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ORIGINAL PAPER

When standard DNA barcodes do not work for species identification: intermixed mitochondrial haplotypes in the *Jaera albifrons* complex (Crustacea: Isopoda)

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Abstract

Here, we characterise the standard "Folmer region" of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) marker and a fragment of nuclear 28S marker in four species of the *Jaera albifrons* complex. *Jaera albifrons* (Leach, 1814), *Jaera ischiosetosa* Forsman, 1949, *Jaera praehirsuta* Forsman, 1949, and *Jaera forsmani* Bocquet, 1950 were collected from localities on the Norwegian coast and identified with morphological characters. We compared DNA sequences with sequences available in GenBank and BOLDsystems and calculated haplotype networks and interspecific versus intraspecific genetic distances. These analyses revealed low interspecific genetic distance (CO1 0.00–1.57%, 28S 0.00–0.39%) and extensive haplotype sharing between *J. albifrons* group species and specimens from both sides of the North Atlantic for both CO1 and 28S. Genetic distances between *J. albifrons* group species and other *Jaera* species, however, exceeded 29% for both CO1 and 28S, with no haplotype sharing. These assessments, together with taxonomically unconstrained analyses with software ABGD and ASAP, show that these markers are unable to distinguish between the *J. albifrons* group of morphospecies. The sequences do, however, clearly identify *J. albifrons* species complex from other *Jaera* species. Thus, a likely hypothesis is that taxa in this complex represent a single species. Our results corroborate previous finds where discordance between mitochondrial gene clusters, AFLP, and other data highlights the potential conflict between different "species criteria" and the well-established distinction between gene trees and species trees. In operational terms, common protocols for metabarcoding will potentially underestimate sympatric species diversity with cases like the *J. albifrons* complex, if the members of this complex indeed represent different species.

Keywords DNA barcoding · Isopoda · DNA barcodes · Species identification · Genetic distance · Haplotype network

Introduction

Several disciplines in biology, such as ecology, evolutionary, and conservation biology, depend on a correct identification of and the knowledge about species diversity provided through taxonomic work (Dayrat 2005). A new species would be described as a morphospecies, that is, a species recognised based on external morphological characteristics. However, an exclusively morphological approach to species identification can potentially fail to detect cases of strong polymorphism (Wares and Cunningham 2001), including life history stages, sexual dimorphism (Naylor and Brandt 2015), cryptic species (Hebert et al. 2004a), or phenotypic plasticity (Hebert et al. 2003a). To resolve such difficulties, the integration of molecular data has proven to be a highly useful assistant in the differentiation of species (Cain 1966). Today, DNA barcoding has become an important tool in the

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performance of more certain species identification (DeSalle 2006; Goldstein and DeSalle 2011), often combined with morphology in an integrative taxonomy approach. The standard method for this approach uses a ca. 658 base pair region, known as the Folmer region (Folmer et al. 1994) of the mitochondrial gene cytochrome *c* oxidase subunit 1 (CO1), to distinguish between congeneric animal species (Hebert et al. 2003b; Raupach et al. 2015). Due to the assumed low intraspecific variation and high interspecific variation of the CO1 gene (Casiraghi et al. 2010), it has been considered the standard marker for identifying animal species through DNA barcoding (Hebert et al. 2003a; 2003b).

DNA barcoding has the potential not only to provide a way of identifying species, but also to correct misidentifications and to unveil the presence of cryptic species (Hebert et al. 2004a; Johnson et al. 2008; Velzen et al. 2007). When using CO1 barcodes, an interspecific genetic distance greater than 2% (Hebert et al. 2003b) or a tenfold difference between mean intra- and interspecific distance has been suggested (Hebert et al. 2004b) as the standard threshold to recognise one species from another. However, traditional species designations are usually not based on genetic distances. Therefore, when new data on genetic distances and/or gene trees appear to challenge current species taxonomies, DNA barcoding can become a tool for species discovery and species delimitation (e.g., DeSalle 2006; Puillandre et al. 2021). A distance-based identification can, however, become difficult in cases of mitochondrial introgression, heteroplasmy, from phylogeographic processes, and incipient speciation (Raupach et al. 2015). True heteroplasmy can be difficult to separate from sequencing errors and NUMTS. Heteroplasmic immigrant mitochondria may also be hard to discover simply due to their low frequency compared with the wild type (Palozzi et al. 2018). Mitochondrial introgression will represent a methodological challenge when inferring evolutionary history from genetic data (Funk and Omland 2003; Hickerson et al. 2006; Bonnet et al. 2017). Such challenges will certainly also have implications for taxonomies based on mitochondrial markers and impair the utility of CO1 as a valid marker for species identification (Keck et al. 2022). For instance, Johnsen et al. (2010) found that 6% of the Scandinavian bird species could not be unambiguously identified with CO1 barcodes.

Amongst the isopods, the genus *Jaera* Leach, 1814 holds several common intertidal species in the North Atlantic (Mifsud 2011). Globally, some *Jaera* species are currently known from few locations with restricted geographical distribution, whereas others possess wider distributions (Linse et al. 2014). Amongst the five species that make up the species complex known as the *Jaera albifrons* group (Bocquet 1954; Naylor and Haahtela 1966), the four species, *Jaera praehirsuta* Forsman, 1949, *Jaera ischiosetosa* Forsman, 1949, *Jaera albifrons* Leach, 1814, and *Jaera forsmani* Bocquet, 1950 are regarded as common on both sides of the North Atlantic, with *J. praehirsuta* and *J. ischiosetosa* also occurring in the Baltic Sea (Jażdżewski 1969). The morphological identification of all members of the *J. albifrons* group relies on characteristics of pereopod setation in males, whilst females are considered morphologically indistinguishable (Bocquet 1954; Forsman 1949; Naylor and Haahtela 1966; Ribardière et al. 2017; Siegismund 2002). Quantitative trait locus (QTL) mapping of the diagnostic characters in *J. albifrons* and *J. ischiosetosa* indicates that carpus shape is controlled by a single locus (or several closely linked loci), whilst numbers of setae are the product of dominance, epistasis, and possible pleiotropic effects of several loci in putative regions of restricted recombination (Mifsud 2011). Given many observations of co-occurring species, considerable work has focused on speciation modes and "isolation mechanisms" for *Jaera* (Mifsud 2011; Ribardière et al. 2017; Ribardière et al. 2019; Siegismund 2002; Solignac 1981). Forsman (1951, cited by Jażdżewski 1969) stated that it was simple to crossbreed *J. albifrons* (sensu stricto) and the North American *J. posthirsuta* in the laboratory. According to Bocquet (1954), hybridization could also readily occur between *J. forsmani* and the other members of the *J. albifrons* group, except for *J. praehirsuta*, and subsequent results of experimental crossbreeds have clarified many aspects of the biology of these species (e.g., Solignac 1981; Ribardière et al. 2018; Ribardière et al. 2019).

Initial DNA sequencing of *Jaera* material from the Norwegian coast showed little to no variation in the CO1 gene amongst the *J. albifrons* species complex as identified with morphology. The BOLD database also displayed this group of species as a case of "bin discordance", which is a colloquial expression for a BIN containing similar sequences with different species names. In this study, we examine partial gene sequences from CO1 and the nuclear 28S rRNA gene produced from specimens that are either *J. albifrons*, *J. praehirsuta*, *J. ischiosetosa*, or *J. forsmani*, based on morphological diagnostics. To understand the observed similarities and differences amongst these species in a broader context, we also compare our new data with available *Jaera* sequences from GenBank (https://www.ncbi.nlm.nih.gov/nuccore). Comparing our results with other publicly available data, we review the literature on taxa in the species complex.

