, are summarized in Table 2.

Table 2. Valid names according to the taxonomic treatments of the present work.

Currently Used Names	Valid Name According to This Paper
New genus	
-	*Isoaeschna* **gen. nov.**
Synonymizations at the generic rank	
Aeschnophlebia Selys, 1883 **syn. nov**.	*Brachytron* Evans, 1845
Epiaeschna Hagen in Selys, 1883 **syn. nov.**	*Brachytron* Evans, 1845
Nasiaeschna Selys in Förster, 1900 **syn. nov.**	*Brachytron* Evans, 1845
Polycanthagyna Fraser, 1933 **syn. nov.**	*Indaeschna* Fraser, 1926
Synonymization at species rank	
Gynacantha hyalina Selys, 1882 **syn. nov.**	*Gynacantha subinterrupta* Rambur, 1842
Synonymization at subspecies rank	
Aeshna juncea atshischgho Bartenef, 1929	*Aeshna juncea crenatoides* Bartenef, 1925
Species to subspecies level	
Aeshna septentrionalis Burmeister, 1839	*Aeshna caerulea septentrionalis* Burmeister, 1839
New combinations	
Aeshna isoceles (Müller, 1767)	*Isoaeschna isoceles* (Müller, 1767) **comb. nov.**
Aeschnophlebia anisoptera Selys, 1883	*Brachytron anisoptera* (Selys, 1883) **comb. nov.**
Aeschnophlebia longistigma Selys, 1883	*Brachytron longistigma* (Selys, 1883) **comb. nov.**
Epiaeschna heros (Fabricius, 1798)	*Brachytron heros* (Fabricius, 1798) **comb. nov.**
Nasiaeschna pentacantha (Rambur, 1842)	*Brachytron pentacantha* **comb. nov.**
Polycanthagyna erythromelas (McLachlan, 1896)	*Indaeschna erythromelas* (McLachlan, 1896) **comb. nov.**
Polycanthagyna melanictera (Selys, 1883)	*Indaeschna melanictera* (Selys, 1883) **comb. nov.**
Polycanthagyna ornithocephala (McLachlan, 1896)	*Indaeschna ornithocephala* (McLachlan, 1896) **comb. nov.**

Abbreviations

bp–base pairs: COI–mitochondrial cytochrome coxidase subunit I (further in the text, this abbreviation is often used as a grammatical modifier in expressions like 'the COI tree' as referring to the COI gene fragment we analysed rather than the whole gene sequence); ITS–internal transcribed spacer, here implying two spacers, ITS1 and ITS2, between sequences coding for rRNA, while 'ITS region' or 'ITS' used as a grammatical modifier refering to the sequence embracing ITS1, 5.8S gene, and ITS2; PCR–polymerase chain reaction.

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