

**A MOLECULAR STUDY OF THE RELATIONSHIP
BETWEEN THE COENAGRIONID GENERA *ERYTHROMMA*
AND *CERCION*, WITH THE CREATION OF
PARACERCION GEN. NOV. FOR THE EAST ASIATIC “*CERCION*”
(ZYGOPTERA: COENAGRIONIDAE)**

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The ribosomal DNA genes (18S, 5.8S) and internal transcribed spacers (ITS1, ITS2) of 5 representatives of “*Cercion*” occurring in East Asia were examined and compared with west-palaearctic “*Cercion*” *lindenii*, *Erythromma najas* (2 populations), *E. viridulum*, some true *Coenagrion* species, and with *Enallagma* and *Ischnura* as outgroups. The molecular phylogenetic tree confirms the position of H. Heidemann & R. Seidenbusch (1993, *Die Libellenlarven Deutschlands und Frankreichs*, Bauer, Keltern) that *Cercion lindenii* belongs in *Erythromma*, and consequently, the binomen *Erythromma lindenii* is accepted. Regarding the “oriental” group for which, under the new situation, the name *Cercion* is no longer available, the genus name *Paracercion* gen. n. is introduced. This is supported by molecular evidence and by some morphological traits. A morphological basis for setting apart the new genus from *Erythromma* is thus achieved, but its delimitation from *Coenagrion* remains to be defined.

INTRODUCTION

Many black-and-blue coenagrionids were originally lumped into the genus *Agrion*, later renamed *Coenagrion*. Things changed when NAVAS (1907: 55) placed *Coenagrion lindenii*, described by SELYS (1840) in honour of Vander Linden and with Belgium as type locality, in a new monotypic genus, *Cercion*. He founded this move on the fact that in *lindenii* males, the superior appendages are longer than the inferiors (Figs 1-4). In a footnote he explains the name of the new genus as meaning: “close to *Agrion* but with forcipate superior appendages, resembling those of a *Lestes*”. Many authors

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originally rejected this position, and kept *lindenii* in *Agrion*, later *Coenagrion*. They argued that, if such a character was to be given generic rank, species like *C. armatum* and *C. glaciale* would also need to be moved to different genera. Only after 1970 (ST. QUENTIN, 1970) the name *Cercion* gained a wider acceptance, and it is currently used in a variety of local and European faunas.

In fact, it is possible to specify additional male characters of a unique nature in *Cercion lindenii*, viz. the apex of the appendix superior is rather finely drawn out but apically bifid (Fig. 1) (a character often hidden by the position of the appendix superior), while the base of the appendix superior is flattened and widened, and ends in a sharp, downwardly curved spine (Fig 4, arrow).

Between 1865 and 1930, a number of species of black-and-blue *Coenagrion*-like zygopteran have been described from the East of Eurasia, between the Russian Primorye, Japan, and down to southern China, Korea, Taiwan, Thailand, India, and the Philippines. All were originally placed in *Agrion* or *Coenagrion* (BRAUER, 1865; SELYS, 1883; RIS, 1916; NEEDHAM, 1930) but, starting with ASAHINA (1950), and specifically for *Coenagrion v-nigrum* Needham (ASAHINA, 1956), they were later moved to *Cercion*, such that the genus is currently composed of one Mediterranean species, and about eight East Asian species.

To complicate matters further, HEIDEMANN & SEIDENBUSCH (1993), in a study of larval morphology, found that *Cercion lindenii* is closely related to *Erythromma*, to the point where they synonymized it with the latter genus. If this is true, the question arises whether the eastern representatives of the genus are true *Cercion* or not. Interestingly, three *Erythromma* (*najas*, *humerales* and *viridulum*) were examined and were found to share strategic male characters with *C. lindenii*: all possess a bifid apex of the appendix superior (much more pronounced than in *lindenii*), and a basal spine (pointed and downturned in *najas* and *humerales*, pointed and upturned in *viridulum*). (see DUMONT, 1991, figs 192-194 for SEMs).

It should be noted that, if *C. lindenii* is indeed an *Erythromma*, the name *Cercion* is not available for the "eastern group", since *lindenii* is the type species of the genus. It follows that the eastern group should then either return to *Coenagrion*, or be given a new status.