Material and methods

Sampling and locations

Specimens were collected from the intertidal zone at 22 locations along the Norwegian coastline (Table 1). Collections were made by turning over rocks, gathering visible individuals with a paintbrush, or by shaking algae over

Table 1 Specimens of *Jaera* included in the present study, with museum voucher ID, sampling location, process ID in the BOLD database, and GenBank accession numbers. Haplotypes correspond to Fig. 6. New sequences obtained in the present study are highlighted in bold. Outgroup sequences are sectioned off at the end of the table. Information not available is denoted N/A

Species	Museum voucher ID	Sampling location	Process ID (BOLD)	cox1 haplotype	28S haplotype	GenBank ac. number	
						cox1	28S
J. albifrons	NTNU-VM 79747	Norway, 58.142N 8.003E	HABFA1467-21	**7**	**1**	**ON601011**	**ON598986**
J. albifrons	NTNU-VM 79966	Norway, 63.599N 10.459E	HABFA1495-21	**4**		**ON601024**	
J. albifrons	NTNU-VM 79970	Norway, 63.599N 10.459E	HABFA1497-21	**4**		**ON601018**	
J. albifrons	NTNU-VM 79971	Norway, 63.599N 10.459E	HABFA1498-21	**4**		**ON601036**	
J. albifrons	NTNU-VM 80026	Norway, 63.599N 10.459E	HABFA1511-21	**4**	**1**	**ON601017**	**ON598988**
J. albifrons	NTNU-VM 80027	Norway, 63.599N 10.459E	HABFA1512-21	**4**	**1**	**ON601058**	**ON598995**
J. albifrons	NTNU-VM 80028	Norway, 63.599N 10.459E	HABFA1513-21	**4**		**ON601063**	
J. albifrons	ZMBN 136697	Norway, 60.447N 6.641E	HABFA1535-21	**4**		**ON601069**	
J. albifrons	ZMBN 136698	Norway, 60.447N 6.641E	HABFA1536-21	**20**		**ON601009**	
J. albifrons	ZMBN 136797	Norway, 69.622N 18.058E	HABFA1544-21	**19**	**1**	**ON601057**	**ON598994**
J. albifrons	ZMBN 136798	Norway, 69.622N 18.058E	HABFA1545-21	**19**	**1**	**ON601039**	**ON598991**
J. albifrons	ZMBN 136799	Norway, 69.622N 18.058E	HABFA1546-21	**19**		**ON601062**	
J. albifrons	ZMBN 136800	Norway, 69.622N 18.058E	HABFA1547-21	**4**		**ON601025**	
J. albifrons	ZMBN 139048	Norway, 68.147N 14.198E	HABFA1575-21	**4**		**ON601042**	
J. albifrons	MT01171	Germany, 53.733N 7.767E	BNSC096-10	23		KT209037	
J. albifrons	MT01173	Germany, 53.733N 7.767E	BNSC098-10	23		KT209171	
J. albifrons	MT01174	Germany, 53.733N 7.767E	BNSC099-10	23		KT209248	
J. albifrons	MT01176	Germany, 53.733N 7.767E	BNSC101-10	23		KT209028	
J. albifrons	MT03135	Germany, 54.169N 7.898E	BNSC405-12	24		KT209388	
J. albifrons	MT03137	Germany, 54.169N 7.898E	BNSC407-12	24		KT208466	
J. albifrons	MT03138	Germany, 54.169N 7.898E	BNSC408-12	25		KT209002	
J. albifrons	MT03139	Germany, 54.169N 7.898E	BNSC409-12	24		KT208400	
J. albifrons	12NJNB0060	Canada, 45.1019N 67.0527W	NBCRU019-12	26		MG936058	
J. albifrons	12NJNB0098	Canada, 45.1019N 67.0527W	NBCRU057-12	27		MG936162	
J. albifrons	L164AR1-12	Canada, 47.957N 69.795W	WW155-07	4		N/A	
J. albifrons	L90AR1-01	Canada, 45.159N 64.359W	WW810-08	4		FJ581736	
J. cf. *forsmani*	NTNU-VM 79748	Norway, 63.457N 10.45E	HABFA1468-21	**6**		**ON601047**	
J. forsmani	NTNU-VM 79925	Norway, 63.594N 9.527E	HABFA1488-21	**9**	**3**	**ON601037**	**ON598990**
J. forsmani	NTNU-VM 79926	Norway, 63.594N 9.527E	HABFA1489-21	**9**	**3**	**ON601064**	**ON598997**
J. forsmani	NTNU-VM 79929	Norway, 63.594N 9.527E	HABFA1490-21	**9**		**ON601007**	
J. cf. *forsmani*	NTNU-VM 79963	Norway, 63.599N 10.459E	HABFA1493-21	**4**		**ON601040**	
J. forsmani	NTNU-VM 79997	Norway, 63.6N 10.461E	HABFA1501-21	**12**		**ON601044**	
J. forsmani	NTNU-VM 80018	Norway, 63.872N 9.739E	HABFA1508-21	**15**	**1**	**ON601065**	**ON598998**
J. ischiosetosa	NTNU-VM 79730	Norway, 63.457N 10.45E	HABFA1461-21	**3**		**ON601033**	
J. ischiosetosa	NTNU-VM 79731	Norway, 63.457N 10.45E	HABFA1462-21	**4**		**ON601049**	
J. ischiosetosa	NTNU-VM 79732	Norway, 63.457N 10.45E	HABFA1463-21	**5**		**ON601006**	
J. ischiosetosa	NTNU-VM 79739	Norway, 58.967N 9.842E	HABFA1464-21	**7**		**ON601067**	
J. ischiosetosa	NTNU-VM 79743	Norway, 58.967N 9.842E	HABFA1466-21	**8**		**ON601053**	
J. ischiosetosa	NTNU-VM 79750	Norway, 59.46N 10.36E	HABFA1469-21	**4**	**1**	**ON601015**	**ON598987**
J. ischiosetosa	NTNU-VM 79751	Norway, 59.46N 10.36E	HABFA1470-21	**4**	**2**	**ON601066**	**ON598999**
J. ischiosetosa	NTNU-VM 79863	Norway, 58.977N 9.825E	HABFA1480-21	**18**		**ON601051**	
J. ischiosetosa	NTNU-VM 79865	Norway, 58.977N 9.825E	HABFA1481-21	**7**		**ON601045**	
J. ischiosetosa	NTNU-VM 79870	Norway, 59.023N 11.015E	HABFA1483-21	**4**		**ON601031**	
J. ischiosetosa	NTNU-VM 79964	Norway, 63.599N 10.459E	HABFA1494-21	**10**		**ON601055**	
J. ischiosetosa	NTNU-VM 79985	Norway, 63.582N 10.418E	HABFA1500-21	**4**		**ON601035**	
J. ischiosetosa	NTNU-VM 80035	Norway, 63.639N 10.587E	HABFA1520-21	**13**		**ON601028**	
J. ischiosetosa	NTNU-VM 80036	Norway, 63.639N 10.587E	HABFA1521-21	**5**	**1**	**ON601046**	**ON598992**

Table 1 (continued)

Species	Museum voucher ID	Sampling location	Process ID (BOLD)	cox1 haplotype	28S haplotype	GenBank ac. number	
						cox1	28S
J. ischiosetosa	NTNU-VM 80037	Norway, 63.639N 10.587E	HABFA1522-21	**10**		**ON601027**	
J. ischiosetosa	NTNU-VM 80038	Norway, 63.873N 9.748E	HABFA1523-21	**14**		**ON601013**	
J. ischiosetosa	ZMBN 147153	Norway, 60.447N 6.641E	HABFA1576-21	**7**		**ON601068**	
J. ischiosetosa		Russia, 69.3191N 34.3495E					MG751085
J. praehirsuta	NTNU-VM 79774	Norway, 58.149N 8.037E	HABFA1471-21	**11**		**ON601023**	
J. praehirsuta	NTNU-VM 79775	Norway, 58.149N 8.037E	HABFA1472-21	**7**		**ON601060**	
J. praehirsuta	NTNU-VM 79998	Norway, 63.6N 10.461E	HABFA1502-21	**12**		**ON601022**	
J. praehirsuta	NTNU-VM 80001	Norway, 63.6N 10.461E	HABFA1503-21	**14**		**ON601048**	
J. cf. *praehirsuta*	NTNU-VM 80003	Norway, 63.6N 10.461E	HABFA1504-21	**9**		**ON601059**	
J. praehirsuta	NTNU-VM 80004	Norway, 63.6N 10.461E	HABFA1505-21	**9**	**3**	**ON601029**	**ON598989**
J. praehirsuta	NTNU-VM 80005	Norway, 63.6N 10.461E	HABFA1506-21	**4**	**3**	**ON601061**	**ON598996**
J. praehirsuta	NTNU-VM 80013	Norway, 63.873N 9.748E	HABFA1507-21	**9**	**3**	**ON601005**	**ON598985**
J. praehirsuta	NTNU-VM 80019	Norway, 63.872N 9.739E	HABFA1509-21	**9**		**ON601012**	
J. praehirsuta	NTNU-VM 80022	Norway, 63.819N 9.622E	HABFA1510-21	**9**		**ON601026**	
J. praehirsuta	NTNU-VM 80030	Norway, 63.872N 9.739E	HABFA1515-21	**9**	**3**	**ON601056**	**ON598993**
J. praehirsuta	NTNU-VM 80031	Norway, 63.872N 9.739E	HABFA1516-21	**9**		**ON601038**	
J. praehirsuta,	NTNU-VM 80032	Norway, 63.819N 9.622E	HABFA1517-21	**9**		**ON601008**	
J. praehirsuta	NTNU-VM 80033	Norway, 63.819N 9.622E	HABFA1518-21	**9**		**ON601043**	
J. praehirsuta	NTNU-VM 80034	Norway, 63.819N 9.622E	HABFA1519-21	**16**		**ON601016**	
J. praehirsuta	ZMBN 132522	Norway, 66.512N 13.217E	HABFA1528-21	**18**		**ON601014**	
J. praehirsuta	ZMBN 132523	Norway, 68.31N 13.679E	HABFA1529-21	**4**		**ON601054**	
J. praehirsuta	ZMBN 136699	Norway, 60.447N 6.641E	HABFA1537-21	**21**		**ON601032**	
J. praehirsuta	ZMBN 136700	Norway, 60.447N 6.641E	HABFA1538-21	**22**		**ON601050**	
J. praehirsuta	ZMBN 139045	Norway, 71.05N 25.913E	HABFA1573-21	**7**		**ON601020**	
Jaera sp.	NTNU-VM 79826	Norway, 63.457N 10.45E	HABFA1476-21	**5**		**ON601010**	
Jaera sp.	NTNU-VM 79834	Norway, 58.967N 9.842E	HABFA1477-21	**18**		**ON601041**	
Jaera sp.	NTNU-VM 79842	Norway, 58.967N 9.842E	HABFA1478-21	**7**		**ON601034**	
Jaera sp.	NTNU-VM 79859	Norway, 58.977N 9.825E	HABFA1479-21	**18**		**ON601030**	
Jaera sp.	NTNU-VM 79958	Norway, 63.594N 9.527E	HABFA1492-21	**9**		**ON601021**	
Jaera sp.	NTNU-VM 79968	Norway, 63.599N 10.459E	HABFA1496-21	**4**		**ON601019**	
Jaera sp.	ZMBN 132516	Norway, 68.609N 16.565E	HABFA1526-21	**17**		**ON601052**	
J. caspica		Caspian Sea					MG751082
J. hopeana		Montenegro, 42.4237N 18.7610E					MG751084
J. massiliensis		Montenegro, 42.2844N 18.8442E					MG751083
J. sarsi	MJR-ISO-0021	Germany, 51.7115N 7.25573E	BISCE121-17	1		MT521209	
J. sarsi	MJR-ISO-0022	Germany, 51.7115N 7.25573E	BISCE122-17	2		MT521121	
J. sarsi	MJR-ISO-0023	Germany, 51.7115N 7.25573E	BISCE123-17	1		MT521284	
J. sarsi	MJR-ISO-0024	Germany, 51.7115N 7.25573E	BISCE124-17	1		MT521242	
J. sarsi	MJR-ISO-0025	Germany, 51.7115N 7.25573E	BISCE125-17	1		MT521245	

a white tray. All specimens collected for this study were preserved in 96% ethanol and deposited at the NTNU University Museum (NTNU-VM) (Bakken et al. 2023) and the University Museum of Bergen (ZMBN). A map of sampling locations was produced using QGIS version 3.22.3 (Fig. 1).

Morphological identification

Only males, recognised by the inverted T-shape of the preoperculum (see Figs. 3, 4, and 5), carry the morphological characters needed to identify *J. albifrons* group species.

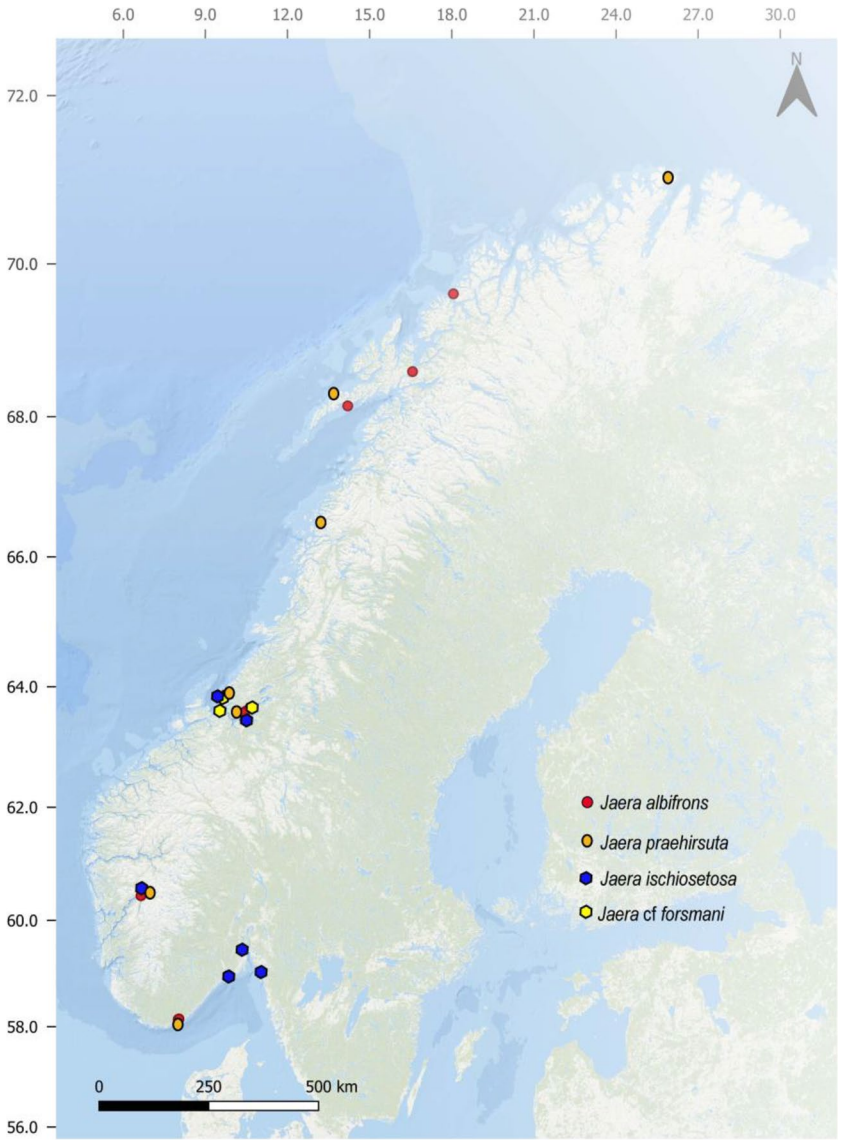

Fig. 1 Sampling locations of morphologically identified *Jaera* species

Each specimen was identified to species based on the morphological characters described by Forsman (1949) (Fig. 2) using a Leica M165C stereo microscope, and a Supra 55VP scanning electron microscope (SEM). Photographs of complete specimens were taken with a Leica DFC420 camera attached to a Leica MZ16A stereo microscope. All specimens identified as *J. albifrons* clearly displayed the diagnostic carpal lobe on pereopods 6 and 7 (Fig. 3) whereas specimens identified as *J. ischiosetosa* and *J. praehirsuta* displayed a comb of setae ventrally on the ischium of pereopod 6 and 7 (Fig. 4), and dense setation of ventral side of carpi, proximal half of propodi, and distal half of meri of pereopods 1–4 (Fig. 5) respectively. The identification of *J. forsmani* relies on the presence of a carpal spine in pereopod 6 and 7. Such a spine was believed to be present in seven specimens included in this study. These specimens did not portray any of the morphological characters typical for other *Jaera* species. The possibility that these specimens displayed a hybrid morphology should also be considered.

Extraction, PCR, and sequencing

DNA was extracted from 65 *Jaera* specimens (Table 1) using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. Due to the small size of the animals, entire individuals were used for extractions. Partial sequences of mitochondrial gene cytochrome *c* oxidase subunit 1 (CO1) were amplified using the primer pair CO1_Fbalt (TCAACT AACCATAAGGATATTGG) and CO1_Rbalt (TAAACC TCAGGGTGCCCAAAGAA) (Panova et al. 2017). For the PCR amplification process, 2 µl DNA extract was added to a master mix consisting of 16.35 µl ddH2O, 2.5 µl × 10 Takara reaction buffer, 2 µl dNTPs (200 µM each), 1 µl