What does male morphology tell us? Some eastern "*Cercion*" indeed have superior appendages that are longer than the inferiors (like the species *impar* and *plagiosum*), but in others the two pairs of appendages are about the same length, or the inferior is longer than the superior (like in the Japanese *C. sieboldii* Selys). However, all eastern males share one other character (see DUMONT, 2004): a robust, elongated, often squarish tooth emanates from the base of an appendix superior. The appendix is never apically bifid, and the basal projection is never acutely pointed. This set of characters sets *lindenii* and the *Erythromma*'s apart from the "eastern group", but whether it is sufficient to distinguish it from true *Coenagrion* is uncertain, since some species have the tooth (e.g. *C. lunulatum*) (illustrated in DUMONT, 2003), while others do not (e.g. *C. hastulatum*).

Table I
Gene length, GC content and accession number of the ribosomal 18S and 5.8S genes and the ITS1 and ITS2 spacers, and geographical origin of the species used in this study. Collector's institutional affiliations are provided in the acknowledgments

Species/Subspecies	18S gene		ITS-1 spacer		5.8S gene		ITS-2 spacer		EMBL acc. number	geographical origin	collector
	length	GC %	length	GC %	length	GC %	length	GC %			
<i>Paracercion hieroglyphicum</i>	1865	52.23	226	46.46	165	56.36	219	63.47	AJ488551	Inner Mongolia, China	H.J. Dumont
<i>Paracercion sieboldii</i>	1865	52.23	184	44.39	165	56.36	226	59.73	AJ621056	Kitajima, Japan	K. Inoue
<i>Paracercion v. nigrum</i>	1865	52.23	197	44.16	165	56.36	227	59.91	AJ621057	Primorye, Russia	E. Malikova
<i>Paracercion plagiosum</i>	1865	52.17	198	43.94	164	56.10	215	61.86	AJ488552	Chechuan, China	Su Rong
<i>Paracercion calamorum dyeri</i>	1865	52.23	189	47.62	165	56.36	222	61.26	AJ488549	Asan Lake, India	H.J. Dumont
<i>Erythromma viridulum</i>	1865	52.28	178	46.06	166	56.63	220	69.55	AJ621058	Villa Chay, Azarbaijan	H.J. Dumont
" <i>Cercion</i> " <i>lindenii</i>	1865	52.17	198	50.51	165	56.97	221	66.97	AJ488550	Gard, France	H.J. Dumont
<i>Erythromma najas</i> (Belgium)	1865	52.17	176	44.89	165	56.36	220	65.00	AJ621054	Kalmthout, Belgium	H.J. Dumont
<i>Erythromma najas</i> (Russia)	1865	52.17	176	44.89	165	56.36	220	65.00	AJ621055	St. Petersburg, Russia	V. Alekseev
<i>Coenagrion ornatum</i>	1865	52.06	179	48.04	166	56.02	215	65.58	AJ621059	Erdemli, Turkey	K. Van Damme
<i>Coenagrion australocaspicum</i>	1865	52.06	182	46.15	166	56.63	214	63.26	AJ621060	Sollar, Azarbaijan	H.J. Dumont
<i>Coenagrion pulchellum</i>	1865	52.06	180	46.11	166	55.42	215	63.72	AJ488547	Kalmthout, Belgium	H.J. Dumont
<i>Coenagrion puella</i>	1865	52.06	183	46.45	166	56.02	215	63.72	AJ488546	Kalmthout, Belgium	H.J. Dumont
<i>Ischnura graellsii</i>	1857	52.18	189	41.27	166	56.02	203	59.11	AJ488545	Ifrane, Morocco	H.J. Dumont
<i>Enallagma cyathigerum risi</i>	1857	52.23	194	42.78	166	56.63	205	58.54	AJ420941	Huhhot, China	H.J. Dumont

The present paper is an attempt to clarify the relationship between *Erythromma*, *Cercion lindenii*, the "eastern *Cercions*", and *Coenagrion* using molecular phylogenetic methods.