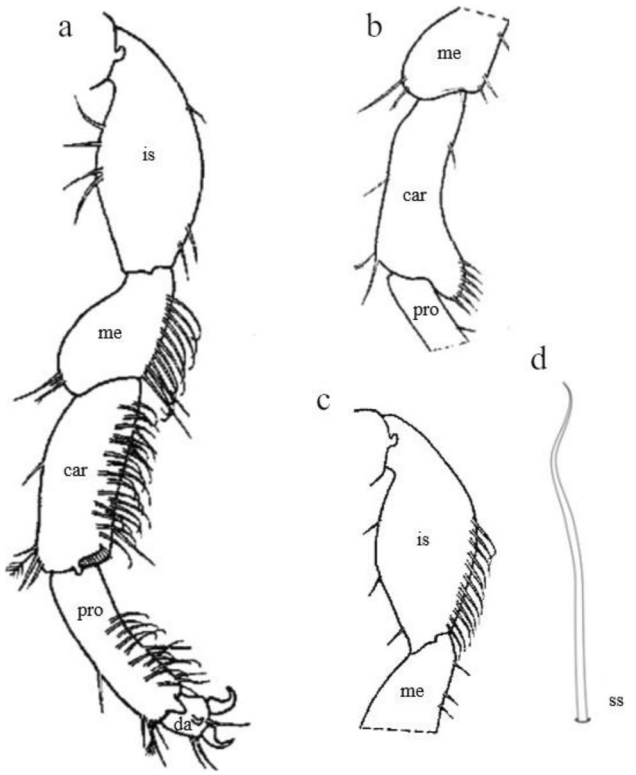

Fig. 2 Illustration of the diagnostic characters used to identify the three *Jaera* species. Abbreviations: is, ischium; me, merus; car, carpus; pro, propodus; da, dactylus; ss, simple setae. **a** Male *J. praehirsuta* pereopods 1–4 showing curved setae ventrally on me, car, and pro; **b** male *J. albifrons* pereopods 6–7 displaying a lobe distoventrally on car; **c** male *J. ischiosetosa* pereopods 6–7 with comb of setae ventrally on is; **d** ss morphology. Modified from Forsman (1949):459 and Riehl and Brandt (2010):12, Fig. 1

forward primer (10 µM), 1 µl reverse primer (10 µM), and 0.15 µl Takara Ex Taq Hot Start DNA Polymerase (5 U/µl) (Takara Bio Inc., Japan), yielding 25 µl per PCR reaction. PCR cycling consisted of one 5 min initiation cycle at 94 °C; five annealing cycles at 94 °C for 45 s, 45 °C for 30 s, and 72 °C for 1 min; 30 extension cycles at 94 °C for 45 s, 50 °C for 30 s, and 72 °C for 1 min; and ending with one elongation cycle for 10 min at 72 °C. PCR amplification results were checked on a 1% TAE gel electrophoresis. Purification and sequencing of PCR products were performed at Macrogen Inc. (Amsterdam, the Netherlands).

Partial sequences of nuclear 28S rRNA were amplified in separate reactions using either the primer pair 28S rd1a (CCCSCGTAAYTTAGGCATAT) and 28Sb (TCGGAAGGAACCAGCTAC), or 28SDKF (GATCGGACGAGATTACCCGCTGAA) and LSU1600R (AGCGCCATCCATTTTCAGG) (Cabezas et al. 2019). PCR cycling was a 4 min initiation cycle at 94 °C; 40 annealing cycles at 94 °C for 20 s, 58 °C for 1 min, and 72 °C for 2 min; and ending with one elongation cycle for 10 min at 72 °C.

Sequence assembly and alignments

A dataset with all specimens used for molecular analysis and metadata can be found in BOLDsystems (Ratnasingham and Hebert 2007) with the dataset name "DS-ISOPODA2 Genus *Jaera* in Norwegian waters".

Forward and reverse sequences were assembled and trimmed using Geneious 11 (https://www.geneious.com). Sequences for the chosen CO1 outgroup species, *Jaera sarsi* Valkanov, 1936 (Raupach et al. 2022), and other available *J. albifrons* sequences (Radulovici et al. 2009; Raupach et al. 2015) from outside of Norway (Table 1), were downloaded from BOLD and aligned with our own sequences using MUSCLE (Edgar 2004) in MEGA11 version 11.0.8 (Tamura et al. 2021). All CO1 sequences were uploaded to BOLD and subjected to the Barcode Index Number (BIN) analysis which clusters sequences with a certain level of similarity (Ratnasingham and Hebert 2013).

For the 28S dataset, *Jaera massiliensis* Lemercier, 1958, *Jaera hopeana* Costa, 1853, and *Jaera capsica* Kesselyak, 1938 were chosen as outgroups. Sequences for these three species, downloaded from GenBank, were assembled with our sequences in Geneious. Other available *J. albifrons* group sequences were also included, except for two *J. albifrons* sequences (< 400 bp) that appeared aberrant at the 3′-end. To examine the 28S *albifrons* group sequences and localise divergent sites, we performed sequence search with RNAcentral (Kalvari et al. 2021) (https://rfam.org/). Associated online R2DT (Sweeney et al. 2021; https://bio.tools/r2dt) was used to initially infer the secondary structure of the sequence fragments. We mapped a sequence of *J. albifrons* to the LSU secondary structure of *Drosophila melanogaster* with R2DT and applied the helix terminology of Petrov et al. (2014), as displayed in the online gallery at http://apollo.chemistry.gatech.edu/RibosomeGallery. Because R2DT did not produce a structure for 367 nucleotides in expansion segment 25ES7 (Fig. S1), we used LocARNA (Will et al. 2007) with the web server at the University of Freiburg (https://rna.informatik.uni-freiburg.de/LocARNA/) to infer the structure of this segment, anchoring the 5′ start and 3′ end in conserved helix 25. We combined the results from LocARNA with those obtained with R2DT to a structure file in parenthesis notation and visualised the structure with input to *forna* (Kerpedjiev et al. 2015) at the University of Vienna bioinformatics server (http://rna.tbi.univie.ac.at/forna/) (Fig. S2). Putative structural base pairings with complements outside the range of the sequence, such as H10, H4, and H2, were additionally marked with reference to their location in *D. melanogaster* (Fig. S3). Whilst the *J. albifrons* group 28S sequences alone aligned automatically with no gaps, the outgroup did not align well. We therefore utilised Geneious Prime 2023.0.4 (https://www.geneious.com) to search for conserved LSU motives. We manually

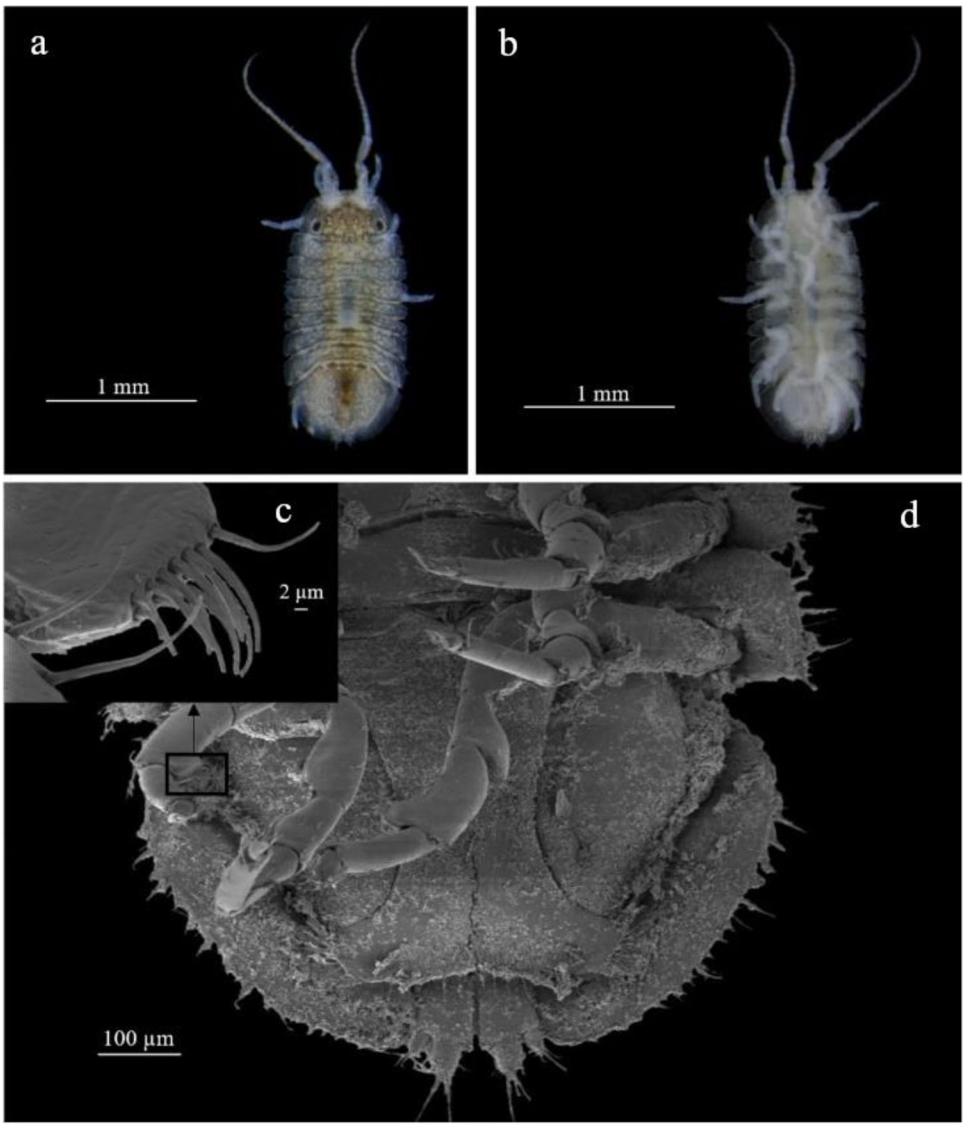

Fig. 3 Male *Jaera albifrons*. **a** Dorsal view; **b** ventral view; **c** display of carpal lobe on pereopod 6; **d** male preoperculum. **a**, **b** August R. Nymoen; **c**, **d** Katrine Kongshavn

aligned these motives and subsequently used them as anchor points for automated alignment of the intermediate sequence regions. We used MUSCLE 3.8.425 with default settings in Geneious to consecutively align the regions between the conserved anchor points. When analysing *J. albifrons* sequences with the outgroup, we trimmed the alignment 3′ end at position 949 (Fig. S3) to accommodate uniform sequence lengths.

Sequence relationships and nucleotide substitution tests

MEGA11 (Tamura et al. 2021) was used to calculate genetic p-distances between and within morphospecies. Calculations were performed assuming uniform and homogenous rates, and with pairwise deletion of gapped positions. We used MEGA to perform Tajima's (1989) test for neutrality and the codon-based Z-test for purifying selection (Nei and Gojobori 1986). McDonald-Kreitman test was performed with ingroup CO1 sequences against *J. sarsi* in DNAsp v6 (Rozas et al. 2017) to examine possible selection on CO1. DNAsp was also used to generate haplotype data files for both CO1 and 28S. In the latter case, we did not include outgroup sequences. Haplotype matrices were uploaded to PopArt (Leigh and Bryant 2015) to produce minimum spanning networks (Bandelt et al. 1999), and TCS networks (Clement et al. 1999, 2002). Neighbour joining trees with 500 bootstrap replicates were calculated from both data sets with Geneious.

Exploring distance gaps

Disregarding morphological characters, we explored the data with Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) and Assemble Species by Automatic

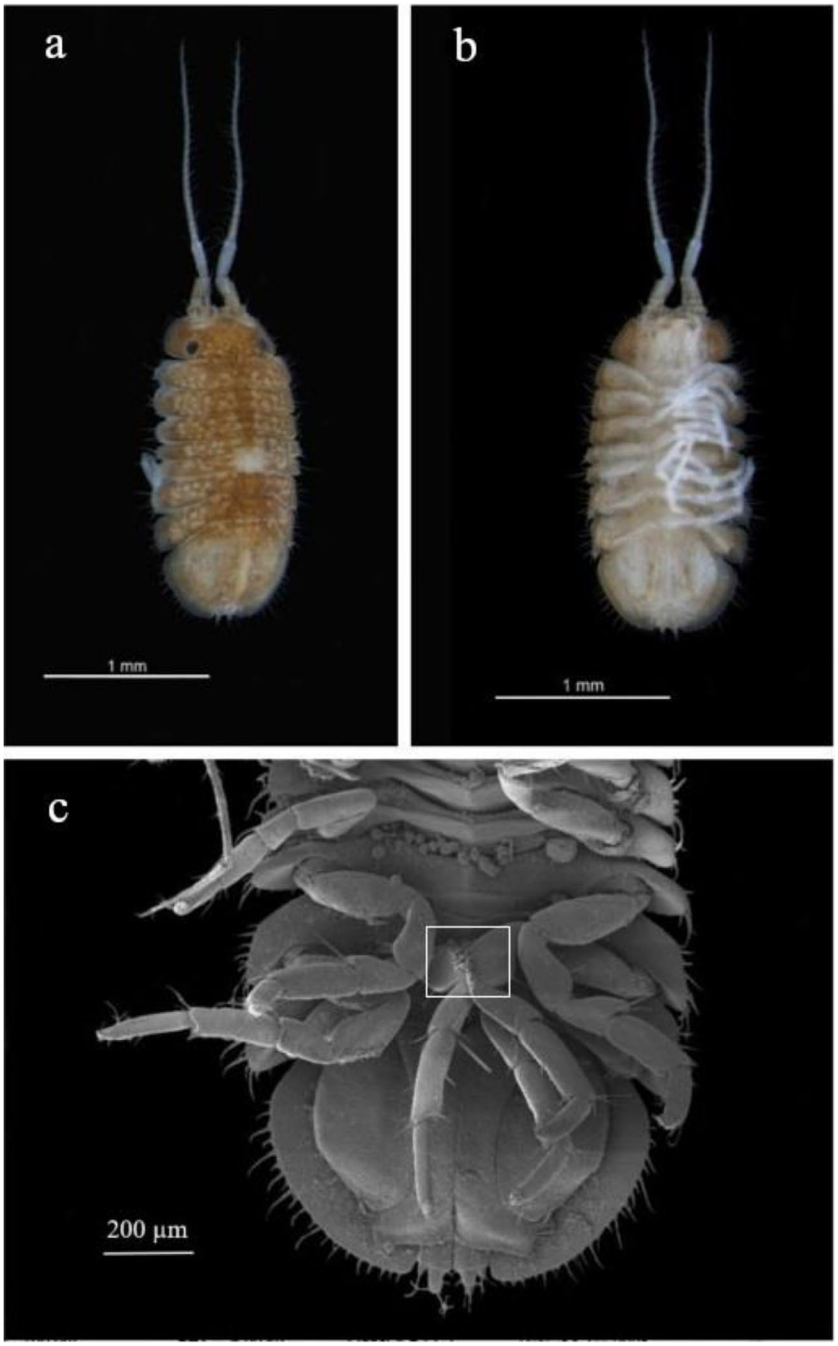

Fig. 4 Male *Jaera ischiosetosa*. **a** Dorsal view; **b** ventral view; **c** comb of straight setae on ventral side of ischium on pereopod 6 (in a white square). **a**, **b** August R. Nymoen; **c** Katrine Kongshavn

Partitioning (ASAP) (Puillandre et al. 2021), employing the respective web servers at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html and https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html. In both sets of analyses, we also included outgroup sequences. ABGD aims to estimate the boundary between intraspecific and interspecific genetic distances by identifying the so-called barcode gap between species. As opposed to other popular methods of species delimitation, it does not require an a priori hypothesis of species to be analysed. However, it needs user input of an expected range of values for maximal intraspecific divergence. We applied a range of priors from 0.001 to 0.2 for both data sets. We used Kimura 80 (K2P) distances for the CO1 data with ASAP because it has been used as a standard in barcoding. For the 28S, we used raw distances (p-dist) computed with complete deletion of gaps/missing data, input as a distance matrix to ASAP. ABGD recursively estimates barcode gaps from the data, based on different values within this defined input range. Because different input values may return different estimates of barcode gaps and different partitions of the data, it may be difficult to validate which of potentially conflicting partitions is "the best". ASAP was motivated