MATERIAL AND METHODS

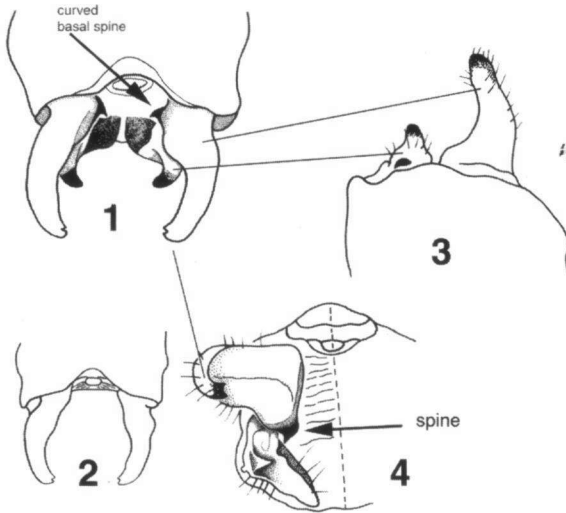
EXPERIMENTAL PROCEDURES. — Thoracic muscular tissue was dissected, and total DNA prepared according to the protocol of the Puregene™ DNA isolation kit, type D-5000A (BIOzym, Landgraaf, the Netherlands). Nuclear ribosomal genes (18S, 5.8S, partial 28S) and spacers (ITS1, ITS2) were amplified by PCR, using Qiagen DNA polymerase (Westburg, Leusden, the Netherlands) as previously described (SAMRAOUI et al., 2003). PCR products were used for direct sequencing using the BigDye™ technology, the protocol of the ABI Prism BigDye terminator cycle sequencing ready reaction kit, and thereafter analyzed on an ABI Prism 377 DNA sequencer (PE Applied Biosystems) as described earlier (SAMRAOUI et al., 2003).

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS. — Sequences of the 18S-ITS1-5.8S-ITS2-28S (partial) region were aligned automatically using CLUSTALW (THOMPSON et al., 1994), but visually optimized using Genedoc (NICHOLAS et al., 1997). The complete gene and spacer

region was used in the phylogenetic analysis. Sequence divergence values were computed by the Kimura 2-parameter model (KIMURA, 1980) using the DNADIST program of PHYLIP (FELSENSTEIN, 1989). Phylogenetic reconstruction was performed using the neighbor-joining method and the JIN & NEI (1990) correction method of Treecon 1.3b (VAN DE PEER & DE WACHTER, 1994); bootstrap values were calculated with the same program (FELSENSTEIN, 1985) to assess the stability of each branchpoint.

RESULTS

DNA ANALYSIS. — Complete and unambiguous sequences for the 18S-ITS1-5.8S-ITS2-28S (partial) region were obtained for all ingroup (*Cercion*, *Coenagrion*, *Erythromma*) and outgroup taxa (*Ischnura*, *Enallagma*). The length of the 18S gene is 1865 bp for the ingroup taxa and 1857 bp for the outgroup taxa, but the GC content for all taxa is in the same range (52.06-52.23%) (Tab. I). The length of the 5.8S gene for all taxa varies between (164-166 bp), also the GC content shows some variation (55.42-56.97%). The ITS1 and ITS2 show length variation for all taxa, ranging between 176-226 bp (ITS1) and 203-227 (ITS2). Also the GC content varies, 41.27-50.51% (ITS1) and 58.54-69.55 (ITS2), with the outgroup taxa having the lowest GC content (Tab. I). The highest inter-specific sequence diversity



Figs 1-4. Some structural features of male *Coenagrion lindenii*: (1) dorsal view of terminalia of specimen from Ifrane, Morocco; — (2) same, specimen from Wellemeersen, Belgium; — (3) lateral view of specimens from Ifrane; — (4) posterior view of specimen from Ifrane.

between all taxa investigated is 7.21%, while for the ingroup alone it varies between 0.37-6.23% (Tab. II).