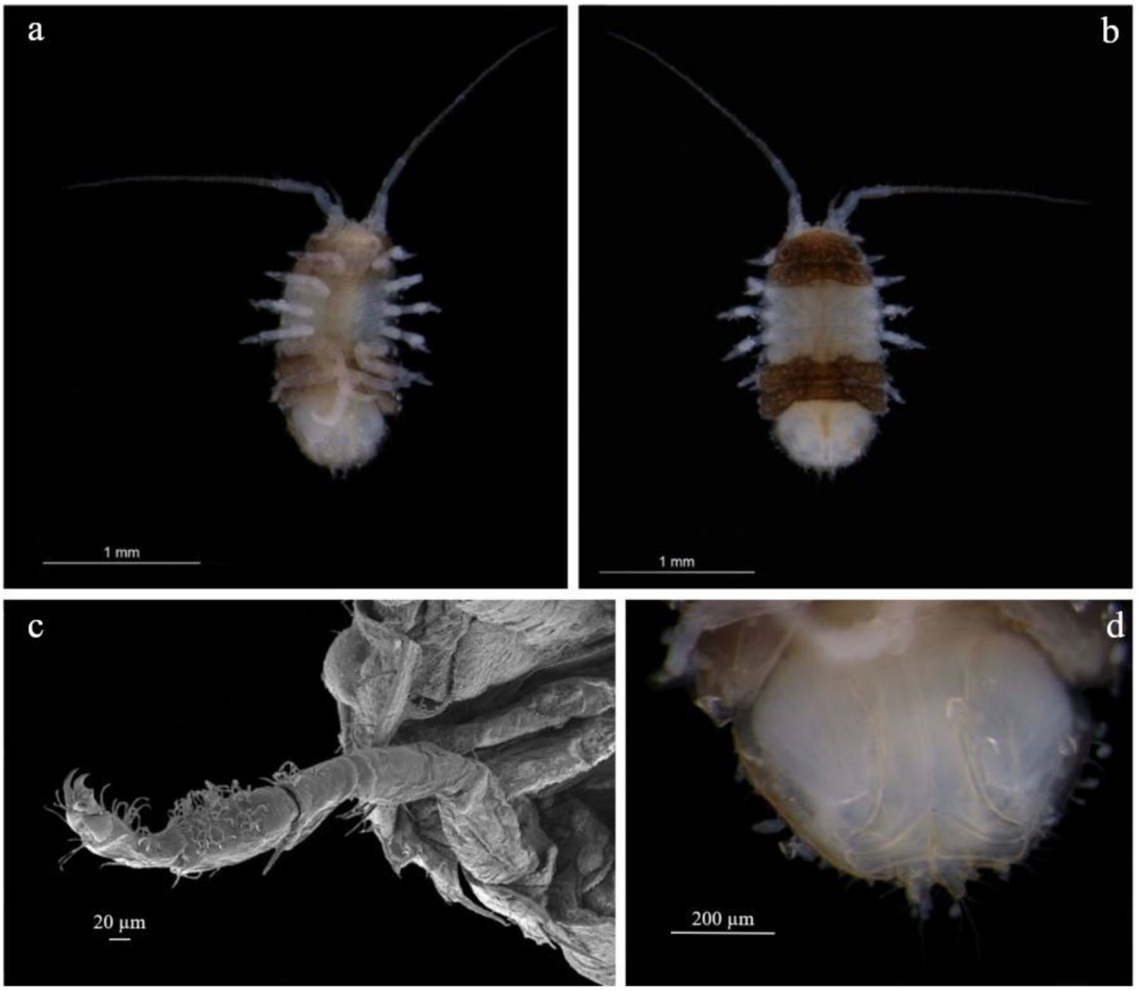

Fig. 5 Male *Jaera praehirsuta*. **a** Ventral view; **b** dorsal view; **c** display of curved setae on propodus, carpus, and merus on pereopod 3; **d** male preoperculum. **a**, **b**, **d** August R. Nymoen; **c** Katrine Kongshavn

by Puillandre et al. (2021) from this suspected shortcoming. The algorithm starts with pairwise distance measures between all sequences and successively clusters them into larger, more inclusive groups, based on ranked pairwise distances. The best set of groups is indicated by the ASAP score, which combines an estimate for the relative barcode gap width (w) and a *p*-value indicating that each group in the partition is a single (panmictic) species. The lowest score is considered the best partition. ASAP was run with default settings.

Results

We identified four morphospecies of *Jaera* from Norwegian waters, all belonging to the *albifrons* species group: *J. albifrons*, *J. ischiosetosa*, *J. praehirsuta*, and *J. forsmani*. Three specimens with incomplete pereopod spines were indicated with uncertain identifications given as cf., two *J. forsmani*, and one *J. praehirsuta* (Table 1). A total of 65 CO1 sequences (length between 575 and 658 bp) and 15 28S sequences (length between 995 and 997 bp) were produced for Norwegian specimens, representing all four morphospecies (Table 1). Specimens identified as *Jaera* sp. in the dataset correspond to females which cannot be identified based on morphology.

The aligned CO1 data included 575 sites of which 26 were variable in the *J. albifrons* group. There were two cases of variation in a 1st codon position whilst the remaining variability was in 3rd codon positions. All mutations in the ingroup were synonymous. However, a singleton case of base call ambiguity could alternatively be interpreted as a non-synonymous mutation. We did not consider this possibly ambiguous alternative in haplotype analysis and other calculations. Neutrality of CO1 was not rejected ($p > 0.10$) by Tajima's test, D being 0.28 in *J. praehirsuta* and had negative values (−0.11 to −0.37) in the other species (Supplementary Table 1a). McDonald-Kreitman test (MK)

indicated positive selection judged from NI values < 1 in all *J. albifrons* groups when based on comparison with *J. sarsi* (Supplementary Table 1b). However, the test was significant (Fisher's exact test *p*-value: 0.029; G test *p*-value: 0.012) only when all *J. albifrons* group sequences were pooled, returning a neutrality index (NI) of 0.138 and an alpha value of 0.862, indicating a high proportion of substitutions driven by adaptation (Bazin et al. 2006). The neutrality index (NI) is computed from the ratio of nonsynonymous to synonymous within species ($ka = \pi_N/\pi_S$) and between species ($ks = d_N/d_S$). Because all substitutions in *J. albifrons* group were synonymous, the index could not be computed by comparing amongst the ingroup morphospecies. The codon-based Z-test significantly indicated purifying selection within species, as well as in the combined set of ingroup sequences (Supplementary Table 1c).

Automatic alignment of the 28S sequences from the *J. albifrons* group alone was trivial and showed point mutations at three positions only: C/T in position 392, A/T in 467, and G/A in 575. All variable sites are associated with expansion segments 25ES7, and the latter of the three mutations appears as a compensating substitution, paired with a U in a long helix stem (Fig. S2). When including outgroup *Jaera* sequences in the data set, it was difficult to compute an alignment that appeared reasonable in the downstream part, from about the 5′ start of helix 25. The alignment problem, due to hypervariability of this region, was also reflected in our search for similar sequences when RFam reported a short series of Ns (Fig. S1) where LocArna modelled a complex secondary structure in expansion segment 25ES7 (Fig. S2: positions 320–688). Gapped regions were not included in the distance calculations.

Both markers show a high level of genetic similarity between *J. albifrons* group species and a clear distinction from the outgroup of *Jaera* species. CO1 p-distances between *J. albifrons* group morphospecies are all maximum of 1.57% (Table 2). Within-group distances are much on the same scale, clearly smoothing over an initially anticipated barcode gap. The BIN analysis in BOLD also clustered all sequences belonging to the *J. albifrons* group into the same BIN BOLD:AA17930, reflecting the low level of variation between sequences. By contrast, the outgroup *J. sarsi* differed from the *J. albifrons* group by 23.5–24.5%.

The 28S interspecific genetic distances showed a maximum of 0.39% between morphospecies and a maximum within-group distance of 0.13% in *J. forsmani* (Table 3). Distances between *J. albifrons* group species and other *Jaera* species were all more than 25%.

The short distances were mirrored in neighbour joining trees from each of the data sets, showing short branch lengths and little topological resolution of *J. albifrons* group species (Fig. S4).

Table 2 Minimum and maximum raw CO1 distances (as %) within and between morphologically identified species of *Jaera*

CO1	Within gr		Between gr							
			J. albifrons		*J. forsmani*		*J. ischiosetosa*		*J. praehirsuta*	
	min	max	min	max	min	max	min	max	min	max
J. albifrons	0.00	1.74								
J. forsmani	0.00	1.22	0.00	1.57						
J. ischiosetosa	0.00	1.22	0.00	1.57	0.00	1.21				
J. praehirsuta	0.00	1.39	0.00	1.57	0.00	1.39	0.00	1.39		
J. sarsi	0.00	1.74	23.5	24.0	23.7	24.5	23.7	24.5	23.5	24.2

Table 3 Minimum and maximum raw 28S distances (as %) within and between group morphologically identified species of *Jaera*

28S	Within gr		Between gr											
			J. albifrons		*J. forsmani*		*J. ischiosetosa*		*J. praehirsuta*		*J. caspica*		*J. hopeana*	
	min	max	min	max	min	max	min	max	min	max	min	max	min	max
J. albifrons	0.00	0.00												
J. forsmani	0.00	0.13	0.00	0.13										
J. ischiosetosa	0.00	0.13	0.00	0.13	0.13	0.26								
J. praehirsuta	0.00	0.00	0.23	0.26	0.00	0.13	0.26	0.39						
J. caspica	0.00	0.00	25.28	25.28	25.55	25.55	25.39	25.39	25.55	25.55				
J. hopeana	0.00	0.00	29.41	29.41	29.41	29.41	29.52	29.52	29.52	29.52	20.70	20.70		
J. massiliensis	0.00	0.00	32.94	32.94	32.94	32.94	32.79	32.94	32.94	32.94	31.81	31.81	35.67	35.67

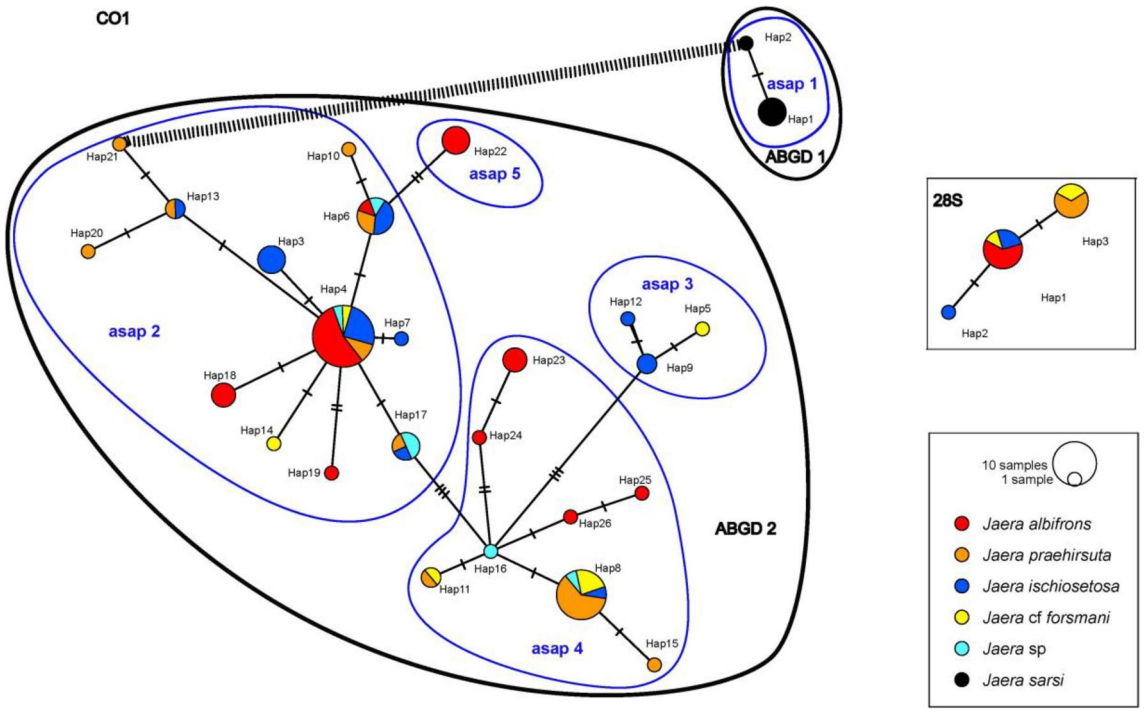

Fig. 6 Nearest neighbour network of CO1 and 28S (inset) haplotypes. Circle area proportional to number of sequences. Circumscribed haplotypes are groups computed with ABGD (in black) and ASAP (in blue)

Minimum spanning networks (Fig. 6) and TCS (not shown) produced very similar results. Whilst *J. sarsi* is clearly separated from the *J. albifrons* group by 134 mutations in CO1, extensive haplotype sharing can be observed between all *J. albifrons* group species. Of the 26 haplotypes, six are shared amongst two or more species. The dominant haplotype, Hap4, is shared by 5 specimens of *J. ischiosetosa*, 11 *J. albifrons*, 2 *J. praehirsuta*, 1 *J. forsmani*, and 1 *Jaera* sp. sequences. Remarkably, all four morphotypes with Hap4 were collected on the same beach in Trondheimsfjorden. *Jaera albifrons* haplotypes are separated with up to 10 mutations, but there was no obvious geographical structure in the network. For instance, *J. albifrons* specimens from Germany did not cluster together but were separated in haplotypes Hap22, Hap23, and Hap24 (Fig. 6). The Canadian specimens were assigned to either Hap4, which is represented on both sides of the Atlantic, and to haplotypes Hap25 and Hap26, which are five to six mutations different from Hap4. The ASAP score (Supplementary Table 2) indicated that these haplotypes of *J. albifrons* belong to three different groups, namely asap2, asap4, and asap5. However, only one of these groups, asap5, came out as apparently monospecific (Fig. 6).

The 28S haplotype network yielded three haplotypes (Fig. 6, insert) based on the mutations that were identified in the secondary structure model (Fig. S2). Hap1-28S is shared by specimens of *J. albifrons*, *J. ischiosetosa*, and one specimen of *J. forsmani*. Hap2-28S was exclusively observed in one *J. ischiosetosa*. *J. forsmani* and *J. praehirsuta* specimens shared the third haplotype (Hap3-28S).

ABGD analysis divided the CO1 data in two groups (Fig. 6, Supplementary Table 2), and the 28S data in four groups, in both cases displaying all the *J. albifrons* group as one single species. The best ASAP score from the CO1 data indicated five groups with a threshold distance of 0.436%. These groups corresponded well to the clusters obtained with haplotype networks (Fig. 6, Fig. S4). However, again, these groups had no concordance with the groups of morphospecies. The best ASAP score for the 28S data indicated three groups (Supplementary Table 3), one of which was composed by the *J. albifrons* species, the others by *J. massiliensis* and *J. hopeana* plus *J. caspica* (Fig. 6).

Discussion

We found that CO1 distances between morphospecies of the *Jaera albifrons* species complex do not comply with the criteria for distance-based species identification as suggested by Hebert et al. (2003b, 2004b). All specimens appear as members of the same BIN in BOLDsystems. Our results from ASAP with CO1 would indicate four different (panmictic) species in the *J. albifrons* complex, but these groups do not correspond with the morphology-based identifications, and the barcode

gap threshold distance is as low as 0.4%. The 28S comparison indicated a distance threshold of 23% between the examined *Jaera* species, and that the *J. albifrons* group is comprised by one species only. Therefore, a likely hypothesis from our results is that the *J. albifrons* complex represents a single species.

Species barcodes and taxonomic resolution

A BIN discordance, as we found, may certainly have different reasons (Radulovici et al. 2021; Keck et al. 2022), one of which is a priori mislabelling of the barcoded specimens. We believe that identifications of *J. albifrons* group males stand in accordance with descriptions in the literature. Therefore, given that these nominal taxa are valid species, the molecular standard barcode marker does not provide a means to identify them. Overlaps between interspecific and intraspecific genetic distances, as well as low divergence rates based on DNA barcoding with CO1, have been documented by previous studies on groups of butterflies and odonates (Elias et al. 2007; Rach et al. 2017). A solution to such cases often entails the inclusion of nuclear markers. We sequenced partial 28S to search for an alternative species barcode. Whilst comparison with outgroup species revealed a hypervariable sequence segment that is difficult to align with other *Jaera*, the *J. albifrons* group sequences are conserved across morphospecies with only three variable sites. Haplotype 3 of 28S is shared by *J. praehirsuta* and *J. forsmani*, and haplotype 1 by *J. albifrons* and *J. ischiosetosa*. These pairs of taxa have been believed to represent sister species (Solignac 1981). However, haplotype 1 is also found in a specimen of *J. forsmani*, which interrupts monophyly of each of the two proposed sister groups (Fig. 6, Fig. S4). Thus, our data from 28S do provide an alternative diagnostic tool to identify the *J. albifrons* complex. However, the marker cannot discriminate the alleged species within the complex.

Jaera albifrons sensu stricto, *J. ischiosetosa*, and *J. praehirsuta* were first described by Forsman (1949) as sub-species of *J. albifrons*. Bocquet (1953, 1954) observed additional morphotypes and argued that five distinct units should be considered as species-level taxa. Technically, that taxonomic decision parallels what Isaac et al. (2004) call "taxonomic inflation", i.e., when subspecies are raised to the level of species. Bocquet characterised *J. albifrons* sensu lato (as *Jaera marina* (Fabricius)) as a "superspecies", much in the philosophical spirit of Mayr (1942), who defined a superspecies as "… a monophyletic group of closely related and largely or entirely allopatric species." He speculated extensively on speciation mechanisms and evolutionary history of these taxa, and much of his perspectives were focused on micro-geographical separation of species that, according to his observations, are separated by ecological barriers over a physical gradient that projects perpendicularly to the shoreline. Hence, *J. ischiosetosa*, he stated, is found closest to terrestrial conditions, whilst *J. praehirsuta* is found in the deepest part of the littoral, and the other species in between these spatial ends. Bocquet's views prepared the ground for much of the later work on the *J. albifrons* group, and research has been directed towards identifying effects of ecological and behavioural barriers to gene flow amongst putative (biological) species. We were not able to observe such detailed habitat distributions. However, subsequent studies have shown that the habitat preferences of the different morphospecies are less specific than originally envisioned by Bocquet (1954). The degree of habitat overlap between putative species has been found to vary amongst different locations (Bocquet 1954; Naylor and Haahtela 1966; Ribardière et al. 2017), and populations have therefore been described as either monospecific, regularly zoned, or intermingled sympatric species (Naylor and Haahtela 1966; Ribardière et al. 2017; Siegismund 2002; Solignac 1981; Wenzel et al. 2018). Other studies suggest more strict habitat preferences amongst the species, claiming that mixed populations are rare and that interbreeding is virtually non-existent (Jones and Naylor 1971). Our samples from Norway do not show a pattern of allopatry, since morphospecies co-occur at the same sampling sites. For example, in the Trondheimsfjorden (at 63.599° N, 10.459° E), we recorded *J. albifrons*, *J. ischiosetosa*, and *J. forsmani* specimens together. About 150 m away, on the same beach, we collected both *J. forsmani* and *J. praehirsuta*. Similar mixed species observations were also reported from several localities in Denmark (Siegismund 2002), Britain (Naylor and Haahtela 1966), and France (Ribardière et al. 2017; Ribardière et al. 2019). This indicates that separate habitats and niche diversification are inconclusive and questions the complex that represents more than one species, supporting our genetic results. Our data do not support insight in effects of glaciation effect from ice ages, as other studies have demonstrated resulting in population structuring (e.g., Rossel et al. 2020).