PHYLOGENETIC ANALYSIS, using the method described above, was used to construct a phylogenetic tree. Of 2543 aligned positions of all taxa, 314 sites were variable, 229 of which were phylogenetically informative. The 18S-ITS1-5.8S-ITS2-28S (partial) sequence data used to construct a neighbor-joining tree using the Kimura 2-parameter correction method (Fig. 5), show a monophyletic ingroup (bootstrap support = 100%) with three well-separated clades; the eastern *Cercion*, hereinafter renamed *Paracercion* n.gen. (bootstrap support = 97%), *Erythromma* spp. (bootstrap support = 99%) and *Coenagrion* spp. (bootstrap support = 100%). The *Erythromma* clade, harbouring "*Cercion*" *lindenii*, is sistergroup to *Paracercion*.

DISCUSSION AND CONCLUSIONS

SEQUENCE VARIATION

The length of the 18S (1857-1865 bp) and 5.8S (164-166 bp) genes of all taxa is in the same range as for other dragonflies (DUMONT et al., 2003; SAMRAOUI et al., 2002, 2003). The internal transcribed spacers ITS1 (176-226 bp) and ITS2 (203-227 bp) are short but are in the same range as reported for other dragonflies (DUMONT et al., 2003; SAMRAOUI et al., 2002, 2003; WEEKERS et al., 2001). The GC content of the two genes (18S, 5.8S) and the spacers (ITS1, ITS2) of all taxa is within the normal range for dragonflies (DUMONT et al., 2002; SAMRAOUI et al., 2002, 2003; WEEKERS et al., 2001) and other insects (data from Genbank/EMBL. However, the GC content in the ITS2 is in general higher than in the ITS1.

PHYLOGENETIC RELATIONSHIPS; INTER- AND INTRASPECIFIC IMPLICATIONS

Our phylogenetic tree shows the relationship between three well-separated monophyletic clades. The *Erythromma* clade, which encapsulates "*Cercion*" *lindenii* (bootstrap support = 99%), is sistergroup to the *Paracercion* clade (bootstrap support = 97%), with *Coenagrion* spp. as basal clade. All clades and individual taxa are well separated by genetic distance (Tab. II).

This analysis confirms the position of HEIDEMANN & SEIDENBUSCH (1993), that "*Cercion*" *lindenii* is an *Erythromma*. It is in fact a closer ally of *E. najas* (Hansemann,

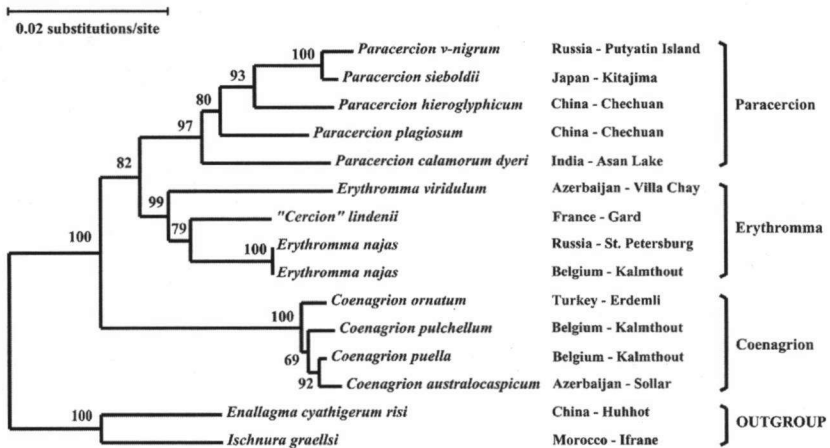


Fig.5. Distance tree inferred from ribosomal gene (18S, 5.8S) and spacer (ITS1, ITS2) sequence data showing phylogenetic relationships between selected *Cercion*, *Coenagrion* and *Erythromma* taxa. Evolutionary distances calculated by neighbor-joining using the Jin & Nei correction method in Treecon. The tree is rooted using *Enallagma risi* and *Ischnura graellsii* as outgroup. Bootstrap values are based on 1000 replicates and expressed as a percentage.

Table II

Pairwise comparison of all ingroup (*Paracercion*, *Erythromma*, *Coenagrion*) and outgroup (*Ischnura*, *Enallagma*) taxa in the analysis. Above the diagonal are absolute nucleotide differences. Below the diagonal are distances calculated for the combined 18S, 5.8S, ITS1 and ITS2 regions, using the Kimura 2-parameter model as correction method

Species	Phiero	Psiebo	Pvniig	Pplag	Pedye	Eviri	"Clind"	Ena-B	Ena-R	Corna	Caust	C.pulc	Cpuel	Igrae	Ercis
<i>P. hieroglyphicum</i>	---	49	50	61	72	114	85	94	94	129	131	126	128	152	154
<i>P. sieboldii</i>	0.0204	---	14	58	70	103	88	97	97	137	141	138	138	160	160
<i>P. v. nigrum</i>	0.0207	0.0057	---	60	75	110	93	102	102	144	145	142	143	163	166
<i>P. plagiolum</i>	0.0255	0.0242	0.0250	---	72	96	73	85	85	123	130	130	127	154	152
<i>P. c. dyeri</i>	0.0301	0.0292	0.0313	0.0302	---	114	90	101	101	107	112	110	108	155	155
<i>E. viridulum</i>	0.0485	0.0438	0.0467	0.0409	0.0485	---	79	74	73	129	137	135	133	139	147
" <i>Cercion</i> " <i>lindenii</i>	0.0357	0.0371	0.0391	0.0307	0.0379	0.0333	---	47	47	115	121	121	117	128	130
<i>E. naias</i> (Belgium)	0.0398	0.0412	0.0432	0.0632	0.0429	0.0312	0.0196	---	0	112	117	116	113	132	132
<i>E. naias</i> (Russia)	0.0398	0.0412	0.0432	0.0362	0.0429	0.0308	0.0196	0.0	---	111	116	115	112	131	131
<i>C. ornatum</i>	0.0553	0.0590	0.0619	0.0528	0.0457	0.0555	0.0489	0.0480	0.0476	---	19	18	14	149	147
<i>C. australocaspicum</i>	0.0562	0.0607	0.0623	0.0559	0.0478	0.0591	0.0516	0.0502	0.0479	0.0078	---	18	9	150	148
<i>C. pulchellum</i>	0.0539	0.0594	0.0609	0.0559	0.0470	0.0583	0.0516	0.0497	0.0493	0.0074	0.0074	---	13	148	144
<i>C. puella</i>	0.0548	0.0593	0.0613	0.0545	0.0460	0.0573	0.0498	0.0483	0.0479	0.0058	0.0037	0.0054	---	144	142
<i>I. graellsii</i>	0.0661	0.0699	0.0709	0.0672	0.0677	0.0606	0.0554	0.0575	0.0570	0.0651	0.0655	0.0645	0.0627	---	69
<i>E. risii</i>	0.0668	0.0698	0.0721	0.0662	0.0675	0.0640	0.0560	0.0574	0.0570	0.0640	0.0645	0.0627	0.0618	0.0290	---

1823) than of *E. viridulum* (Charpentier, 1840). Morphological characters of adult males (a long and somewhat forcipate superior appendix, with a bifid apex, and with a basal pointed tooth) are thus supported by DNA-based evidence and *vice versa*. The "eastern *Cercion*" has non-forcipate, non-bifid superior appendages, and a non-acutely pointed basal tooth descending between the inferior appendages. Females have a lower apophysis at the mid-hind ridge of the pronotum (for details, see DUMONT, 2004). Based upon these characters and molecular support, we unite the eastern group under the genus name *Paracercion* gen.n. The type species is *Paracercion hieroglyphicum* (Brauer, 1865); other species reliably included in this new genus are *P. melanotum* (Selys, 1876) (teste MAY, 1997) *P. calamorum* (Ris, 1916), *P. plagiolum* (Needham, 1930), *P. v-nigrum* (Needham, 1930), *P. impar* (Needham, 1930), and *P. barbatum* (Needham, 1930). The geographical range of the genus is sejnct and extends from the Pa-

cific coast of Russia (Primoriye and Amurland) and Japan across China to India and the islands of Taiwan and the Philippines. A full morphological treatment with keys to both sexes will be published separately (DUMONT, 2004). *Coenagrion*, in our molecular phylogeny, stands well apart from *Paracercion*, but more extensive molecular and morphological work is needed to clarify its true position.

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