As habitat separation has been somewhat downplayed with new observations of spatial co-occurrence of morphotypes, it has been suggested that mate choice is a primary barrier for genetic exchange between *J. albifrons* group species in mixed populations (Mifsud 2011; Ribardière et al. 2017; Ribardière et al. 2019). Males have been observed performing what is believed to be species-specific brushing movements with their pereopods to seduce females. Still, female mate selection does not always guarantee the same species partner (Khaitov et al. 2007; Ribardière et al. 2019). Ribardière et al. (2017) found that populations of *J. albifrons* and *J. praehirsuta* appear to be sexually isolated in some mixed populations, but that hybridization does occur in other cases of habitat sharing. When hybridization does occur, morphological characters also become blurred (Solignac 1981; Ribardière et al. 2017). We observe that *J. albifrons* and *J. praehirsuta* morphotypes share both haplotypes 4 and 6 of CO1 in our material (Fig. 6).

Conflicting molecular data

When comparing three enzyme loci from Danish populations, Siegismund (2002: Fig. 2) found that *J. albifrons*, *J. ischiosetosa*, and *J. praehirsuta* were completely mixed in an unrooted phylogenetic tree, somewhat similar to our haplotype network for CO1. However, his *gpi* (glucose phosphate isomerase) gene produced a different tree, that is a good match with the tree produced from AFLP data by Mifsud (2011). Here, *J. albifrons* and *J. praehirsuta* are sister groups, whereas in the phylogeny based on allozymes (Solignac 1981), *J. albifrons* and *J. ischiosetosa* are sister species with *J. praehirsuta*, *J. forsmani*, and *J. posthirsuta* combined in a sister clade. With parenthesis notation, the two different three topologies appear like this: 1. [(*J. albifrons*, *J. praehirsuta*), *J. ischiosetosa*] (Siegismund 2002:gpi; Mifsud 2011:AFLP). 2. [(*J. albifrons*, *J. ischiosetosa*), ((*J. praehirsuta*, *J. forsmani*), *J. posthirsuta*)] (Solignac 1981:allozymes).

Mifsud (2011) discovered phylogenetic incongruence between mitochondrial and nuclear data and ascribed the mismatching 16S to introgression. Of totally 12 different haplotypes in 16S sequences, the one haplotype with the highest frequency was shared between all four species of the group. By contrast, a neighbour joining tree computed from amplified fragment length polymorphism (AFLP) displayed clades that were mostly in concordance with the morphospecies, except in one case of paraphyly that was interpreted as a misidentification (Mifsud 2011). We found that there is no correspondence between CO1 sequence clusters and morphologically defined species of the *J. albifrons* complex. As can be seen from genetic distance values (Table 2, Table 3), haplotype networks (Fig. 6), and NJ-trees (Fig. S4), neither CO1 nor 28S can be used to diagnostically distinguish between the different morphospecies of the *J. albifrons* complex. There is almost no difference between intraspecific and interspecific genetic distances for each putative species, and there are several examples of different morphospecies having identical sequences.

Because mitochondria are evolutionary units with their own characteristics and evolutionary potentials, they may distort the cladogenetic speciation history (Chan and Levin 2005; Funk and Omland 2003; Ballard and Whitlock 2004; Palozzi et al. 2018; Toews and Brelsford 2012) and accordingly obliterate the use of mitochondrial genes as species identification markers. Whilst it is also tempting to interpret the mitochondrial paraphyly (Mifsud 2011; this paper: Fig S4) as incomplete lineage sorting, our observations of shared haplotypes between sympatric individuals of putative morphospecies are also a typical signature of introgression (Funk and Omland 2003; Ballard and Whitlock 2004). Although successful interspecific crossings have been obtained particularly in "no-choice" experimental situations, natural hybridization is traditionally believed to be exceptional (Solignac 1981). Therefore, the strongly intermixed mitochondria amongst the species seem confusing, but rare hybridization may still have profound effects, and mitochondria have been inferred to introgress faster and more often than nuclear genes (Chan and Levin 2005). Introgression of mitochondria can come about if biological species borders are "semipermeable" (Harrison and Larson 2014), which seems to be the case in some interspecific crossings (Ribardière et al. 2017). This will allow for incorporation of some extrinsic genes by hybridization and backcrossing. Although it may be difficult to separate hybridization from lineage sorting in relatively young species (Holder et al. 2001), "mitochondrial capture" by hybridization has been inferred in several animal studies (e.g., Avise 2004). In extreme cases, collectively called "massively discordant mitochondrial introgression (MDMI)" by Bonnet et al. (2017), most of a population may hold mitochondria from another species whilst there are still few signs of introgression in the nuclear genome. Amongst alternate explanations for easier introgression of mitochondria, they may be less functionally linked to nuclear genes that would be strongly selected against in a new "immigrant genome". Our 28S data did not provide clues of introgression, because the species had very similar sequences.

The neutrality index (NI), computed from the ratio of nonsynonymous to synonymous within species ($ka = \pi N/\pi S$) and between species ($ks = dN/dS$) is believed to indicate deviations from neutrality. We obtained $NI < 1$ in comparisons with *J. sarsi*, indicating adaptive evolution in the *albifrons* group (Bazin et al. 2006; Meiklejohn et al. 2007). The Z-test (Nei and Gojobori 1986) was significant for purifying selection in all species except in *J. sarsi*. Tajima's test, however, was not significant for non-neutrality of the CO1 sequences. Because all substitutions within *J. albifrons* group were synonymous, the neutrality index could not contrast between the ingroup morphospecies to detect indication of selection amongst the haplotypes of the *albifrons* complex.

Alternatives to selection-driven introgression have been characterised by Bonnet et al. (2017) as "mt-neutral". They usually imply some sort of sex-biased asymmetry in gene flow across species borders. This opens for scenarios that would perhaps fit with published observations of mating behaviour, hybrid survival, and sex ratios in the *J. albifrons* complex. Our simple tests of the CO1 segment suggested that the gene is not neutral, however. Maternally biased introgression should be largely the result of the females of the least abundant species in a mixed mate with males of the common species, although the sex ratio is skewed towards female dominance (Chan and Levin 2005; Wirtz 1999).

Concluding remarks

Since the pioneering work of Bocquet (1953), observations of karyotypes, ecological separation, mate recognition, and experimental hybridization have produced some fascinating clues to reproductive isolation between members of the *J. albifrons* complex, which are central in the different versions of biospecies concepts. However, hybridization has also been observed, and recent evidence for introgression of nuclear genes comes from locus mapping (Mifsud 2011) and analyses of microsatellites (Ribardière et al. 2017). Mitochondria are showing even stronger interspecific sharing of haplotypes. These observations are challenging the isoenzyme-based species tree (Solignac 1981) that has been guiding the notion of species in this group. Allowing for a relaxed version of the biological species concept that would accept these nominal species of the group as members of a "Darwinian continuum" (Mallet 1995; Mallet 2008) would highlight the problem of "secondary species criteria" (de Queiroz 2007), and at present, there does not seem to be any operational diagnostic criteria that unambiguously identify specimens to any of these nominal species. Whereas one of the more conspicuous effects of DNA barcoding has been the discovery of unexpected genetic divergence and detection of cryptic species, the *J. albifrons* group stands out as a special case of taxonomic ambiguity with its intermixed mitochondrial markers. In DNA barcoding context, the situation in the *J. albifrons* complex is one of Keck et al.'s (2022) "*seven challenges of taxonomic reference databases in metabarcoding analyses*", characterised as a *sequence conflict*, where several different taxa are assigned to the exact same genetic sequence. When this is observed, the reason is, according to Keck et al. (2022), that the barcode region is either not sufficiently diverged to discriminate between two or more species, or it may be because the genomes have experienced introgression. However, there is also a third possibility, namely that of an over split taxonomy. That problem may become uncovered by barcoding, when sequences show that scientists use different names on units with little genetic divergence. Also, genetic population studies may find population structures that would not be considered as different species, unless some additional corroborating data were available. Despite substantial accumulation of evolutionary and systematic studies of the *J. albifrons* group over the years, our work here suggests that the species taxonomy is still somewhat suspect and that not only females but also males cannot be unambiguously identified to alleged species. Such observations certainly do not devaluate DNA barcoding as a methodological approach. Rather, it should inspire more studies to get our taxonomy right.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval No animal testing was performed during this study.

Sampling and field studies Sampling and field studies were commissioned by relevant Norwegian authorities and are compliant with CBD and Nagoya protocols.

Data availability Data are available in the paper and Supplementary files. Specimen data with all details on vouchers for included specimens (Table 1) with their CO1 and 28S sequences are available as a dataset in the BOLD database "DS-ISOPODA2 Genus *Jaera* in Norwegian waters".

Author contribution ARN, TB, and JAK conceived and designed the research. ARN, JAK, and TB conducted fieldwork. ARN and JAK conducted lab sorting and identification, sequence editing, and BOLD handling. ARN and EW analysed data. ARN wrote the manuscript with TB, with contributions from EW and JAK. All authors read and approved the manuscript.

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